Common microplastics in aquatic systems and their effects on fish

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Summary

The abundance of microplastics in the environment is rising with increasing anthropogenic mass production of plastics. Unintentional released plastic items and mismanaged plastic waste disintegrate into smaller microplastic fragments, which spread through wind, with wastewater and runoffs. This results in a global distribution of microplastics in the atmosphere, terrestrial, and aquatic environments. The high persistence of microplastic items that can last hundreds of years leads to their progressive accumulation in the environment. The increasing occurrence of plastics in the environment raised concerns that organisms inhabiting aquatic systems are negatively affected by microplastics, which could induce disturbances of communities and ecosystems. To determine the actual risks of microplastics in the environment, information about current and potential future concentrations of microplastics in nature together with knowledge on its toxicity thresholds in individuals, communities, and ecosystems are required. Therefore, the present thesis addresses, which microplastic types are relevant for aquatic systems and investigates their potential toxicity and health effects on different life stages of fish in laboratory studies.

With the first chapter, the present thesis seeks to investigate common types of microplastics in the environment and their previously determined effects on aquatic organisms based on a literature review. Improvements in methods for detection and analysis led to the recognition that the majority of microplastics in the environment are fibers and to some extent irregular-shaped fragments. In contrast, most effect studies conducted so far used microplastic spheres. The commercially available, spherical reference particles were often applied in exposure studies since other microplastic components, in particular fibers, pose additional challenges in handling and characterization in laboratory settings, for example due to their potential to entangle and agglomerate. Therefore, a limited number of studies investigated effects of microplastic fibers on aquatic organisms and the potential risk by environmentally relevant microplastic shapes was not determined up to date.

In order to close the gap between abundance of microplastic fibers in nature and their largely unknown effects on aquatic organisms, methods were developed how to work with microplastic fibers in laboratory settings in the frame of the present thesis. The following chapters describe laboratory effect studies that were conducted with the model organism three-spined stickleback (*Gasterosteus aculeatus*) to investigate the potential effects of common textile microplastic fibers on different life stages of fish.

In chapter II, potential effects of microplastic fibers in the rearing water on the fertilization process of fish eggs and the development of early life stages of fish were assessed. *In vitro* fertilization was used to compare the fertilization rate of eggs that derived from the same adult breeding pair with and without fibers present in the water. The results

showed that the experimental fibers in the water did not affect *in vitro* fertilization rates, hatch rates, and the early development of sticklebacks at concentrations even higher than currently observed in nature.

The subsequent chapters address whether direct ingestion of fibers as feed additives negatively affects three-spined sticklebacks. At first, a method to create fish feed that is supplemented with different amounts of fibers was developed in chapter III. The use of ethanol during the manufacturing process of the feed facilitated to overcome the problems of entanglement of fibers and promoted their homogeneous distribution in the feed.

In chapter IV, the produced experimental feeds were fed to subadult sticklebacks to investigate the potential effects of ingestion of microplastic fibers. Measured endpoints were compared with the ingestion of fibers from natural material that were also included in treatment diets, since natural fibers are frequently encountered by fish in nature. Growth, gonad development, and immune parameters were analyzed after nine weeks. None of the fibers ingested with the diet impaired growth performance, body condition, gonad development, and the immune system of exposed fish – even at concentrations orders of magnitude above levels occurring in nature. The efficient excretion of ingested fibers likely prevented the fish from deleterious impacts on their health.

Together, those results demonstrate that common microplastic textile fibers do not affect fish of different life stages in laboratory studies. While previous effect studies report deleterious effects of microplastics on organisms, those were often conducted with extremely high concentrations and microplastic components that are not common in the environment. The absence of negative effects of common textile fibers, as observed in the present thesis, demonstrates the need to consider the environmental relevance when interpreting effect studies in terms of an overall environmental risk assessment of microplastics. At current environmental concentrations the actual toxicological risk of (often fibrous) microplastics in aquatic systems seems to be lower to fish than suspected in earlier microplastic research. Accordingly, future risk assessments of microplastics in aquatic systems should not be driven by every impact reported but by realistic interpretations of effect studies.

Zusammenfassung

Mit zunehmender Massenproduktion von Plastik steigt ebenfalls die Menge an Mikroplastik in der Umwelt stetig an. Unbeabsichtigt freigesetztes Plastik sowie unsachgemäß entsorgter Plastikmüll verbreiten sich durch Wind, Oberflächenabfluss und Abwässer und führen zu einer weltweiten Verteilung von Plastik und Mikroplastik in der Atmosphäre, in terrestrischen und in aquatischen Systemen. Die Beständigkeit von Kunststoffen über hunderte von Jahren und die dadurch verursachte zunehmende Akkumulation in unserer Umwelt löste die Besorgnis aus, dass aquatische Organismen durch Plastik und Mikroplastik beeinträchtigt sein könnten. Negative Einflüsse könnten sich nachfolgend auch auf Organismen-Gemeinschaften und ganze Ökosysteme auswirken. Um das konkrete Risiko von Mikroplastik in der Umwelt bewerten zu können, sind neben Kenntnisse über die aktuellen und potenziell zukünftigen Konzentrationen von Mikroplastik in der Umwelt ebenso Kenntnisse zu Belastungsgrenzen von Individuen, Gemeinschaften und Ökosystemen nötig. Demzufolge wird in der vorliegenden Dissertation untersucht, welche Mikroplastik-Komponenten relevant für aquatische Systeme sind und anschließend mittels Laborstudien erforscht, welche Auswirkungen diese auf die Gesundheit verschiedener Lebensstadien von Fischen haben können.

Das erste Kapitel der Dissertation adressiert als Literatur-Rezension die Frage, welche Mikroplastik-Komponenten häufig in der Umwelt zu finden sind und welche Effekte auf aquatische Organismen durch diese schon beschrieben wurden. Fortschritte in den Detektions- und Analysemethoden von Mikroplastik führten zu der Erkenntnis, dass der überwiegende Anteil an Mikroplastik in der Umwelt aus Fasern und teils irregulär geformten Plastik-Fragmenten besteht. Im Gegensatz dazu wurden die meisten Effekt-Studien mit kommerziell erwerblichen Mikroplastik-Kugeln durchgeführt. Die runden Referenz-Partikel wurden vorwiegend benutzt, weil andere Mikroplastik-Komponenten, vor allem Fasern, zusätzliche Schwierigkeiten im Handling und der Charakterisierung im Labor mit sich bringen. Für Fasern ist dies beispielsweise ihr Potential sich zu verwickeln und zu agglomerieren, weshalb bislang nur sehr wenige Studien zu Effekten von faserartigem Mikroplastik auf aquatische Organismen durchgeführt wurden und dementsprechend das Risiko durch Mikroplastik-Fasern in der Umwelt nahezu unerforscht ist.

In den folgenden Kapiteln der Dissertation wurden daher Methoden zum Umgang mit Mikroplastik-Fasern in Laborstudien entwickelt. Anschließend wurden Labor-Effektstudien mit dem Modell-Organismus Dreistachliger Stichling (*Gasterosteus aculeatus*) und häufig in der Umwelt vorkommenden Textilfasern durchgeführt, um die Auswirkungen von Mikroplastik-Fasern auf die verschiedenen Lebensstadien von Fischen genauer zu untersuchen.

Kapitel II ermittelt mögliche Effekte von Mikroplastik-Fasern im Wasser auf die Befruchtung und Entwicklung früher Lebensstadien von Stichlingen. Mit Hilfe von *in vitro* Befruchtung wurden die Befruchtungsraten von Eiern eines bestimmten Brutpaars mit und

ohne Mikroplastik-Fasern im Wasser bestimmt. Dabei hatte die Anwesenheit von Mikroplastik-Fasern im Wasser keine negativen Auswirkungen auf *in vitro* Befruchtungsraten, Schlupfraten und die Entwicklung früher Lebensstadien von Stichlingen, selbst wenn die Konzentrationen höher sind als gegenwärtig in der Umwelt detektiert wurden.

In den folgenden Kapiteln der Dissertation wird untersucht, ob die direkte Aufnahme von Fasern mit dem Futter die Stichlinge beeinträchtigt. Kapitel III beschreibt dazu zunächst eine Methode, die entwickelt wurde, um Fischfutter mit unterschiedlichen Mengen an Fasern kontrolliert herzustellen. Durch die Verwendung von Ethanol im Produktionsprozess konnte das Verwickeln der Fasern verhindert werden und eine homogene Verteilung der Fasern im Futter gewährleitet werden.

In Kapitel IV wurden die auf diese Weise mit Fasern hergestellten Futterpellets an subadulte Stichlinge verfüttert, um die Folgen der oralen Mikroplastik-Faseraufnahme zu untersuchen. Da Fische in der Umwelt häufig auch natürlichen Fasern ausgesetzt sind, dienten die Aufnahme von Fasern aus natürlichem Material, welche ebenfalls in Versuchsfutter eingebracht wurden, als Vergleich für potenzielle Auswirkungen aufgenommener Fasern. Nach neun Wochen Exposition wurden das Wachstum, die Gonaden-Entwicklung und Immunsystem-Parameter der Versuchsfische analysiert. Es zeigte sich, dass keine der Fasern, die direkt mit der Nahrung aufgenommen wurden, negative Auswirkungen auf Wachstum, Körperkonditions-Parameter, die Gonaden-Entwicklung und die analysierten Immunsystem-Parameter hatten – auch bei deutlich höheren Konzentrationen als in der Natur gegenwärtig vorkommen. Die effiziente Ausscheidung der aufgenommenen Fasern ist vermutlich der maßgebliche Grund, der die Fische vor schädlichen Auswirkungen durch aufgenommenes Mikroplastik schützt.

Insgesamt zeigen die Ergebnisse, dass die häufig in der Umwelt vorkommende Textilfasern Fische verschiedener Lebensstadien im Laborversuch nicht beeinträchtigen. Frühere Laborstudien, die von starken Effekten von Mikroplastik auf aquatische Organismen berichten, wurden hingegen häufig mit extrem hohen Konzentrationen von Mikroplastik sowie Mikroplastik-Komponenten, die selten in der Umwelt vorkommen, durchgeführt. Das Ausbleiben negativer Effekte von Mikroplastik-Fasern auf Fische, wie es in der vorliegenden Dissertation beobachtet wurde, zeigt die Notwendigkeit, die Ergebnisse von Effekt-Studien immer in Bezug zu ihrer Umweltrelevanz zu interpretieren, wenn auf das generelle Risiko von Mikroplastik für die Umwelt gefolgert werden soll. Bei gegenwärtigen Mikroplastik-Konzentrationen in der Umwelt ist das tatsächliche Risiko durch – häufig faserartiges – Mikroplastik in aquatischen Systemen für Fische voraussichtlich geringer als in der frühen Mikroplastikforschung angenommen. Demzufolge sollten zukünftige Einschätzungen zum Risiko von Mikroplastik in aquatischen Systemen nicht auf sämtlichen beschriebenen Auswirkungen basieren, sondern auf der realistischen Einordnung der Effektstudien.

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Abbreviation list

BLE	Federal Office for Agriculture and Food
BMEL	Federal Ministry of Food and Agriculture
D	Descriptor
EU	European Union
FTIR	Fourier Transform Infrared spectroscopy
GES	Good environmental status, according to the Marine Strategy Framework Directive
HK	Head kidney
HKL	Head kidney leucocytes
MSFD	Marine Strategy Framework Directive
PA	Polyamide
PBS	Phosphate buffered saline
PE	Polyethylene
PES	Polyester
PET	Polyethylene terephthalate
POP	Persistent organic pollutant
PP	Polypropylene
PS	Polystyrene
PVA	Polyvinyl alcohol
PVC	Polyvinyl chloride
pyr-GC-MS	Pyrolysis gas chromatography-mass spectroscopy
R-90	90% RPMI-1640 medium diluted with 10% water
RLU	Relative luminescence units
RLUarea	Integrated oxidative burst activity in relative luminescence units
ROS	Reactive oxygen species
WWTP	Wastewater treatment plant

General introduction

Aquatic environments under anthropogenic impacts

Our world is a blue planet since about 71% of the earth's surface is covered with water. Almost all the area belongs to marine systems while ice sheets and freshwater systems together make up 2-3%. The global oceans play a major role in regulating the world's climate and in biogeochemical cycles. Marine and freshwater systems inhabit many species and are thus important sectors of global biodiversity, serve as major food sources, and provide valuable ecosystem services (CBD, 2001). Furthermore, aquatic systems are beneficial for humans in regard to transportation and recreation services. Global aquatic systems provide thus multiple services for humans and should be preserved.

Inhabiting aquatic organisms constantly interact with their surrounding environment in terms of habitat structure, food availability, and other organisms, thereby influencing population dynamics, food webs, and ecosystem functioning. External stressors can lead to disintegration of these natural balances and affect aquatic ecosystems. Humans can cause such stressors, for example the loss of biodiversity was connected to several human activities such as chemical pollution and eutrophication, exploitation, and habitat destruction (CBD, 2001). Anthropogenic driven invasions of exotic species as well as climate change, with rising temperature and ocean acidification, are an additional burden to freshwater and marine ecosystems (CBD, 2001; Halpern et al., 2008).

For centuries it was common sense to dump anything that is not needed by humans anymore into the sea. So-called pollutants are substances or energy introduced into the environment that have undesired effects (Weis, 2015). Known pollutants in aquatic systems are excessive nutrients, metals, petroleum hydrocarbons, pesticides, industrial organic chemicals, pharmaceuticals, litter, nanomaterials, but also, radioactivity, suspended solids, light, and noise (Kennish, 1997; Weis, 2015). The concentrations of pollutants vary on a spatial and temporal scale since factors such as distribution with currents, sedimentation, remobilization from sediments, their availability to aquatic organisms, and the subsequent transfer along the food chain influence their prevalence. Accordingly, effects of pollutants vary in their severity in different regions of the world. So-called persistent pollutants exist almost everywhere in aquatic environments and also within inhabitant species (Andrady, 2015; Jepson & Law, 2016).

A change in attitude towards the reduction of our continuous input of pollutants and its environmental impact started only within the late 20th century. In the European Union (EU), the Marine Strategy Framework Directive (MSFD) was established in 2008 to develop and progressively implement policies and measures that protect the marine environment from

pollution (EC, 2008). The aim is to achieve and maintain the so-called "good environmental status" (GES) that considers the whole ecosystem (ecosystem approach). The good environmental status is achieved when seas and oceans provide a clean, healthy, and productive environment, which is ecologically diverse and dynamic. The risk of introduced pollutants must be assessed, evaluated, and minimized to protect and conserve the marine environment with its resources and ecosystem services it offers to humans. In order to assess the good environmental status, eleven descriptors (D) have been defined that address pollutants, such as contaminants (D8, D9), marine litter (D10), and underwater noise (D11), but also varying aspects of biodiversity (D1, D2, D6), the status of commercially exploited fish and shellfish (D3, D4), eutrophication (D5), and alterations of hydrological conditions (D7) (EC, 2008, 2017).

Plastics became part of the marine litter (D10) problem in the last decades. First reports of plastics that accumulate in some areas of the sea and might threaten the health of marine ecosystems were published in the late 1960s and early 1970s. Yet, research interest rapidly increased only after alarming reports of mid-ocean garbage patches (Moore et al., 2001) and with the recognition of the pervasive nature of plastics and its fragments (Bergmann et al., 2015). In the future, the mismanaged plastic waste is expected to grow with population growth and the consumer preference for plastic products – even in scenarios with higher investment in reduction of plastic waste and in waste management infrastructure (Lebreton & Andrady, 2019). The leakage of plastics into the environment will likely follow a similar trend.

The term "plastics" refers to a diverse group of synthetic polymers, which were invented and fabricated from the late 19th century onwards. Mass production of plastics started in the 1950's and substantially increased in the recent decades to a global production of 367 million tons in 2020 – and is expected to increase even further (Crawford & Quinn, 2016; PlasticsEurope, 2021; Ryan, 2015). Plastics are ideal for a wide range of manufacturing and packaging applications due to their versatile properties, such as the low density, durability, excellent barrier and insulation properties, toughness, and relatively low cost. However, strength and durability are, at the same time, the properties that hamper plastic degradation in the environment and make inappropriately handled plastic waste a pollutant of environmental concern (Crawford & Quinn, 2016; Ryan, 2015). Early research already demonstrated negative impacts on large animals such as marine mammals once they get entangled in plastic nets or ingest plastic debris (Gregory, 2009). This raised concerns about the potential negative impacts of smaller plastic fragments that are available for ingestion by a wide range of organisms. Their ingestion could cause physical injuries or interact with and disturb physiological processes within organisms. While

research started to investigate effects of microplastic reference particles on aquatic organisms, the potential effects of environmentally relevant microplastics is not conclusively understood up to date and will be addressed in the present thesis.

Plastic & microplastics in aquatic environments

Plastic waste in the environment derives from various land-based point and diffuse sources, such as landfills, construction sites, dispersed littering, and sewage water. Furthermore, plastic waste items can origin from ships and other installations at sea (Galgani et al., 2015). In the environment, the discarded or released plastic waste undergoes different disintegration and fragmentation processes, which result in smaller plastic pieces. Thermal degradation, photo-oxidative degradation by light, and mechanical degradation due to ocean currents, waves, as well as collisions and abrasions from rocks and sand result in smaller plastic pieces (Crawford & Quinn, 2016; Jahnke et al., 2017). Plastic particles that are smaller than 5 mm are commonly referred to as microplastics and below 1 µm in size they are called nanoplastics (Crawford & Quinn, 2016). While some plastic particles are already manufactured in small sizes, e.g. for personal care products, cosmetics, and paints ('primary microplastics'), most microplastics derive from weathering and fragmentation of bigger plastics ('secondary microplastics').

The creation of secondary microplastics happens on land, at beaches and shorelines, as well as in the water. Microplastics that are created on land or formed on beaches can enter aquatic systems via waves, wind, rivers, and wastewater and drainage inflows (Figure 1). Due to their small size, microplastics can stay afloat over long distances, become entrapped in sediments for a long time, or are available for ingestion by and interaction with aquatic organisms (Figure 1), (Wong et al., 2020). Though microplastics got into focus of pollution research in the past two decades and knowledge about their occurrence, characterization, bioavailability, and (potential) impact on organisms continuously increases, their overall environmental risk is still not assessed and evaluated.
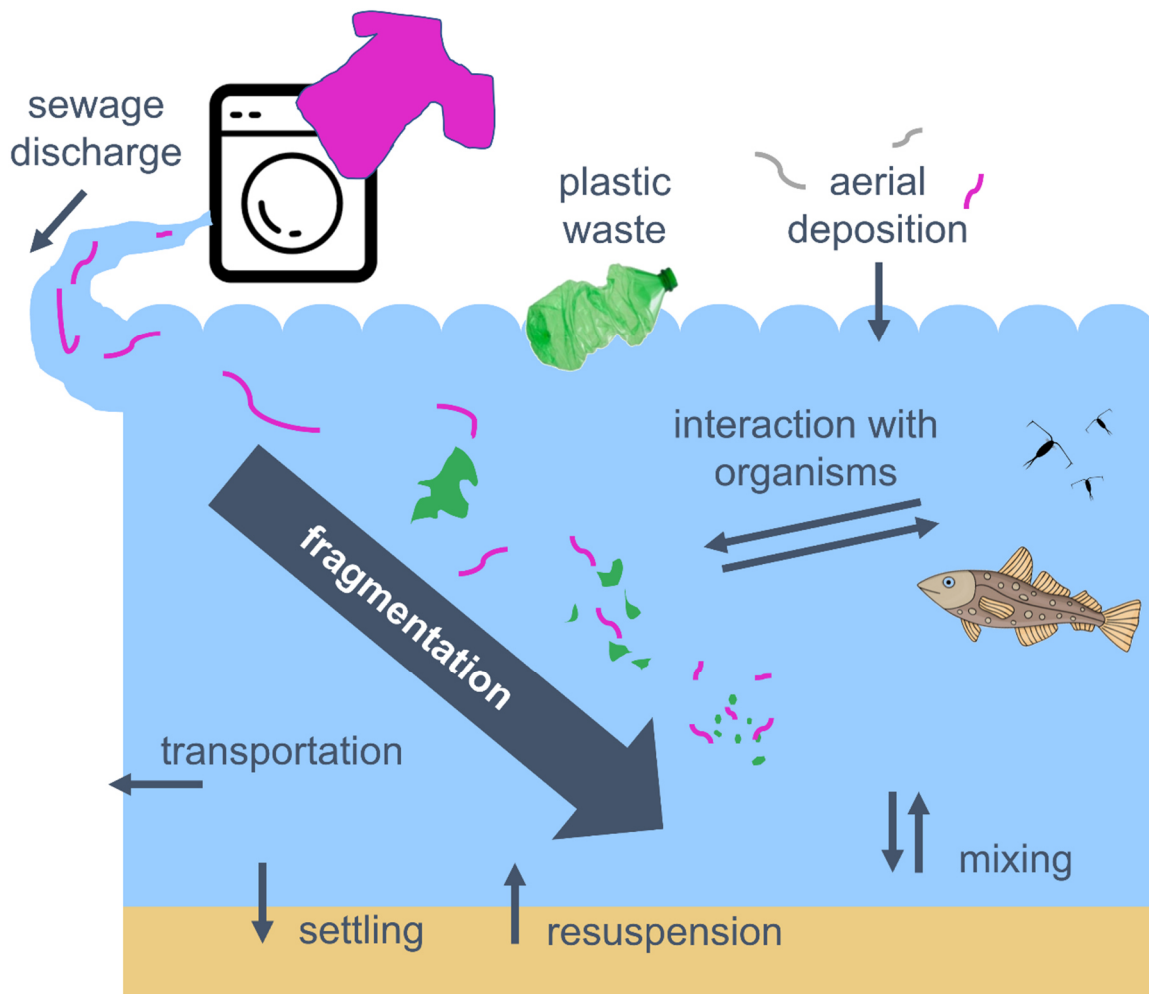
Figure 1. Different paths of plastics into and within aquatic environments.

Occurrence

Microplastics are present from terrestrial ecosystems to freshwater and marine systems, from surface waters to the open ocean, and in sediments (Rillig & Lehmann, 2020; Waldschläger et al., 2020; Wong et al., 2020). Their widespread distribution extends even towards remote regions such as polar oceans including sea ice (La Daana et al., 2020; Ross et al., 2021), the deep sea (Choy et al., 2019; Kane et al., 2020; Peng et al., 2018), and secluded lakes on the Tibetan Plateau in the Himalaya region (Feng et al., 2020). Despite the omnipresence of microplastics, some areas are clearly more polluted than others with highest concentrations of microplastic reported at shorelines, in enclosed seas (e.g. Mediterranean Sea, China Sea, Black Sea), and accumulated in garbage patches in the middle of the big ocean basins (Waldschläger et al., 2020). Furthermore, microplastic concentrations increase with closer distance to coastal and urbanized areas due to their anthropogenic origin (Galgani et al., 2015). Overall, concentrations of microplastics are driven by numerous environmental and anthropogenic factors. Wind, currents, waves, and other environmental factors, such as seasonal aspects (e.g. rain events), can affect

microplastic abundance on beaches and in the water (He et al., 2020; Kim et al., 2015; Kukulka et al., 2012; Zhao et al., 2019). The abundance of microplastics, at the same time, shapes their bioavailability to aquatic organisms in the different water bodies (Zheng et al., 2019).

Overall, high variations in microplastic concentrations with time and space together with different methods used for sampling and analysis hamper the assessment of universal valid microplastic concentrations. The high variety of microplastic concentrations and thus bioavailability in the environment brings about the need for a differentiated assessment of potential risks caused by microplastic presence in the water.

Characterization

The term microplastics refers to a heterogenous mixture of particles (all <5 mm) of various shapes, polymers, colors, and size classes. Overall, most microplastics detected in freshwater und marine samples had an elongated fibrous shape (= fibers, 52%), followed by irregular-shaped fragments (29%), while all other shapes, such as spheres/ beads, films, and foams, make up only a small proportion, as reviewed by Burns & Boxall (2018). Common polymers reported from the water column were polyethylene (PE, 28%), polyethylene terephthalate (PET, 15%), polyamide (PA, 15%), polypropylene (PP, 13%), polystyrene (PS, 5%), polyvinyl chloride (PVC, 2%), and polyvinyl alcohol (PVA, 1%) (Burns & Boxall, 2018).

The composition of microplastics in the different water bodies is determined by its adjacent sources and varies in different regions. Microplastic fibers, for example, can originate from a variety of sources, such as textiles, carpeting, upholstery, or synthetic fishing nets and ropes (Browne et al., 2011; Gago et al., 2018). Fibers that get released during the production, usage, and washing of textiles account for a significant amount of microplastics released into global oceans (Boucher & Friot, 2017). Accordingly, microplastic fibers detected in the sea are typically made of polyester (PES, fibrous form of PET), PA (=nylon) and PP (Gago et al., 2018), which coincides with the main fiber types used in the textile industry (Carr, 2017). The present thesis will focus in particular on fibers as most frequent microplastic shape in aquatic environments.

During the plastic production process, additives are frequently supplemented to the polymeric raw material. The added chemicals can give plastic features like color and transparency, or enhance their performance in terms of resistance to degradation by temperature, light radiation, bacteria and humidity, or mechanical and electrical resistance (Hahladakis et al., 2018). Common additive types are fillers, plasticizers, flame retardants, antioxidants, acid scavengers, light and heat stabilizers, lubricants, pigments, and antistatic agents (Hahladakis et al., 2018). Microplastics in the environment are thus often a complex

mix of polymer material and different chemicals. The incorporated additives can desorb from the microplastics. Whether microplastics might thereby act as a vector for chemical substances that can affect organisms is still under debate.

The variety of features microplastics can possess makes their analysis and characterization challenging. Moreover, characteristics of microplastic items in the environment can change over time due to degradation, fragmentation, and biofouling (Jahnke et al., 2017; Liu et al., 2020). The diverse characteristics of microplastics also complicate their quantification. The quality of quantification and characterization of microplastics in samples depends on the equipment used for sampling, extraction, and analysis, together with the accuracy of used methods. Several authors developed guidelines for analyzing microplastic abundance in water and biota (Hale et al., 2022; Hermsen et al., 2018; Löder & Gerdts, 2015; Primpke et al., 2020). Those include measures such as the use of as fine mesh sizes as possible for capturing and extracting microplastics since small microplastics otherwise slip through the mesh and are not accounted for, which is particularly relevant for elongated fibers that have a small diameter (Ryan et al., 2020). Furthermore, the authors recommend the use of chemical identification via spectroscopic methods to ensure plastic identity, the processing of procedural blanks during all conducted steps, and the need to report all analytical details obtained to make it easier to compare studies even when they were conducted with different methods.

Due to a lack of standardization and use of appropriate methods in (earlier) microplastic research, existing data on environmental microplastic concentrations and characteristics should be interpreted carefully and always in relation to the methods used to obtain the data. In principle, more recent studies can provide a better insight into environmental relevant microplastic components and concentrations.

Environmental risk assessment perspective

Risk assessments are a common tool to evaluate the environmental impact of pollutants. In general, the assessment determines the risk of individual organisms first, and can be extended to population and community levels.

An organism is at risk when its individual growth and fitness, accordingly reproductive success, are reduced. Environmental pollutants can damage individual organisms directly by causing an increase in their mortality rates or by interfering with resource acquisition and uptake processes, and thereby reducing reproduction rates (Walker et al., 2006). On the other hand, organisms might avoid or restrict damage on the cellular or organ level by

the use of detoxification and repair mechanisms (e.g. detoxifying enzymes, DNA repair), which consumes energy that is therefore not available for growth and reproduction (Walker et al., 2006). Adverse impacts of pollutants on individuals can result in slower population growth or even population decline due to the close linkage of responses to pollutants at the different organizational levels (Figure 2). Ecotoxicological risk assessments are thus required to evaluate the overall risk by microplastics in the environment.
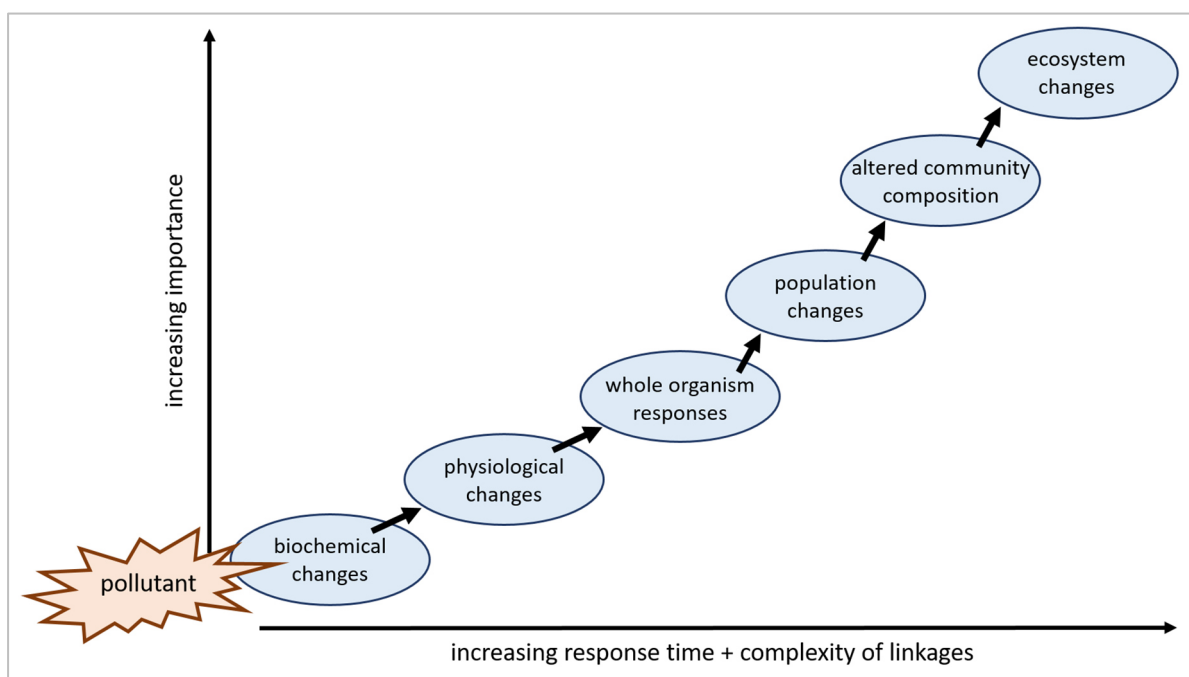

Figure 2. Schematic relationship of linkages between responses from the cellular up to ecosystem levels.

Risk assessments are based on a comparison of two specifications: the toxicity of a compound and the anticipated exposure of an organism to this compound (Walker et al., 2006). In terms of microplastic pollution, data are needed on the current (and potential future) environmental concentrations of microplastics, their bioavailability, and the toxicity concentrations of microplastics on biological endpoints that reduce the individual's fitness and health.

Up to date, most studies that investigated potential affected endpoints and mechanisms of how microplastics could impact aquatic organisms did not interpret their results in the light of environmental relevance of described effect concentrations. The variety of microplastics impacts reported from laboratory exposure studies (in detail in the next section) led to and fostered growing concerns of microplastics in the environment. Whether those concerns are justified in terms of environmental microplastic components and concentrations is often not examined and must be looked at in more detail.

The variety of microplastics in terms of polymer, size, shape, chemical additives, and their concentration in different locations complicates a general risk assessment of

microplastics and results in the necessity of a differentiated analysis of effects of different microplastic types and organisms. Overall, effect studies should pay attention to known (local and global) environmental conditions to assess reasonable scenarios and come to realistic estimations.

Effects of plastic on aquatic organisms

Plastic items are encountered and taken up via different mechanisms by nearly all aquatic organisms from primary producing algae up to top predators, such as seals. They were also detected in species living from and next to aquatic environments, such as seabirds. While bigger organisms are probably more affected by macroplastics than microplastics, smaller organisms are more prone to suffer from microplastics pollution. The microplastic burden in organisms at lower trophic levels is often higher than in animals higher up in the food chain, which contain fewer microplastics per gram body weight (Walkinshaw et al., 2020). Yet, all aquatic organisms are connected in complex food webs and the overall impact of microplastics in aquatic systems is unknown up to date. Within this thesis the focus will be in particular on fish, which serve as food and feed source to humans and have an important role for biodiversity and ecosystem functioning. Fish are often intermediate species in food webs that belong to and link different trophic levels (Pikitch et al., 2014). Plastic ingestion by fish is widespread and global observations demonstrate it is increasing (Savoca et al., 2021).

Microplastic encounter and uptake routes

In general, aquatic organisms show a variety of routes and mechanisms in which they interact intentionally and unintentionally with the microplastics they encounter. Small organisms, such as zooplankton, can get affected by microplastic encounter already due to physical contact when microplastics adhere externally or even damage appendages, which can cause impairment of their locomotion (Cole et al., 2013).

The second relevant interaction between organisms and microplastics is the (oral) uptake of plastic items, which can happen via different mechanisms. While direct uptake from the water column is the most probable for filter feeders and zooplankton (Wright et al., 2013), higher trophic levels have more options. Indiscriminate feeders, such as filter feeding bivalves and some fish species, capture particles, including microplastics, without selection and in proportion to their environmental availability (Walkinshaw et al., 2020). Passive intake happens in fish also during breathing, in particular for microplastic fibers (Li et al., 2021). Laboratory studies revealed that most fibers taken up during breathing flow

out over the gills without getting caught, while some fibers were observed on the gill filaments and some fibers in the mouth cavity were inadvertently swallowed (Bour et al., 2020; Hu et al., 2020; Jabeen et al., 2018; Li et al., 2021).

Discriminate feeders, such as predatory crustaceans and most fish, capture microplastics actively from the water column. This can be intentionally, when plastics are mistaken for food due to their appearance (e.g. size, color) (Ory et al., 2018; Potocka et al., 2019; Talley et al., 2020) or accidentally during foraging (de Sa et al., 2015; Desforges et al., 2015; Li et al., 2021; Roch et al., 2020). When fish recognize ingested hard microplastics as unpalatable particles, they are able to spit them out (Jabeen et al., 2018; Li et al., 2021). Other microplastics are swallowed and passed on to the gastrointestinal system of fish.

Another conceivable way of microplastic ingestion is the indirect ingestion when microplastics are attached to food items or were previously consumed by prey organisms. Though the mechanisms of trophic transfer have been observed in laboratory exposure studies (Bour et al., 2020; Cedervall et al., 2012; Chae et al., 2018), environmental observations and meta-analyses of microplastic occurrence in wild organisms do not support enrichment of microplastics in the food web (= biomagnification) (Gouin, 2020; Walkinshaw et al., 2020).

In a few species other uptake mechanisms than oral ingestion were observed. Microplastic spheres (0.5 – 20 µm) and fibers (mean length of 57 µm) were (after attachment) able to enter the body wall of sea cucumbers through pores in the outer surface during respiration (Mohsen et al., 2022). Yet, oral uptake of microplastics was also observed in sea cucumbers (Mohsen et al., 2020) and seems to be the most relevant uptake mechanism for almost all taxonomic groups.

Microplastics in the lower size range (< 5-10 µm) can transfer from the gastrointestinal tract into cells and tissues, mainly via endocytosis (Browne et al., 2008; Ribeiro et al., 2020; Zeytin et al., 2020). However, transfer into other tissues could not be confirmed for larger microplastic items ($\geq$ 10 µm) in exposure studies feeding microplastic supplemented feed (Kim et al., 2020). Therefore, transfer into body tissues seems to be unlikely for most microplastic size classes.

A global meta-analysis reported that 49% of all wild-caught and analyzed fish had microplastics in their gastrointestinal tract and the average plastic load was 3.5 ± 0.8 plastic pieces per fish (Wootton et al., 2021). Yet, significant variations were observed between studies and in particular between regions. Multiple field studies support that uptake of microplastics by organisms is closely related to the abundance and availability of

microplastics in the environment (Gove et al., 2019; Kumkar et al., 2021; Savoca et al., 2021).

It is assumed that the level of microplastic uptake by fish is driven by several environmental but also biological factors. Habitat preferences of fish might correlate with higher concentration of microplastics in parts of the water column, selective consumption of food conceivably favors the uptake of similar looking microplastics, and microplastic ingestion is likely favored when the size of the fish's mouth is bigger than the ambient microplastic particles. However, no clear relationships of microplastic ingestion with trophic position, feeding strategy or habitat preference are proven up to date (Avio et al., 2020). Some studies support that demersal fish ingest more plastics than pelagic fish (Jabeen et al., 2017; Bimali Koongolla et al., 2020), while others rather support the opposite (Rummel et al., 2016), or report no difference in ingestion of microplastics due to the feeding habitat of fish (Campbell et al., 2017; Lusher et al., 2013). Similarly, some studies show that observed omnivorous fish to take up more microplastics than herbivores and carnivores (Kasamesiri, 2020; Mizraji et al., 2017), whereas a recent meta-analysis reported a significant higher plastic load in detrivorous fish than in carnivorous, omnivorous, or herbivorous species (Wootton et al., 2021). The microplastic-biota interactions seem to be far more complex than currently understood (Scherer et al., 2018) and the range of feeding types in combination with the different degrees of selective feeding complicate the generalization of microplastic uptake patterns.

In general, freshwater fish ingested higher quantities of microplastics than marine and estuarine fish in a global synthesis, which was related to the higher abundance of microplastics in freshwaters than marine environments (Wootton et al., 2021). Moreover, higher microplastic ingestion was reported closer to shore and in urbanized areas (Murphy et al., 2017; Peters & Bratton, 2016; Steer et al., 2017), which indicates the anthropogenic influence on microplastic encounter and uptake by aquatic organisms. Yet, it is currently unknown whether the higher microplastic load reported from fish is associated with any or even more negative effects of ingested microplastics.

Analysis of freshwater fish from the Chicago region, which were preserved in museum collections, revealed that microplastic contamination of fish increased from 1950 onwards (Hou et al., 2021). This coincides with rates of plastic production, population growth, and plastic pollution documented in ecosystems. All microplastics extracted from the historic and contemporary fish, water and sediment samples from that area were fibers (Hou et al., 2021). This correlates with other studies that report dominance of fibers as microplastic morphotype found in aquatic organisms, as well as in surface waters, the water column, and benthic sediments worldwide (Avio et al., 2020; Barrows et al., 2018; Koongolla et al.,

2020; Li et al., 2021; Zheng et al., 2020). Furthermore, the common environmental polymers PP, PE, PA, and PES/ PET are among the most abundant microplastic polymers detected in aquatic organisms (Avio et al., 2020; Bessa et al., 2018; Kumar et al., 2021; Pozo et al., 2019). Overall, the environmental abundance and potential uptake of microplastics by organisms are closely related.

Effects of microplastic encounter and ingestion

There exist several hypotheses how microplastic encounter and uptake could affect organisms. One assumption is that ingested plastic particles, which have no nutritional value, cause starvation because there is not enough capacity for nutritious items anymore or by complete gut blockage. Furthermore, ingested foreign particles might disturb tissues, cells, and physiological processes by their (physical) presence and might even enter cells and tissues when small enough. Another way of disturbance considered for microplastics is chemical toxicity either due to inherent added compounds or potentially by chemical pollutants adsorbed in the environment. Furthermore, it is speculated that microplastics adsorb not only pollutants but also potential pathogens, which can consecutively be transferred to organisms. In the larger context, microplastics are discussed to alter feeding behaviors and even ecosystem functioning.

In general, one factor that matters for almost all conceivable impacts is the actual retention time of microplastics within the body once ingested. Adult gilthead seabream, which were fed a diet with different virgin microplastic fragments, were able to effectively eliminate the microplastics without any accumulation and did not show impacts such as stress induction or altered growth (Jovanović et al., 2018). Yet, characteristics of microplastics, such as size, type, and composition can alter the retention time within organisms (Au et al., 2015; Gray & Weinstein, 2017; Qiao et al., 2019). A meta-analysis of 175 ecotoxicological studies on terrestrial and aquatic organisms revealed that the physicochemical heterogeneity of the used plastic particles influenced the organisms' responses and distinct differences were attributed to polymer type, size, morphology and surface alterations (Gomes et al., 2022). The heterogeneity and complexity of the microplastic pollution problem thus demands a more targeted approach to analyze and evaluate in particular the potential risks of microplastic components that are relevant in the aquatic environment.

Microplastic fibers that are a major component of microplastic pollution, in particular the common polymers, should receive greater attention and relevance in risk assessments of microplastics in the environment. However, fibers were often ignored due to difficulties in sampling, analysis, and experimental handling when investigating environmental

abundance (Avio et al., 2015; Cózar et al., 2014; Kühn et al., 2018) and when conducting effect studies (Burns & Boxall, 2018; Jacob et al., 2020).

Microplastics as potential vectors

If adverse impacts are induced by either the physical properties of the microplastics or the chemicals incorporated, is often debated and can vary depending on the plastic polymer (Zimmermann et al., 2020). In the past, plastics were considered as biochemically inert (Bern, 1990). Yet, incorporated additives can desorb from the polymer. Mere chemicals typically used as additives, such as plasticizer, brominated flame retardants, and the antioxidant bisphenol A, can affect reproduction, energy metabolism, stress-related defense, neurotoxicity, and hepatotoxicity in aquatic organisms (Gunaalan et al., 2020; Liu et al., 2020). When incorporated in plastics, the desorption rates of those substances depend on many factors such as pore sizes in the plastic matrix, the amount and type of additive used, and environmental factors such as salinity and pH (Liu et al., 2020). Yet, exposure studies with irregular shaped PVC particles and marine medaka (*Oryzias melastigma*) embryos revealed that the chemical toxicity of used microplastics seemed to be insignificant, while the physical contribution was the main toxicity mechanism (Xia et al., 2022). In general, short retention times of microplastics in the digestive tract make the risk of adverse effects due to additive leaching negligible for many species (Koelmans et al., 2022; Koelmans et al., 2014).

Combined effects of microplastics and other environmental pollutants are ambivalently discussed. Pollutants such as persistent organic pollutants (POPs) can adsorb to plastic particles (Cormier et al., 2021). In laboratory settings, microplastics spiked with organic contaminants showed deleterious impacts on early life stages of fish while virgin microplastics did not (Le Bihanic et al., 2020). The difference to additive chemicals is, that POPs that adhere to microplastics are already widespread in the environment, while additives associated with plastics are only around since the (mass) production of plastics in the 1950s (Hammer et al., 2012). Though microplastics might be a vector for plastic-associated chemicals, POPs present in the environment are presumably taken up by organisms in substantially larger magnitudes via pathways such as food and water compared to microplastics (Hanslik et al., 2021; Hoellein et al., 2021; Koelmans et al., 2022; Koelmans et al., 2016; Lee et al., 2019).

Reported effects of microplastics

In field conditions, it is difficult to distinguish possible adverse effects on organisms due to exposure to microplastics from those caused by other stressors. Though some studies detected lower body condition along with higher concentration of ingested microplastics in wild fish, it was unclear if higher ingestion of microplastics led to lower body condition or individuals with lower body condition are more prone to microplastic ingestion (Mizraji et al., 2017; Sbrana et al., 2020). In this respect, laboratory studies are conducted to analyze and characterize the potential ecotoxicological risk of microplastics and make predictions for their environmental risks. However, most laboratory studies conducted on microplastic effects so far used spheres, a microplastic type that is not very common in nature. This must be kept in mind when reading the following paragraphs on already known impacts of microplastics, which yet represent mainly laboratory studies conducted with microplastic components that are not prevalent in the environment.

Meta-analyses indicate that effects of exposure to microplastics are highly variable across taxa and vary from negative to neutral even within the same species (Bucci et al., 2020; Burns & Boxall, 2018; Foley et al., 2018; Gomes et al., 2022). Organisms at the base of the food web such as copepods and amphipods often show more severe impacts. Observed adverse effects on zooplankton reach from damage of appendages due to physical contact, to decreased feeding rates and reduced growth and reproduction after ingestion of microplastics, up to higher rates of mortality (Au et al., 2015; Cole et al., 2013, 2019). However, not all exposure studies with microplastics showed adverse impacts. The freshwater crustacean *Daphnia magna*, for example, was not affected by long-term exposure to different concentrations of PE in the water in terms of mortality, reproduction, body length, lipid content, feeding, and immune responses (Jemec Kokalj et al., 2021).

Likewise, neutral and various negative effects were observed in filter-feeding bivalves exposed to microplastic items in the water column (at different concentrations). The microplastic presence led e.g. to alterations in antioxidant capacity, immune system responses, neurotransmitter systems, reproductive function, and filtering activity in different bivalve species, which affected their metabolism, respiration, and growth rate (Gardon et al., 2018; Sussarellu et al., 2016; Teng et al., 2020). Yet, other studies that used only low concentrations of microplastics detected no physiological differences between oysters from the control treatment and oysters exposed to virgin microplastics (Fabra et al., 2021; Revel et al., 2020).

Analysis of 46 fish exposure studies conducted with virgin microplastics revealed that only 32% of analyzed endpoints demonstrated significant adverse effects (Jacob et al.,

2020). Most negative effects were observed for behavioral, sensory, and neuromuscular function indicators such as feeding and nervous system (overall 57% negatively affected) (Jacob et al., 2020). Exposure to microplastic particles caused also some structural alterations in the gills and the digestive tract in fish, which were observed in histological sections (Hu et al., 2020; Jabeen et al., 2018). The accumulation of microplastics in the gastrointestinal system of fish and induced damage in some studies provoked energetic costs that subsequently caused alterations in the metabolism and affect individual fitness. Jacob et al. (2020) outlined that several exposure studies observed adverse effects on digestive enzymes, lipid metabolism, and oxidative stress, while mortality, blood components, sex hormones, and the detoxification system were rarely affected. In addition, a few studies detected alterations in the microbiome of exposed fish, which were associated with disorders in the metabolism, immune system, intestinal permeability changes, and oxidative stress (Jin et al., 2018; Qiao et al., 2019b). Overall, the inherent variability associated with the physiological and behavioral traits of organisms complicates the potential to mechanistically characterize the effects of microplastics on fish.

Significant adverse effects on the endpoint growth were reported mainly in larval and juvenile life-stages (Jacob et al., 2020). Accordingly, significant adverse effects of exposure to microplastics on consumption and feeding were reported for larval and juvenile fish but not for adults in another meta-analysis based on 43 studies (Foley et al., 2018). Those observations suggest a higher vulnerability of younger life stages of fish towards microplastic encounter than for adults. Yet, the low number of exposure studies conducted previously with early life stages makes an overall evaluation regarding the risk of microplastics to early life stages difficult.

Overall, the majority of exposure studies conducted so far used unrealistically high concentrations of microplastics and often microplastic spheres made of polystyrene, which are more easily manageable in the laboratory but not the shape and polymer common in the environment (Burns & Boxall, 2018; Phuong et al., 2016; Rozman & Kalčikova, 2021). The reported negative effects on aquatic organisms reinforced the concerns of microplastics in the environment regardless of the environmental relevance of conducted experiments. Toxicity in conducted exposure studies typically occurred at concentrations that exceeded those observed in the natural environment by several orders of magnitude (Burns & Boxall, 2018). While microplastic concentrations vary in nature in different regions and it is thus reasonable to test a range of concentrations when investigating toxicity effects, the tested concentrations should be somehow environmentally relevant and realistic, and not manifold orders of magnitude higher.

Due to the differences in effects of the different microplastic characteristics (e.g. type, size, morphology) (Gomes et al., 2022), the main research focus should be placed on potential effects of commonly occurring microplastics. In the present thesis, biological endpoints that were affected in fish in previous exposure studies – conducted with mainly spherical microplastic components – were analyzed in exposure studies with common fibrous microplastics.

Biological endpoints and biomarkers used in pollution research with fish

Mortality and growth are important biological endpoints that are investigated for risk assessments of exposures to pollutants as they reflect the whole organism level. Yet, changes in the condition or health of an individual are reflected by biological parameters on lower levels that are termed 'biomarkers' in toxicology (Chambers et al., 2002). Biomarkers can be biochemical, physiological, histological, morphological, and behavioral measurements (Walker et al., 2006). The analyses of biomarkers are valuable since they provide information about the effect mechanisms of pollutants, which can be important with regard to potential remediation strategies.

Biomarkers can be rather unspecific (e.g. growth performance, oxidative stress) or more specific (e.g. vitellogenin levels demonstrating endocrine disruption) (Walker et al., 2006). Both, specific and non-specific biomarkers are of value in risk assessments and are frequently used in microplastic exposure studies. Below, biological endpoints investigated within the present thesis are outlined.

Mortality and growth performance

At the extreme, unfavorable environmental conditions can be so hostile that fish are not able to maintain their metabolic and structural integrity and die (Wootton, 1984). The lethal level marks the border of the zone of tolerance towards an environmental factor such as a pollutant, at which metabolic processes are unable to compensate fully for the breakdown in the integrity of the fish (Wootton, 1984). Mortality can occur after a short exposure, as tested with acute toxicity tests, and after a time interval needed to cause the lethal effect (examined in chronic toxicity tests) (Fry, 1971).

Pollutants might not be lethal per se, but can require an additional metabolic demand for repair reactions that maintain constant internal conditions (Fry, 1971). The additional metabolic demand reduces the energy that can be partitioned off to other components such as growth, activity, and reproduction. As fish grow relatively rapid within the first few

months of their life (Wootton, 1984), growth performance is often used as a read-out in exposure studies conducted with fish, in particular for young and subadult life stages. Growth is the change in size of a fish and can be measured in length, in weight, or in total energy content of the fish (Wootton, 1984). Food consumption and concomitant energy assimilation leads to growth of fish, which is represented partly by an increase in body dimensions, partly by an increase in energy reserves stored in their body, and partly by an increase in the size of the gonads (Wootton, 1984). Within the present thesis, growth rates and body condition parameters were analyzed, such as the condition index and organosomatic indices (hepatosomatic index and gonadosomatic index), which reflect growth performance and indicate the physiological health status of fish.

Immune system of fish

When organisms experience stress, a number of responses are induced involving all three regulatory systems: the neural, the endocrine, and the immune system (Tort, 2011). The immune system of fish is known to be highly sensitive, and it can therefore serve as an early indicator of responses to environmental stressors (Tort, 2011). Acute stressors often stimulate an enhanced innate immune response, while chronic stressors enhance the chance of an infection due to suppressive effects on the immune system (Tort, 2011). Stimulation of the immune system is caused by exogenous and endogenous disturbances, such as microorganisms, toxic pollutants, or malignant cells (Biller & Takahashi, 2018) and might also happen when microplastics are encountered or ingested.

The immune system of fish consists of a set of cellular and humoral components, which defend the body against foreign materials. Two defense systems, the innate and the acquired immune system, counteract invaders and induce defensive processes (Biller & Takahashi, 2018). The innate immune system forms the first defense barrier, which acts quickly and continuously. It consists of all protective components present before the pathogen invasion, such as the skin barrier (physical barrier), an antimicrobial enzyme system (humoral defense), and nonspecific mediators, such as interferon, interleukins and organic defense cells (cell-mediated defense). Defense cells, such as granulocytes, monocytes, macrophages, and natural killer cells produce highly reactive oxygen species (ROS), which contribute to the destruction of microorganisms by unspecific attacks to their membranes (Biller & Takahashi, 2018). The specific or acquired immune response is triggered when receptors in the membrane of immunocompetent cells (T lymphocytes and B lymphocytes) detect invading agents. Activated cells will then stimulate an increase of circulating antibodies specific to the according invaders, and promote the immune memory (Biller & Takahashi, 2018).

Within the frame of this thesis, non-specific cellular immune parameters in the head kidneys of fish were analyzed. The head kidneys are the major lymphatic organ of fish, the site of leucocyte proliferation, and play a vital role in immune responses (Bjørgen & Koppang, 2021).

Biological endpoints used in early life stages of fish

Diverse endpoints are used when pollutant effects on ontogenesis and growth are studied in early life stages of fish. Commonly, toxicity potentials are assessed by egg and embryo mortality and hatching success in embryos, while growth performance and malformations are used for larvae (Hallare et al., 2005; Ribeiro et al., 2020; Zhang et al., 2020). Another frequently investigated parameter is the alteration in the heart rate of embryos. Disturbances (mainly decreases) result from impaired oxygen exchange, which leads to a reduced oxygen and energy supply to the tissues and ultimately in delayed or disturbed embryonic development. In this thesis, fertilization and hatching success, the heart rate of embryos, the growth and potential malformations of early life stages, and mortality of embryos and larvae were observed.

Three-spined sticklebacks as model organism

The three-spined stickleback (*Gasterosteus aculeatus*) is a small teleost fish of the family Gasterosteidae with an exceptionally wide geographic distribution (Figure 3). Sticklebacks inhabit marine, estuarine, and freshwater environments in wide areas around North America, the North-Eastern Asia region, and Europe – including the North Sea, river mouths and rivers further inland (Paepke, 1983). Their widespread distribution, ease in being maintained under laboratory conditions, no commercial value, and well-documented biology, made sticklebacks one of the best-studied species of fish (Ostlund-Nilsson, 2006). They are frequently used as vertebrate model organism for endocrine disrupting effects (Katsiadaki et al., 2010), behavior (Gill & Hart, 1994; Norton & Gutierrez, 2019), host-parasite interactions (Barber & Scharsack, 2010), as well as ecological and evolutionary studies (Cresko et al., 2007). Yet, sticklebacks are also a useful sentinel species in water quality assessments and environmental pollutant studies (Katsiadaki, 2006; Katsiadaki et al., 2007; Sanchez et al., 2005). Sticklebacks can be used in freshwater and marine exposure studies. Since plastic is a pervasive pollutant in freshwaters, as well as brackish and marine waters worldwide, sticklebacks pose an ideal model species to assess and compare potential effects of microplastics on a global scale.

As dietary generalists (omnivores), sticklebacks feed to satiation and possess a well-developed gastrointestinal tract (Bolnick et al., 2014; Gill & Hart, 1998). The gastrointestinal tract consists of the buccal cavity, esophagus, stomach, intestine, and rectum, where processed food is egested as feces. Differentiated morphological features include the pyloric sphincter at the posterior part of the stomach to control release of food matter into the intestine and intestinal folds to aid digestion and absorption of nutrients (Wootton, 1984). Their omnivorous feeding habit to satiation might make sticklebacks more prone to microplastic ingestion, and their developed gastrointestinal tract could lead to higher retention time of ingested plastic items.
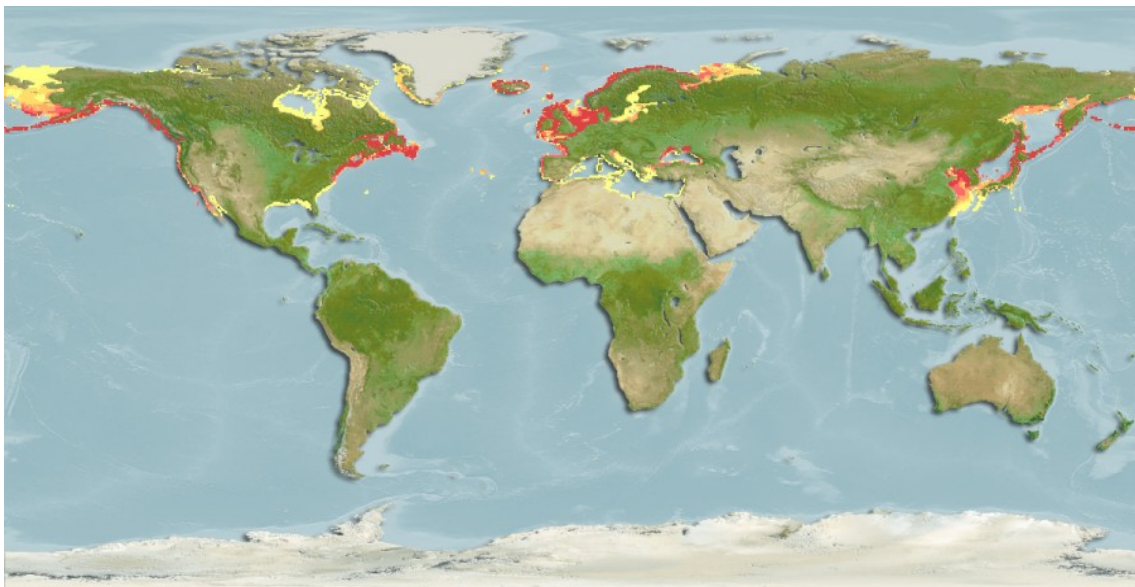

Figure 3. Computer generated native distribution map for three-spined sticklebacks (*Gasterosteus aculeatus*). Retrieved from https://www.aquamaps.org, accessed at 10th of August 2021 (CC-BY-NC) (AquaMaps, 2019).

Aim and outline of the thesis

The present thesis was completed within the frame of the project "PlasM" (**Plas**tic litter and **M**arine fish) that addressed the plastic litter problem in the North and Baltic Seas region, the occurrence of microplastics in fish, and the potentially negative effects of (micro-)plastics on fish health. The investigations were related to the demand of an environmental risk assessment for plastic pollution, which is requested by the European Marine Strategy Framework Directive.

The present thesis addresses the effects of microplastics on fish health. The focus was set on microplastic fibers as one of the most common microplastic components in the environment. Despite their ubiquity, microplastic fibers were rarely investigated in previous impact studies conducted with microplastics and aquatic organisms. Their (potential) effects were thus an undefined variable for environmental risk assessments. The following chapters analyze and assess the potential risk of microplastic fibers on fish.

First, a literature review was conducted to identify fibers as microplastic shape of high environmental relevance. Subsequently, laboratory exposure studies were carried out to investigate potential impacts of microplastic fibers on different life stages of fish. Thereby, methods were developed to work with microplastic fibers in experimental settings since their handling poses additional challenges compared to the use of other microplastic shapes, such as high potential to entangle and difficulties to keep them homogeneously spread in experimental settings. The thesis results are structured according to the following research questions:

Chapter I: Environmental relevance of microplastic fibers and their potential effects on aquatic organisms

Rising awareness of microplastic accumulation in the environment led to an exponential growth of studies published that analyze the occurrence, characteristics, and fate of microplastics within the last decade (Sorensen & Jovanović, 2021; Zhou et al., 2021). Initially, researchers focused on microplastic spheres and fragments in their approaches when developing analytical methods and exposure systems to investigate the fate of microplastics. Yet, those are not the only microplastics that occur in the environment and do not reflect common environmental conditions. Microplastic fibers were particularly challenging in sampling, analyses, and handling, and thus often neglected or deliberately omitted. With advancing methods and knowledge on microplastics, the discrepancy of the prevalence of microplastic fibers in the environment and the lack of knowledge about their occurrence, characteristics, and environmental impact came into focus in the second half of the last decade (Gago et al., 2018; Liu et al., 2019; Mishra et al., 2019, 2020).

Yet, most studies focused on the environmental abundance, sources, and transport mechanisms of microplastic fibers rather than their bioavailability and potential impact on aquatic organisms. Effect studies were still mostly conducted with microplastic spheres and fragments, and not fibers (Jacob et al., 2020; Phuong et al., 2016; Rozman & Kalčikova, 2021). The negligence of fibers hampers an overall risk assessment of microplastics in general due to the large share of fibers in the environment. Therefore, Chapter I aimed at pointing out the relevance of microplastic fibers in the environment, summarizing already existing knowledge, identifying knowledge gaps as well as missing links of the environmental fate of microplastic fibers, whereby the focus was laid on aquatic organisms.

Chapter II: Potential effects of microplastic fibers in the water on early life stages of fish

Early life stages of fish were exposed to microplastic fibers in laboratory studies to gain knowledge on the potential risk of fibers as environmental relevant microplastic component in the water on sensitive organisms. Early life stages of fish and invertebrates are generally more vulnerable to many toxicants than adult life stages (Cormier et al., 2021; Mohammed, 2013). Yet, effect studies of microplastics on fish were mostly conducted with adults and juveniles instead of early life stages (Jacob et al., 2020). A few studies reported that microplastics that adhered to the surface of fish eggs can impair oxygen exchange, which presumably caused observed delays and distortions in the development of early life stages (Chen et al., 2020; Cheng et al., 2020; Duan et al., 2020). However, no studies investigated potential effects of microplastic presence in the water even before fertilization of fish eggs and fibrous microplastics were rarely used in embryo exposure studies. Microplastic fibers that have a large surface area compared to fragments and spheres might conceivably block the micropyle of fish eggs and thereby prevent fertilization when present in the water. Furthermore, microplastic fibers might attach to eggshells and hinder the embryonic development. Finally, hatched larvae might be susceptible to microplastic fibers in their environment. As a consequence, an exposure study was conducted to investigate whether the presence of microplastic fibers in the water column influences fertilization success and early development of three-spined sticklebacks.

Chapter III: Development of fish feed supplemented with microplastic fibers

In the environment fish encounter and frequently ingest microplastic fibers (Bessa et al., 2018; Jabeen et al., 2017; Koongolla et al., 2020; Sun et al., 2019). Experimental laboratory studies facilitate to detect and quantify potential effects of microplastic ingestion. Previous short-term effect studies that exposed fish with fibers via the water column and via the feed indicated negative effects of fibers on fish health after oral uptake (Jabeen et al., 2018; Qiao et al., 2019). Potential health impacts due to direct ingestion of microplastic fibers can

be studied in detail when microplastics are supplied within the diet of fish. Yet, the only study that provided fish with fibers via pellets, inserted each fiber (0.7-5 mm in length) manually in the pellets (Jabeen et al., 2018), which is not manageable for smaller fiber size classes. Methods to handle microplastic fibers in such a way to provide fish with feed that is supplemented with microplastic fibers in small sizes and produce the feed more easily in higher amounts were missing. To this end, a method for producing fish feeds that contain homogeneously distributed microplastic fibers for small experimental fish was developed.

Chapter IV: Potential effects of ingestion of microplastic fibers by sub-adult fish

The few studies that investigated potential effects of ingestion of in particular fibrous microplastics by fish addressed mostly mature adult life stages (Hu et al., 2020; Jabeen et al., 2018; Qiao et al., 2019; Zhao et al., 2021). In addition, most effect studies exposed fish to fibers via the water column, while older life stages of fish frequently ingest microplastic fibers mistaken for food or with their prey. If direct ingestion of microplastic fibers can affect sexually immature subadult fish in terms of growth, body condition, and gonadal development, was not addressed so far. Ingestion of microplastic fibers might lead to false satiation, intestinal damage, and disturbed gut microbiota, which can subsequently affect body condition parameters, maturation, and the health of fish.

In addition, the aspect of regular encounter and ingestion of natural particles and fibers in the wild was neglected in most exposure studies conducted with microplastics up to date (Halstead et al., 2018; Mateos-Cárdenas et al., 2021; Ogonowski et al., 2018) but could change the perspective of ingestion of unpalatable items by organisms. Therefore, Chapter IV presents an exposure experiment conducted with sticklebacks that were provided microplastic fibers via their diet for nine weeks to analyze potential effects on fish growth, maturation, and health. The fiber size class was chosen to emulate textile fibers released during washing. Furthermore, cotton fibers were included as additional treatments of natural origin. Biological endpoints analyzed were growth performance, body condition parameters, gonad development, and immune parameters of the fish.

Chapter I. Microplastic fibers – underestimated threat to aquatic organisms?

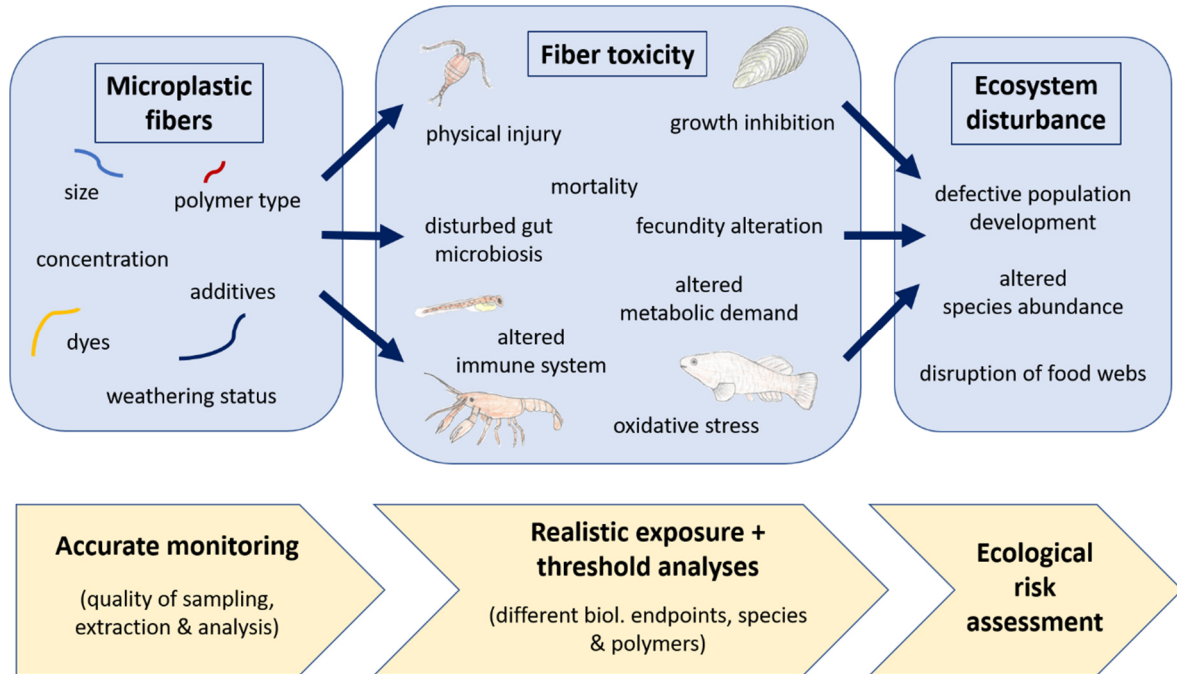Anja Rebelein*, Ivo Int-Veen, Ulrike Kammann, Jörn Peter Scharsack

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Review article

Graphical abstract

Review

Microplastic fibers — Underestimated threat to aquatic organisms?

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HIGHLIGHTS

- Microplastic fibers are an underestimated threat to aquatic environments.
- Microplastic fiber sampling and analysis requires specific methods.
- Microplastic fibers are the most frequent microplastic type ingested.
- Microplastic fibers affect especially lower trophic levels.
- Organisms affected by microplastic fibers can cause ecosystem disturbances.

GRAPHICAL ABSTRACT
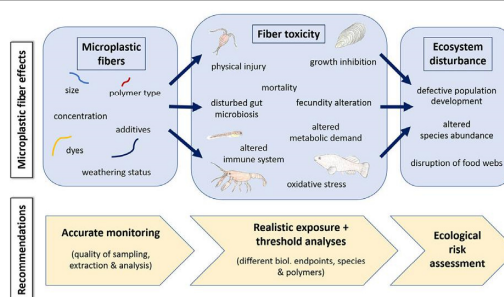
ABSTRACT

Awareness of microplastic pollution in aquatic environments increased strongly during the last decade. Environmental monitoring studies detected microplastic items in every tested water body and found them in various aquatic organisms. Yet, many studies conducted so far, refer to microplastic particles and spheres but not fibers. Microplastic fibers are often not considered due to methodological issues and high contamination risk during sampling and analysis. Only a few of the microplastic exposure studies with aquatic organisms were conducted with microplastic fibers. Recent effect studies demonstrated several negative impacts of microplastic fibers on aquatic organisms, which include tissue damage, reduced growth, and body condition and even mortality. Such negative effects were predominantly observed in taxa at the basis of the food chain. Higher taxa were less heavily affected in direct exposure experiments, but they presumably suffer from negative effects on organisms at lower food chain levels in the wild. Consequently, ongoing and future pollution with microplastic fibers may disturb the functioning of aquatic ecosystems. The present review outlines the current state of knowledge on microplastic fiber abundance in nature, bioavailability, and impacts on aquatic animals. Based on these findings, we recommend inclusion of microplastic fibers in prospective monitoring studies, discuss appropriate methods, and propose to conduct exposure studies with – as well as risk assessments of – these underestimated pollutants.

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1. Introduction

Since the 1950's, pollution of the environment with plastic is an increasing ecological problem due to substantial industrial production and usage of plastic materials (Barnes et al., 2009). Over the last decades, plastic production increased exponentially and reached a global plastic production of 359 million tons in 2018 (Plastics.Europe, 2019). Plastic items are shed into the environment from various sources, by general littering, illegal dumping, and losses e.g. of waste from landfill sites, recycling facilities, plastic mulching in agriculture, or tire and textile industry (Duis and Coors, 2016; Mishra et al., 2019). Over time, plastic items fragment into microplastic particles (Andrady, 2015) and given their durability, microplastics persist and accumulate in the environment (Barnes et al., 2009). In terrestrial ecosystems, recent research showed that coupled photochemical and biochemical processes, facilitate the breakdown of plastics (Ward et al., 2019). Furthermore, biodegradation of plastics was observed in combination with mechanical degradation (Han et al., 2020). Yet, photo- and temperature induced degradation of plastics is slower in the marine environment (Ganesh Kumar et al., 2020) and so far, exact timescales of plastic degradation and fate of degradation products in the oceans are not well understood (Jacquin et al., 2019; Ward et al., 2019).

The term microplastic commonly refers to plastic items <5 mm (Andrady, 2015; Barnes et al., 2009; Cole et al., 2011). Primary microplastics are manufactured as particles in microscopic size, while secondary microplastics derive from larger plastic items via physical or chemical disintegration (Auta et al., 2017; Cole et al., 2011). UV radiation, fluctuating temperatures, abrasion on beaches, but also salinity and physical stress such as wave action are factors influencing weathering of plastic items, which facilitates fragmentation into microplastic particles (Cole et al., 2011; Jahnke et al., 2017). Once submerged in water, lower temperatures and reduced UV light slow down the disintegration processes and microplastics persist in the aquatic environment (Jacob et al., 2020; Kershaw and Rochman, 2015).

Weathering and breakdown of plastic material into (secondary) microplastics happens mainly in terrestrial and beach environments (Andrady, 2011), from which secondary, as well as primary, microplastics are carried into aquatic systems. Studies report that terrestrial sources of plastic account for up to 80% of the debris entering the ocean (Barnes et al., 2009; Jambeck et al., 2015). Principal pathways are industrial and domestic sewage water, including wash water and laundry effluent. Wastewater treatment plants (WWTP) remove microplastics with varying efficiencies in different countries (Hongprasith et al., 2020; Mintenig et al., 2014), but can reach up to 99% removal of the particle load before the water is discharged (Talvitie, 2018; Waldschläger et al., 2020). However, WWTP are not able to retain all microplastic items and leftovers are consequently shed into the aquatic environment (Talvitie et al., 2015). Furthermore, on a global scale only about 20% of the industrial and municipal wastewater is cleaned before it is discharged in the environment (WWAP, 2018).

The microplastic input into rivers and seas is determined by population densities and the amounts of incorrectly handled waste (Jambeck et al., 2015; Lebreton et al., 2018; Waldschläger et al., 2020). Overall, marine microplastics pollution from inland sources via rivers accounts for about 15–20% of the total global input into oceans (Jambeck et al., 2015; Lebreton et al., 2017; Schmidt et al., 2017). According to mathematical modeling, ten top-ranked rivers (with respect to microplastic catchment) contribute 88–95% of the global load of mismanaged plastic waste entering the sea via rivers (Schmidt et al., 2017).

To a lesser extent, microplastics are transported via the atmosphere (Gasperi et al., 2018) and introduced to water systems at contact surfaces and by precipitation (Dris et al., 2016).

Waldschläger et al. (2020) reviewed the information on microplastic concentrations in different water bodies on earth. Many studies reported low (0.1–10 items/m^3) or even lower (<0.1 items/m^3) concentrations of microplastic items (Waldschläger et al., 2020). High concentrations of microplastic (>10 up to 10^4 items/m^3) were observed in the garbage patches in the middle of the big ocean basins (Cózar et al., 2014) and also in the North Sea, the Black Sea, the South China Sea, and in the Mediterranean Sea (Waldschläger et al., 2020), (Table 1). Overall, microplastics are omnipresent in aquatic environments, but vary spatially in their concentration (Table 1).

Microplastics are composed of a wide range of particle sizes (<5 mm), shapes, colors, and polymers due to their difference in sources and production. Many plastic and microplastic items contain a number of substances, termed additives, for enhancing polymer properties and prolonging their life (Hahladakis et al., 2018). Additives, such as plasticizers, stabilizers, flame retardants, pigments, antioxidants and antimicrobials, can leach from plastic material and are substances that show the potential to pose risks to the environment (Gunaalan et al., 2020; Teuten et al., 2009). Accordingly, persistent microplastic items and any associated additives in aquatic systems are environmental pollutants and require a comprehensive risk assessment (Burns and Boxall, 2018; Gunaalan et al., 2020).

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Table 1

Microplastic concentrations in different marine and limnetic habitats on a global scale. Values are given in microplastic items (MPI) per m³, as range between min – max numbers or as mean (± standard deviation). Proportions of microplastic fibers (MPF) and microplastic particles (MPP), and sampling equipment and sample type are specified (n.r. – not reported).

Area	Range (mean) [MPI/m³]	Percent MPF of total MPI [%]	Percent MPP of total MPI [%]	Mesh/net size, Sample type	Reference
Open & coastal ocean					
Atlantic Ocean	Range of means (0.01–2.4)			Divers, Surface waters	Reviewed by Waldschläger et al. (2020)
Northeast Atlantic Ocean	0–22.5	95.9	3.7	Pump (250 μm), Surface waters down to 3 m	Lusher et al. (2014)
Atlantic Ocean transect	13–501	40	n.r.	Pump (10 μm), Surface waters down to 3 m	Enders et al. (2015)
Pacific Ocean	Range of means (0.017–7.25)			Divers, Surface waters	Reviewed by Waldschläger et al. (2020)
Northeast Pacific Ocean	8–9180 (2080)	>70	n.r.	Pump (62.5 μm), Surface waters at 4.5 m	Desforges et al. (2014)
North Pacific, offshore	0.43–2.23	n.r.	n.r.	Manta net (333 μm), Surface waters	Moore et al. (2005)
North Pacific, inshore	5–7.25	n.r.	n.r.	Manta net (333 μm), Surface waters	Moore et al. (2005)
South Africa	258–1215	>90	n.r.	Neuston net (80 μm), Surface waters	Nel and Froneman (2015)
Mariana Trench, deep sea	2060–13,510	n.r.	n.r.	Seawater filtered through 0.3 μm filter, Depth 2673–10,903 m	Peng et al. (2018)
Artic water + Arctic sea ice	0–18 2000–17,000	89 79	n.r. 21	Surface water (250 μm) + Ice cores	La Daana et al. (2020)
Enclosed ocean areas					
Jade system, North Sea	0–650,000 (88000)	57.9	42.1	Surface water filtered with 40 μm	Dubaish and Liebezeit (2013)
Baltic Sea	0–0.8 0–6.8	*Fibers most common (plastic + natural)*	n.r.	Manta net (330 μm) + Pump (100 μm), Surface waters	Setala et al. (2016)
Black Sea	(1100 ± 900)	49.4	20	Neuston net (200 μm) Surface waters	Aytan et al. (2016)
Qatar's Exclusive Economic Zone	0–3 (0.7)	23.3	76.7	Tow net (120 μm), Surface waters	Castillo et al. (2016)
Gulf of Mexico	4.8–18.4	15.8	77.2	335 μm, Surface waters down to 15 m	Di Mauro et al. (2017)
Estuaries					
Changjiang Estuary, China	(157 ± 76)	77.8–91.6	0–6	Pump (60 μm), Surface waters	Zhao et al. (2019)
East China Sea	(113 ± 51)	83.4–91.5	0–10.2		
Estuaries worldwide	Range of means (0.1–100)			Mesh sizes 330 or 333 μm	Reviewed by Zhao et al. (2019)
Freshwater					
Neckar, Germany	8–59.3	0–12.5	82–100	Manta trawl (300 μm), Surface waters	Heß et al. (2018)
Rhine, Germany	2.9–214.2	0–9.9 *(one site 85.1)*	63.8–99.4		
Donau, Germany	9.8–150.8	0–3.4	93–100		
Rhine River	0.1–141.6	2.5	95.9	Manta net (300 μm), River surface samples	Mani et al. (2015); Triebskorn et al. (2019)
Inland freshwaters, China (rivers & lakes)	1660–8925	52.9–95.6	n.r.	Pump (50 μm), Surface waters	Reviewed by Wang et al. (2017b)
Three Georges Reservoir, China	1597–12,611 (4703)	28.6–90.5	2.4–72.2	Pump (48 μm), Surface waters	Di and Wang (2018)
Saigon River, Vietnam	10–519,000	n.r. *(conc. 172,000–519,000 MPI/m³)*	n.r. *(conc. 10–223 MPI/m³)*	Surface water filtered onto 10 μm + manta trawl (300 μm)	Lahens et al. (2018)
Great Lakes, United States	0.05–32	71 *(macro- & microplastics)*	17 *(macro- & microplastics)*	Neuston net (333 μm), Surface waters	Baldwin et al. (2016)
Karst water system, Illinois, USA	0–15,200	100	0	Water samples filtered with 0.45 μm	Panno et al. (2019)

For microplastic items in the environment, variation in particle shape coincides with their distribution characteristics (e.g. sinking velocity) (Khatmullina and Isachenko, 2017), and thus bioavailability in the aquatic environment. The two most common microplastic shapes in the aquatic environment are fragments, further-on referred to as "microplastic particles", and fibrous microplastics, termed "microplastic fibers" (Barrows et al., 2018).

The actual contamination of aquatic environments with microplastic fibers is suspected to be much higher than that with microplastic particles (Gago et al., 2018a; Woods et al., 2018). Despite their dominance in abundance in the aquatic environments, microplastic fibers received only little attention so far. Aspects of their bioavailability and potential impacts to aquatic environments and organisms are not well understood yet. Given their elongate shape, fibers have the potential to

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entangle with appendages, gill filaments, and within the gastrointestinal system of organisms, which may harm the organisms directly once attached or result in negative physiological effects. An open question is whether microplastic fibers affect organisms similar to microplastic particles or induce even more severe effects.

With the present review, we will first outline that the majority of microplastic items in aquatic ecosystems are fibers and that these fibers were often neglected or underestimated in environmental studies. We will then present current knowledge of bioavailability, uptake into organisms and biological effects of microplastic fibers to aquatic organisms. Based on the current knowledge and exposure experiments executed so far, we will indicate potential risks caused by microplastic fibers for organism health and environmental condition, and finally suggest seminal research directions.

2. Environmental prevalence of microplastic fibers

2.1. Sources of microplastic fibers

A large number of materials in our daily life (e.g. furniture, textiles etc.) are made of synthetic and natural fibers (Gago et al., 2018a). Abrasion and release of fibers from synthetic fabrics is a major contributor to microplastic pollution. Shedding of >1900 microplastic fibers from washing of an individual polyester garment resulted in >100 fibers per liter effluent water (Browne et al., 2011). In comparison, polyester-cotton-blends loose substantially less fibers compared to pure acrylic or polyester fabrics (Napper and Thompson, 2016).

Polyester, the fiber form of polyethylene terephthalate, is widespread due to its durability, excellent physical properties, and unique wear advantages (Carr, 2017). Polyester is predominantly used in fabrics for apparel and other finished textile goods, and sales accounted for almost 50% of the global fiber market (Carr, 2017), (Fig. 1). Other synthetic textile fibers are polylactide, nylon (polyamide), rayon (semi-synthetic), acrylic, and polypropylene. Overall, 60% synthetic fibers, 30% cotton, and 10% other non-synthetic fibers, such as animal wool, are used for textile fiber production (Carr, 2017).

Of all primary microplastic items released into global oceans, the two biggest sources are microplastic fibers derived from laundering (semi-) synthetic textiles (35%) and tire erosion particles (28%) (Boucher and Friot, 2017). Total city dust (fibers and particles) is estimated to account for up to 24%, but the contributing groups were not calculated separately (Boucher and Friot, 2017). Microplastic fibers are shed to the environment during the production process, when laundering the end products, or due to disintegration of textiles and non-laundering fabrics (e.g. flags, sails, furniture, carpets, or mattresses). Other sources are household and office dust, and abrasions of insulating materials from construction sites (Mishra et al., 2019; Sundt et al., 2014; Waldschläger et al., 2020).

2.2. Abundance of microplastic fibers in aquatic environments

Microplastic fibers are present in every marine habitat around the world (Gago et al., 2018a), (Table 1). Absolute concentrations of microplastic fibers recorded depend on the region, the sampling equipment and strategy, and analytical methods used (see following section on methodological issues). Accordingly, relative proportion of microplastic fibers reported depend on the sampling of 'total' microplastic items in the environment and must be interpreted with caution. Reported relative proportions of microplastic fibers range from 2.5 to 95.9% of all microplastic items in the water column
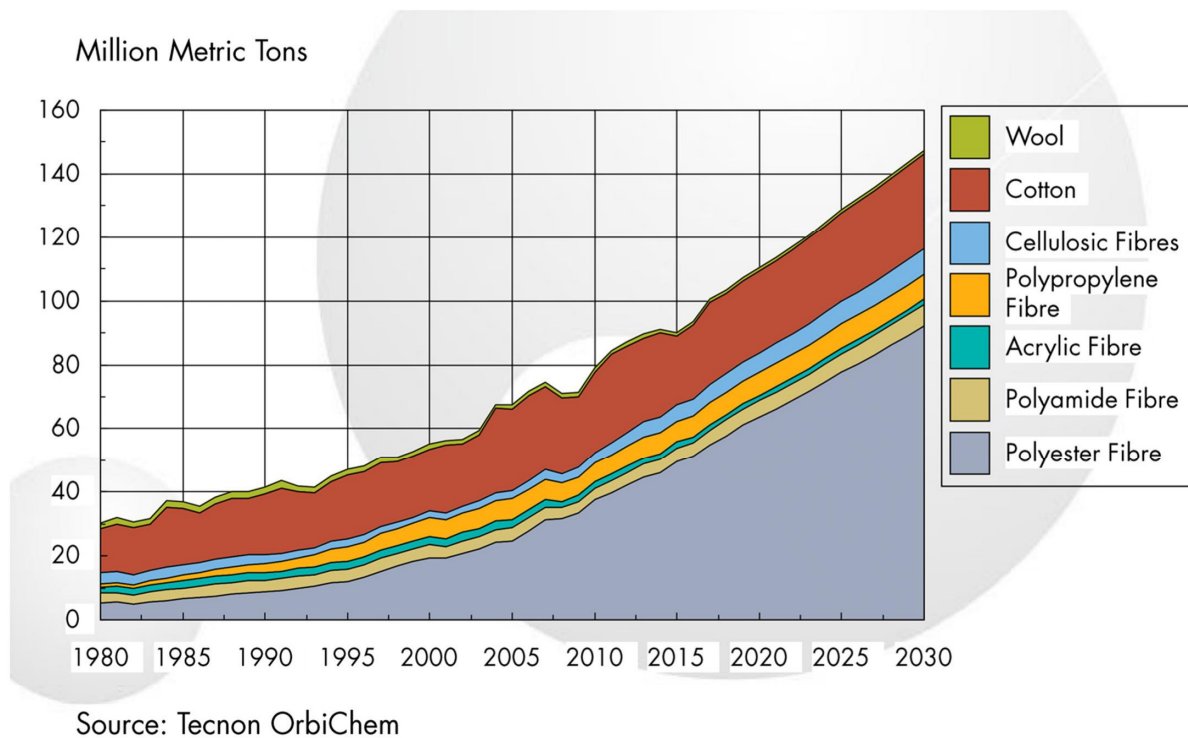

Fig. 1. World fiber production 1980–2030.
(Source: Tecnon OrbiChem, reprinted with permission)

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(Table 1), but in many studies that already included fibers those are the dominant microplastic shape.

On a global scale, absolute microplastic fiber concentrations vary similar to microplastic particles (Enders et al., 2015). Closer to shore lines, higher total microplastic fiber concentrations are reported than offshore (Desforges et al., 2014; Lusher et al., 2014; Nel and Froneman, 2015), which was related to industrial effluent and laundry water (Browne et al., 2011; Henry et al., 2019). Abundance of textile industry is an important factor for the amount of microplastic fibers shed to the environment. Asian countries are the leading synthetic fabrics producers and are globally a major source of microfiber pollution (Mishra et al., 2019). Absolute and relative concentrations of microplastic fibers recorded in Asian freshwater and estuarine systems were much higher compared to microplastic fiber concentrations reported e.g. from European rivers (Table 1).

Fibers occurring in the environment are not only plastic material and detailed chemical analysis is necessary to assess the actual microplastic fiber abundance. In the Mediterranean Sea, concentrations of 5100 fibers/m^3 were observed (Musso et al., 2019). However, most of the fibers were natural materials, such as cotton, cellulose, and wool, just 6.85% (equals about 349 fibers/m^3) were microplastic fibers. In contrast, in the Saigon river, Vietnam, 92% of the detected fibers were microplastic fibers (Lahens et al., 2018). On a global scale, chemical characterization of marine surface water samples identified two thirds of the predominantly fibrous items to be microplastics, whereas 31% were natural fibers (Barrows et al., 2018). While natural fibers degrade, microplastic fibers persist in the environment (Zambrano et al., 2019).

2.3. Methodological issues in microplastic fiber detection

2.3.1. Issues during microplastic fiber sampling

Methodological issues led to underestimation of the amount of microplastic fibers in the aquatic environment. General problems with detection and quantification of microplastic items in the water are the use of different units (e.g. mass or particle numbers per area, length, or volume) and a lack of standardization concerning sampling methods, extraction, and analysis of microplastics, as is already emphasized and discussed (Gago et al., 2018b; Hermsen et al., 2018; Löder and Gerdts, 2015; Primpke et al., 2020; Qiu et al., 2016). Furthermore, several studies report total microplastic concentrations and do not discriminate between microplastic particles and fibers (see Table 1).

In most microplastic monitoring studies, surface water samples were taken with neuston nets, which usually have a mesh size of 300–333 μm (Table 1). Thus, items smaller than 300 μm are not considered and actual total microplastic load is likely underestimated (Conkle et al., 2018; Waldschläger et al., 2020). Higher concentrations of microplastics (particles and fibers) of several orders of magnitude were collected with smaller mesh sizes (5–50 μm) than with neuston nets (300 or 330 μm) in comparable regions (Table 2). Especially for

the detection of microplastic fibers, low mesh sizes are needed as fibers may orient longitudinal and pass through nets. Furthermore, major losses of microplastic fibers from water samples can happen when using surface tow nets, as large volumes of water sampled facilitate to wash fibers through coarse meshes (Ryan et al., 2020). Consequently, increasing concentrations of microplastic fibers were detected in water sampled with decreasing mesh sizes of filters (Ryan et al., 2020).

Further challenges to overcome during sampling are the non-homogeneous spread of microplastic fibers in the water column. Sea surface samples and 5 m sub-surface samples at the same site deviated in total microplastic fiber concentrations from one another (Ryan et al., 2020). Most reliable insights about microplastic fiber concentrations were achieved with fine filters (<25 μm) used for multiple surface samplings at each site – and at different depths (Ryan et al., 2020). Overall, sampling of microplastic fibers requires a well thought-out study design with thorough considerations of sampling mesh sizes, sampling position in the water column and water volume filtered.

2.3.2. Issues with microplastic fiber characterization

Since reliable identification and characterization of microplastic fibers (from water and biota) is difficult, several studies intentionally excluded fibers from microplastic analysis (Avio et al., 2015; Cózar et al., 2014; Foekema et al., 2013; Kühn et al., 2018). So far, most studies examining microplastic fibers in the environment use optical tools for fiber analysis (Gago et al., 2018a), which facilitates confusion of microplastic fibers with natural fibers and artificial, natural-based fibers (e.g. rayon) (Wang et al., 2017a). Visual identification methods of microplastic fibers do not allow the determination of polymer type (Hermsen et al., 2018; Song et al., 2015). More costly and elaborate validation techniques such as Raman or Fourier Transform Infrared (FTIR) spectroscopy, or pyrolysis gas chromatography–mass spectrometry (pyr-GC–MS) are recommended for a profound qualitative and quantitative analysis of microplastics – at least for verification of a subsample (Brander et al., 2020; Hermsen et al., 2018; Löder and Gerdts, 2015). Additional challenges of fiber characterization relevant for all analytical methods are precise counting and length measurements, especially of twisted, entangled, and overlaying fibers (Primpke et al., 2019). Furthermore, airborne fibers are a steady source of potential contamination during sampling or analysis in the laboratory. Textile fibers of lab coats and clothes underneath can accidentally get introduced in samples and make contamination control strictly necessary when analyzing microplastic fibers (Wesch et al., 2017).

Overall, methodological difficulties in sampling, processing, detecting and characterizing fibers lead to underestimation of the abundance of microplastic fibers in the aquatic environment (Barrows et al., 2018; Conkle et al., 2018). To assess global microplastic fiber pollution in water and biota, future studies should use appropriate methods for sampling, extracting and analysis according to recent suggestions (Brander et al., 2020; Hermsen et al., 2018; Koelmans et al., 2019;

Table 2
Detected total microplastic concentrations vary depending on sampling equipment and location. Values of microplastic items (MPI) are given as range (min – max) or as mean (± standard deviation). Sample type refers to surface water (upper 16–30 cm) or sea surface microlayer (SML) samples in the upper 1000 μm.

Area	Range or mean [MPI/m^3]	Sample type	Sampling method, mesh size	Reference
Baltic Sea	0–0.8	Surface water	Manta net (330 μm)	Setala et al. (2016)
	0–1.25	Surface water	Pump (300 μm)	
	0–6.8	Surface water	Pump (100 μm)	
Sea of Japan, South Korea	47 ± 192	Surface water	Manta net (330 μm)	Song et al. (2014)
	1143 ± 3353	Surface water	Hand net (50 μm)	
	16,727 ± 13,457	SML	Filtered with 0.75 μm	
Korean west coast waters	0.06–0.45	Surface water	Trawl net (330 μm)	Chae et al. (2015)
	10–4227	Surface water	Hand net (20 μm)	
	48,092–359,748	SML	Filtered with 0.75 μm	
Rhine River	0.1–141.6	River samples	Manta net (300 μm)	Mani et al. (2015); Triebskorn et al. (2019)
Elbe River, Germany	$1*10^5$–$9*10^5$	River samples	Pump (5 μm)	Triebskorn et al. (2019)

O'Connor et al., 2019; Ryan et al., 2020). For standardized monitoring protocols, fiber sampling and analysis should be performed under strict contamination control. Glass and metal lab ware, cotton protective clothing and gloves, for which polymer composition was checked with the analytical device, should be used. All steps besides environmental sampling should be conducted in clean conditions, such as clean air facilities. For monitoring fiber concentrations in water bodies, multiple samples should be taken with fine mesh sizes at each sampling site. Water and biological samples need to be pretreated at low temperatures and the microplastic fibers analyzed with polymer validation techniques. Negative as well as positive (fiber-spiked) control samples should be implemented along the monitoring procedure whenever possible.

3. Effects of microplastic fibers on aquatic organisms

3.1. Microplastic fibers in biota samples from the wild

Increasing numbers of studies report the organismic ingestion of microplastic fibers in the field (reviewed by Gouin (2020). Higher awareness of textile fibers as pollutants and general progress in analytical methods were proposed as cause. Absolute amounts of microplastic items taken up by organisms show a high temporal and spatial variability (Gouin, 2020). Yet, relative amounts of microplastic fibers are high. In zooplankton microplastic fibers accounted for 43.9–93% of microplastic items (Desforges et al., 2015; Sun et al., 2018; Zheng et al., 2020), which is similar to proportions identified in other taxa, such as clams, shrimps and fish (Table 3). While in small-sized zooplankton less than one microplastic item (fragment and fiber) per organisms was detected, most higher organisms studied contained one or more items (Table 3). Overall, microplastic fibers often resemble the most common microplastic shape detected for all studied organisms.

The prevalent microplastic fiber polymers in seawater and sediments are polypropylene, polyethylene, and polyester (Gago et al., 2018a). The polymers of microplastic fibers detected in biota coincide with fibers detected in water samples of their habitats (Pozo et al., 2019; Zheng et al., 2019). According with fiber distribution patterns, uptake of microplastic fibers by fish from the Mediterranean Sea was positively correlated to coastal human population, river inputs and shipping lanes (Sbrana et al., 2020).

In general, a greater proportion of suspension feeders takes up microplastic particles and fibers compared to more selective feeding taxa, such as crustaceans or fish (Table 3). Suspension feeders are expected to encounter microplastics in high numbers, as their feeding mode concentrates food particles from large volumes of water (Desforges et al., 2015).

Other organisms, such as fish, take up suspended microplastic fibers accidental while foraging (Lusher et al., 2013; Roch et al., 2019), or by active ingestion if microplastic items resemble their prey in size, shape or color (Talley et al., 2020; Walkinshaw et al., 2020). Intestines of the visual predator fish Amberstripe scad (*Decapterus muroadsi*) were enriched for blue microplastic items, presumably because they were confused with blueish copepods, their natural prey items (Ory et al., 2017). Correspondingly, high numbers of red microplastic fibers (79% of all fibers ingested) were found in an omnivorous fish species (*Girella laevifrons*) since their diet range includes red algae (Mizraji et al., 2017). Lower body conditions were observed in specimens with a higher content of microplastic fibers in their digestive tract (Mizraji et al., 2017), which might imply fitness impacts due to ingested fibers.

Ingestion of microplastic fibers by fish was observed for different life stages from larvae to adults in the field (Gove et al., 2019; Kühn et al., 2018; Mizraji et al., 2017; Steer et al., 2017). Larvae and juveniles took up fibers as the principal microplastic shape (Table 3), which raises the concern that earlier life stages might be more prone to ingest

microplastic fibers specifically. If early life stages of invertebrate taxa ingest microplastic fibers actively in nature still needs to be determined.

Indirect uptake of microplastic items by selective feeding crustacean or fish happens when they stick to the outside or appendages of food sources (e.g. copepods) (Cole et al., 2013), and when microplastics were ingested previously by the prey (Walkinshaw et al., 2020). These uptake routes raise the question of biomagnification of microplastics along the food chain. While bioaccumulation specifies a higher uptake than egestion of microplastics within an organism, biomagnification refers to an increase in concentrations of microplastics in organism compared to the level in the prey (Miller et al., 2020). However, recent literature reviews state that microplastics do not biomagnify via trophic transfer in marine food webs based on field observations that did not support an increase in body burden of microplastics in higher trophic levels (Gouin, 2020; Miller et al., 2020; Walkinshaw et al., 2020).

Though within individuals retention and bioaccumulation of microplastic fibers were not detected in bogue (*Boops boops*) captured in the Mediterranean Sea, individuals that ingested higher numbers of predominantly microplastic fibers had lower body condition (Sbrana et al., 2020). Similar to lower body condition of omnivorous fish, which contained higher amounts of microplastic fibers, mentioned above (Mizraji et al., 2017), those observations could indicate negative effects of microplastic fiber consumption. The actual causes for reduced body condition remain unclear and might be coincidental. Fish that had lower body condition anyways, might be less competitive for the best food sources and therefore ingested more microplastic fibers (Sbrana et al., 2020). On the other hand, chronic consumption of a diet rich in indigestive microplastic fibers, might have deprived nutrient availability via gut blockage, false satiety sensation, or physical injury of the digestive tracts (Mizraji et al., 2017). Another possible explanation is that associated bioaccumulative organic substances ingested together with the fibers caused stress effects, which had an impact on the fitness of individuals (Mizraji et al., 2017). In environmental monitoring studies, disentangling cause and effect is not always possible and experimental studies might help a deeper understanding of causalities underlying fitness effects of microplastic ingestion.

3.2. Experimental exposure with microplastic fibers

It has become obvious that organisms are exposed to microplastic fibers in the wild. For a better understanding on the effects that microplastic fibers have on organism, laboratory exposure experiments are important tools. The following section highlights current knowledge on how aquatic organism are affected by microplastic fibers in exposure experiments. It starts with small zooplankton organisms, which can be mechanically impacted by microplastic fibers in their environment. As the section moves along different animal taxa, organisms become bigger and more complex and modes of interactions with microplastic fibers change, whereby the most obvious is the intake during foraging activity.

For bigger animals, such as sea turtles, sharks and marine mammals, plastic ingestion reported in field samples often refers to meso- and macroplastic items above 5 mm (Alexiadou et al., 2019; Bernardini et al., 2018; Smith, 2018). To our knowledge, exposure studies with microplastic fibers were not conducted with higher taxa than fish so far. Thus, we focus on microplastic fiber effects on aquatic taxa up to fish.

3.2.1. Zooplankton exposed to fibers

Microplastic fibers in the water column can affect aquatic organisms via physical contact. Water flea (*Ceriodaphnia dubia*) were exposed to microplastic fibers and spheres in increasing concentrations, from two up to three orders above environmental levels (Ziajahromi et al., 2017). Water fleas did not ingest polyester fibers from the water, but reduced growth, reproduction, and abnormal swimming behavior were attributed to tactile contact with the fibers. Fibers caused body damage, such as carapace and antenna deformities at concentrations of $4.3 * 10^3$

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Table 3
Microplastics in organismic field samples. Microplastic items (MPI) per individual are given as mean or range (min – max). Proportion of microplastic fibers (MPF) of total MPI are specified (n.r. – not reported).

Taxa	Area	Percentage of animals that ingested MPI [%]	Amount of MPI per individual [MPI/ind.]	Percent MPF of total MPI found [%]	Reference
Zooplankton					
Copepod (*Neocalanus cristatus*)	Northeast Pacific Ocean	*1 MP in 34 copepods,*	0.026	43.9	Desforges et al. (2015)
Euphausiid (*Euphausia pacifica*)		*1 MP in 17 euphausiids*	0.058	68.3	
10 zooplankton species	Bohai Sea, China	n.r.	0.001–0.056	93	Zheng et al. (2020)
10 zooplankton species	Yellow Sea, China	n.r.	0.13 *(for Copepoda)* 0.35 *(for Pteropoda)*	54.6	Sun et al. (2018)
Bivalve					
Manila clams (*Venerupis philippinarum*)	Baynes Sound, British Columbia	100	8.4 ± 8.5	90	Davidson and Dudas (2016)
Blue mussel (*Mytilus edulis*)	French-Belgian-Dutch coastline	100	0.2 ± 0.3 *(MPI per g mussel)*	n.r.	Van Cauwenberghe et al. (2015)
Blue mussel (*Mytilus edulis*)	Halifax Harbour, Nova Scotia, Canada	n.r.	34	100 *(investigated only MPF)*	Mathalon and Hill (2014)
Crustacean					
Norway lobster (*Nephrops norvegicus*)	Clyde Sea, Great Britain	83 *(macro- & microplastics)*	n.r.	100	Murray and Cowie (2011)
Brown shrimp (*Crangon crangon*)	English Channel	70	1.2 ± 1.0	96.5	Devriese et al. (2015)
Caridean shrimp (*Palaemon* sp.)	South Adriatic Sea	42.8	1.2 ± 0.4	76.2	Avio et al. (2020)
Other invertebrate taxa					
Barrel jellyfish (*Rhizostoma pulmo*)	Center Adriatic Sea	28.6	2.0 ± 1.2	37.7	Avio et al. (2020)
Purple sea urchin (*Paracentrotus lividus*)	South Adriatic Sea	27.3	1.3 ± 0.6	72.7	Avio et al. (2020)
	Center Adriatic Sea	42.8	1.7 ± 0.6	42.9	
Fish					
6 planktivorous fish species	North Pacific Central Gyre	35 *(macro- & microplastics)*	2.1 ± 5.8 *(range: 1–83 items ingested)*	~ 3	Boerger et al. (2010)
19 fish species	Yellow sea, China	34	1.0–2.6	67	Sun et al. (2019)
24 fish species	Beibu Gulf, South China Sea	49.1	0.2 ± 0.08	96	Koongolla et al. (2020)
21 fish species	Chinese coastal and freshwaters	100/95.7 *(coastal/freshwater species, respectively)*	1.1–7.2	46.3–100	Jabeen et al. (2017)
9 commercial fish species	Estuary in north Jakarta, Indonesia	97.1	12.2 ± 9.8	89.6	Hastuti et al. (2019)
Tropical freshwater fish (*Hoplosternum littorale*)	Pajeú river basin, Brazil	83 *(macro- & microplastics)*	3.6	46.6	Silva-Cavalcanti et al. (2017)
Sea bass, Sea bream, flounder	Mondego estuary, Portugal	38	1.7 ± 0.3	96	Bessa et al. (2018)
5 pelagic & 5 demersal fish species	English Channel	36.5 *(macro- & microplastics)*	1.9 ± 0.1 *(range: 1–15 items ingested)*	68.3	Lusher et al. (2013)
2 pelagic (herring, mackerel) & 3 demersal (cod, dab, flounder)	North Sea & Baltic Sea	5.5 *(macro- & microplastics)*	0.2 ± 0.6	17.4	Rummel et al. (2016)
5 fish species	Prairie Creek, Canada	73.5	1.4 (fragments) + 1.6 (fibers)	47.7	Campbell et al. (2017)
Larvae & Juveniles					
23 fish species, larvae	Western English Channel	2.9	1–2	83	Steer et al. (2017)
8 fish species, larvae	Surface slick + Ambient waters, Hawai'i Island	8.6 3.4	1–3	93	Gove et al. (2019)
5 fish species, juveniles	Tidal pools, central coast of Chile	n.r.	61 *(omnivores)* 14 *(herbivores)* 10 *(carnivores)*	99	Mizraji et al. (2017)

fiber per liter and above. The (mechanical) impact on zooplankton was more pronounced for suspended fibers than spheres when present in high concentration (1 mg/ L, equals $8.6 * 10^3$ fiber per liter) (Ziajahromi et al., 2017). A reduction of 50% in neonate numbers occurred at fiber concentrations of $3.4 * 10^3$ fibers per liter (Ziajahromi et al., 2017), which is three orders above fiber concentration reported

for e.g. the Atlantic and Asian areas (range 10^0 fibers per liter) (Luo et al., 2019; Ryan et al., 2020; Song et al., 2015).

Survival of the water fleas was affected dose-dependently by microplastic fibers when exposed in an acute bioassay for 48 h. The lethal concentration for 50% of the animals (LC50) was as high as $1.3 * 10^4$ polyester fibers per liter (Ziajahromi et al., 2017). Although

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this is four orders of magnitude higher than mean environmental levels (Luo et al., 2019; Ryan et al., 2020; Song et al., 2015), results indicate that local events of high microplastic fiber pollution can severely affect zooplankton.

Ingestion of microplastic fibers was reported for some zooplanktonic organisms on top of mechanical encounter. Selective feeders, such as copepods or euphausiids, which select their food primarily by size, take up suspended microplastics items actively when they confuse them with their prey (Cole et al., 2013; Desforges et al., 2015). Selective foraging freshwater amphipods, *Hyalella azteca,* were exposed to either polypropylene fibers or polyethylene spheres of increasing concentrations above environmental levels (Au et al., 2015). Ingestion of the microplastic fibers was evaluated by counting fibers in excreted feces. Mortality rates rose with higher amounts of fibers ingested with increasing fiber concentrations in the water. In a 10-day exposure, the LC50 dose for the amphipods was reached for fibers at concentrations 1000-fold lower than for spheres ($7.1 * 10^3$ fibers per liter compared to $4.6 * 10^6$ spheres per liter) (Au et al., 2015). Thus, microplastic fiber ingestion induced higher toxicity than spheres in amphipods (Au et al., 2015). Although the determined LC50 concentration for the amphipods is three orders above mean environmental fiber levels (Luo et al., 2019; Ryan et al., 2020; Song et al., 2015), it is only one order above maximum absolute fiber concentration detected in the highly-polluted Saigon River (Lahens et al., 2018).

Apart from acute toxicity, significantly reduced growth by >50% lower weight compared to the control animals was observed for *H. azteca* exposed to high fiber concentrations ($>4.5 * 10^4$ fibers per liter) (Au et al., 2015). The amphipods retained microplastic fibers in their digestive system significantly longer than spheres or natural food items (Au et al., 2015), which might be a reason for reduced growth, since less energy was available. However, aggregation of microplastic fibers did not occur in experimental amphipods and complete egestion of the polypropylene fibers was possible (Au et al., 2015).

Mortality was observed for the water flea *Daphnia magna*, which ingested polyester fibers present in the water column in a 48-hour exposure, similar to amphipods (Jemec et al., 2016). Gut content analysis identified the ingestion of microplastic fibers. However, for that species survival was not dose-dependent, indicating the heterogeneous impacts of microplastic fibers on different aquatic organisms. Overall lower mortality was detected when *D. magna* were pre-fed with algae before the exposure period (Jemec et al., 2016). In contrast, no acute mortality was observed for *D. magna* and *Artemia franciscana*, when they were exposed to high concentration of polyester fibers (100 mg/L, which equals more than $5 * 10^6$ fibers per liter) for 48 h – although microplastic fibers were present in their guts (Kokalj et al., 2018). Several elements, such as tested concentration, type and size of microplastics, exposure time, but also age, sex, reproductive status, and number of animals in the treatment are discussed as factors influencing the impact of microplastics on organisms (De Sales-Ribeiro et al., 2020). The reason for the difference in mortality of exposed water fleas in previous studies conducted with the same species and similar fibers could not be ascertained, but variability in fiber size and exposure concentrations were proposed as potential reasons (Kokalj et al., 2018).

Copepods (*Calanus helgolandicus*), exposed to either nylon fragments or fibers in the water column along with algae, decreased their food intake in the treatments with suspended fibers but not with fragments (Coppock et al., 2019). Furthermore, copepods in the exposure treatments decreased ingestion of algae similar in size and/or shape to the unpalatable nylon fibers, probably due to avoidance behavior (Coppock et al., 2019). In the long term, reduced food intake and consequential decline in available energy will affect fitness (Watts et al., 2015).

Microplastic fiber concentrations as currently observed in aquatic environments may not cause acute toxicity to zooplankton organisms, as demonstrated in laboratory experiments which have used higher concentrations. However, in local pollution events, which may result in high (local) microplastic fiber concentrations, zooplankton could be affected. When factors such as food depletion occur simultaneously, negative impacts can reduce the fitness of zooplankton populations.

3.2.2. Bivalves exposed to fibers

Indiscriminate suspension feeders, such as mussels and clams, take up suspended microplastics together with their food items (Kolandhasamy et al., 2018). Microplastic items from the water column can be captured and trapped into mucus at the gill surfaces and are subsequently assimilated over the gill epithelium or transported into the digestive system (Li et al., 2019a). Concentration dependent uptake of microplastic fibers was reported for bivalves by field monitoring and exposure studies that determined fiber content in bivalves and surrounding waters (Li et al., 2019a; Li et al., 2019b; Qu et al., 2018). Accordingly, bivalves were promoted as bioindicator species for monitoring microplastics in the environment (Li et al., 2019a).

In exposure studies, blue mussels (*M. edulis*) were exposed to polyester fibers in the range of $3 * 10^3$–$3 * 10^4$ fibers per liter (Woods et al., 2018). Uptake rates of microplastic fibers were calculated based on fiber concentrations measured in the water column (corrected for loss controls). Uptake rates increased up to a concentration of $13 * 10^4$ fibers, above this concentration a constant uptake rate was observed. This suggests that mussels have a maximum of fibers they can take up in a certain time (Woods et al., 2018). However, the maximum uptake rate was observed at fiber concentrations higher than environmental levels.

The ingestion of microplastics needs to be considered concomitantly with egestion rates to provide meaningful interpretation of the actual presence of microplastics in organisms (Burns and Boxall, 2018). In blue mussels, egestion of microplastic fibers occurred at various elimination rates from different tissues (Woods et al., 2018). Fibers in the gills were expelled faster than fibers in the digestive gland and in the digestive system, in which microplastic fibers were still present after three days of depuration (Woods et al., 2018). Since excretion is not fast enough to shed all uptaken fibers from tissues in filter feeding bivalves immediately, microplastic fibers remain in the organisms for some time.

Treatments with fiber presence in the water column led to reduced filtration rates (volume of water filtered per minute) of blue mussels compared to algae presence only (Woods et al., 2018). In the long-term, decreased filtration rates will likely impact the energy budget of the mussels.

Li et al. (2019a) hypothesized that mussels do not ingest every microplastic item that is captured with the gills, as they are able to separate and reject nonnutritive particles as pseudofeces. In Asian clams (*Corbicula fluminea*), physicochemical characteristics of microplastic fibers determined their uptake (Li et al., 2019b). The uptake was investigated for different size ranges of polyester fibers (from 5 to 5000 μm) and six different polymers (polyester-amide, polyester, acrylic, polyamide, rayon, and polyvinyl alcohol). Fiber uptake was determined by chemical digestion and analysis of the soft tissues of the clams. Highest ingestion rates were observed for polyester fibers in the size range 100–250 μm, which is related to the optimum size for their feeding apparatus and its morphological structure (Li et al., 2019b). This coincides with the high abundance (>70%) of microplastics <1 mm in estuary coastal regions in the East China Sea (Luo et al., 2019). Accordingly, in a coastal bivalve habitat in English waters, the microplastic size fraction <250 μm accounted for 30–40% of all microplastics detected (mostly fibers) (Li et al., 2018). In the exposure study with clams, polyester fibers were ingested in higher numbers than other polymers, and rayon and polyvinyl alcohol fibers were not taken up at all (Li et al., 2019b). Fiber softness, measured by elasticity, was positively correlated with numbers of fibers ingested and clams took up the softest polyester fibers in higher numbers than other fibers (Li et al., 2019b). Higher uptake of polyester fibers compared to other fibers is a major concern, since those fibers are prominent in water samples. The variation in uptake

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of different fiber polymers raises the need for impact analyses of the common polymers separately and in specific combinations that are found in the respective habitats of the study organisms. Depending on occurring fiber types, their uptake rates by bivalves, and the ability of certain bivalves to reject fibers, some species may be more affected than others.

3.2.3. Gastropods exposed to fibers

A number of studies fed non filter-feeding, bigger-sized organisms such as gastropods with diets containing defined amounts of microplastic fibers to ensure their uptake for retention and impact analyses (Ehlers et al., 2020; Grigorakis et al., 2017; Jabeen et al., 2018; Watts et al., 2015). When freshwater snails (*Radix balthica*), were provided with a biofilm that contained microplastic fibers, they ingested the fibers during grazing (Ehlers et al., 2020). Subsequent egestion of microplastic fibers via the feces in fiber-free medium happened gradually and was completed after three days (microscopic observation) (Ehlers et al., 2020). However, in depth analysis after chemical digestion of whole tested organisms revealed fibers that were still inside the snails six days after the exposure (Ehlers et al., 2020). This demonstrates the persistence of some ingested fibers in the snails' bodies despite most of microplastic fibers were excreted within a few days following ingestion (Ehlers et al., 2020).

In freshwater snails (*Planorbella campanulata*) exposed to very high concentrations of polyester textile fibers in the water column (50 mg/L), accumulation of fibers close to the snail's mouth and higher mortality rates compared to control animals were observed (Philips et al., 2020). Thus, blockage of food intake and mortality of snails can happen in pollution events with high fiber concentration in local hot spots (Philips et al., 2020). Furthermore, higher rates of offspring occurred in snails in the polyester fiber treatment. This was suspected to be a side effect of mortality causing a concomitant pulse of offspring (terminal investment), or to estrogenic effects of additives leaching from the fibers (not tested) (Philips et al., 2020).

3.2.4. Decapods exposed to fibers

Decapods encounter microplastics in the water column at their gill surfaces during ventilation or ingest them during foraging. While selective feeding decapods can actively feed on microplastic fibers, filter- and deposit feeding decapods ingest microplastic fibers passively.

Daggerblade grass shrimp (*Palaemonetes pugio*) were exposed to different shapes (spheres, fragments and fibers) and sizes (30–165 μm) of microplastics in the water column ($5 * 10^4$ items per liter) (Gray and Weinstein, 2017). All tested microplastic shapes adhered to the gills surfaces due to ventilation and the selective foraging shrimps ingested present microplastics. Despite the short exposure time, mortality of the shrimp occurred within the 3-hour treatment and during the following 96-hour depuration period. Both polypropylene fiber sizes tested (34 μm and 93 μm) caused mortality (55% and 35%, respectively) and mortality rates were higher for fibers than for spheres or fragments of different sizes (mortality rates of 0–40%) (Gray and Weinstein, 2017). Damage of intestinal structures by entangled fibers was suggested as possible reasons for the high toxicity of microplastic fibers (Gray and Weinstein, 2017). Yet, the higher toxicity of fibers could also be linked to their weathered status. Tested fibers were cut from an aged polypropylene rope, which was collected from a marine site. In another study, virgin polyester fibers did not lead to increased mortality in grass shrimp during 96-hour exposure with the same concentration as used by Gray and Weinstein (2017) and Leads et al. (2019). The discrepancy in toxicity could result from the different fiber polymers used or the fibers state of weathering (virgin vs. aged) and demonstrate the variability in impact of microplastic fibers on decapods due to fiber characteristics. A subsequent 2-day challenge assay to bacteria (*Vibrio campbellii*) did not induce mortality in the polyester-exposed shrimp either and was linked to the rather fast excretion of polyester fibers within 48 h, probably limiting their immunotoxicity (Leads et al., 2019).

Pacific mole crabs (*Emerita analoga*) that are filter feeders were exposed to ecological relevant concentrations of 1 mm polypropylene fibers in the water column (3 fibers per liter) for 41 days (Horn et al., 2019). Exposed crabs showed elevated mortality compared to control animals. The uptake of fibers was determined by chemical digestion of the whole crabs. Higher numbers of fibers taken up increased mortality in the crabs. This suggests that Pacific mole crabs – and other non-selective feeders – are unable to differentiate between plastic and food items, and are at reasonable risk when environmental fiber concentrations increase (Horn et al., 2019). Furthermore, exposed *E. analoga* showed decreased retention of egg clutches and increasing variability in embryonic development rates. The authors pointed out, that impaired reproduction may be caused either by ingested microplastic fibers themselves or by potential leaching of the associated dye of the fibers (not analyzed separately) (Horn et al., 2019).

Ingestion of microplastic fibers was analyzed for isopods and predatory crabs by providing them fibers with their diet (Hamer et al., 2014; Watts et al., 2015). No signs of aggregation were observed within the digestive tract when the number of ingested fibers was determined in different sections of the intestinal system and within the feces (Hamer et al., 2014; Watts et al., 2015). However, in langoustine (*Nephrops norvegicus*) that fed on a diet supplied with polypropylene fibers for two months, un-moulted individuals aggregated fibers in their digestive tract (Welden and Cowie, 2016). Moulted animals, on the other hand, had no remaining microplastic fibers in their stomach. Microplastic fibers were detected in the shed gut lining, which indicates that *N. norvegicus* can lose microplastic fibers at ecdysis (Welden and Cowie, 2016). Another way to release ingested fibers was detected in Atlantic ditch shrimp (*Palaemon varians*), which were able to regurgitate ingested polyacrylic fibers (Saborowski et al., 2019). Overall, crustaceans that feed on microplastic fibers seem to be able to excrete fibers quite efficiently via different modes.

Long-term dietary exposure to microplastic fibers affected individuals' fitness in some crustacean species. When crabs (*Carcinus maenas*) were fed a diet containing polypropylene fibers (0.3–1.0% by weight) for four weeks, reduced food consumption compared to control animals and significantly less energy available for growth were measured (Watts et al., 2015). This might be a behavioral response to suboptimal food by consecutively avoiding eating the entire meal offered. Since these crabs are able to choose more favorable food items over others in the field, direct ecological consequences of microplastic fiber pollution are rather unlikely for this particular species (Watts et al., 2015).

Overall, many decapods are able to excrete fibers fast and efficiently and are not vitally affected. However, some species are more vulnerable to microplastic fiber exposure and may be affected when microplastic fiber concentrations increase.

3.2.5. Fish exposed to fibers

In the field fish encounter floating microplastic fibers at their gills and can take them up via active or passive ingestion from the water column. Japanese medaka (*Oryzias latipes*) were exposed to suspended polyester or polypropylene fibers in the water column for 21 days (Hu et al., 2020). The fiber concentration tested was $1 * 10^4$ fibers per liter, which is above environmental fiber levels (Luo et al., 2019; Ryan et al., 2020; Song et al., 2015). Hu et al. (2020) hypothesized that suspended microplastic fibers would entangle with the gills of Japanese medaka, however, after exposure they did not find polypropylene or polyester fibers in the gill apparatus. Even if fluid containing microplastic fibers was flushed through the mouth cavity of the tested medaka, fibers passed through the branchial chamber without becoming entangled in the gill filaments. Nevertheless, scanning electron microscopy revealed structural damages in the gills of exposed Japanese medaka, such as denuding of epithelium on gill arches, fusion of primary lamellae, and increased mucus production after exposure (Hu et al., 2020). Microplastic fibers can thus cause structural alterations in fish gills when encountered via ventilation.

Ingestion of microplastic fibers was determined with histological sections of the fish and by analyzing the fiber content in their feces (Hu et al., 2020). Similar to observations of fish in nature, bioaccumulation and blockage of the intestinal system were not detected in exposed medaka. Scanning electron microscopy of fish intestines revealed that most fibers were orientated longitudinally and encased in food, mucus, and waste material within the lumen. Only rare cases of trapped microplastic fibers were visible in the gut folds. Presumably high mucus production and lubricated gut walls favored rapid passage and excretion of microplastic fibers (Hu et al., 2020). The 90% evacuation time for microplastic fibers in goldfish was less than 35 h (Grigorakis et al., 2017).

In Japanese medaka, mortality and changes in body condition, hepatosomatic and gonadosomatic indices were not detected after exposure with microplastic fibers (Hu et al., 2020). Furthermore, no histological alterations in liver, kidney, thyroid, heart, spleen, pancreas, and gonads were observed. Yet, increased egg production and fertilization rates were detected in medaka after the second week of exposure to microplastic fibers. Endocrine disruption caused by additives of the microplastic fibers, which leach into the water column or within the digestive tract, was suggested as plausible explanation (Hu et al., 2020). Yet, chemical analysis of the pristine fibers was not conducted in the frame of the study (Hu et al., 2020).

Embryos of exposed adult medaka did not show differences in mortality, development or hatching success compared to the control group. However, fertilized eggs were removed from the treatment tanks, cleaned and raised in clean water (Hu et al., 2020). Further studies are needed to determine the (potential) impact of microplastic fibers on embryonic development with fibers present in the water column during ontogenesis.

Zebrafish (*Danio rerio*) ingested polypropylene fibers when exposed to microplastic items in the water column for 21 days (Qiao et al., 2019). The microplastics were counted in dissected guts and higher numbers of fibers were present than spheres and fragments after exposure (Qiao et al., 2019). Fiber concentration tested (680 fibers per liter) was slightly above maximum reported fiber concentration in the Saigon River (519 fibers per liter) (Lahens et al., 2018). Fiber exposure affected growth of the zebrafish and led to lower body condition, in contrast to observations of exposed medaka in the previous study (Qiao et al., 2019). Thus, impact of microplastic fibers on fish seems to vary across species.

Intestinal alterations, such as mucosal damage, higher permeability, and inflammation observed in exposed zebrafish might cause the reported depletion in growth (Qiao et al., 2019). Zebrafish in the fiber treatment had oxidative stress measured by higher superoxide dismutase activity in the gut. In addition, genes that belong to the lipid metabolism, hormone metabolism, and protein secretion were down-regulated in exposed fish indicating metabolic disruption (Qiao et al., 2019). Observed alterations of gut microbiota and intestinal dysbiosis in zebrafish exposed to microplastic fibers might explain why fibers cause intestinal toxicity and metabolic disruption in some fish (Fackelmann and Sommer, 2019; Qiao et al., 2019).

Exposure studies that provide diets supplemented with microplastic fibers to fish were used to analyze fiber uptake via oral ingestion. Selective foraging fish use their sensory systems to pick their food and some fish are able to sense and reject hard microplastic items. Goldfish (*Carassius auratus*) that are able to chew on food pellets were exposed to microplastic items via food pellets (Jabeen et al., 2018). The fish expelled those food pellets that contained hard microplastic spheres or fragments (Jabeen et al., 2018). Pellets with microplastic fibers, in contrast, were chewed and ingested, indicating that fish do not recognize fibers as indigestible (Jabeen et al., 2018), presumably since fibers are common structures in natural food items. This suggests that selectively foraging fish are more likely to ingest microplastic fibers than particles.

The goldfish had fibers in their gastrointestinal tract and in-between their gills when exposed for six weeks (Jabeen et al., 2018). Plausible mechanisms of fiber transfer from the food pellets to the gills were

not specified, but microplastic fibers can possibly be released from the pellets during chewing, reach the buccal cavity, and become entangled in the gill filaments subsequently. Thus, microplastic fibers attached to or incorporated in prey items might possibly reach the gills upon release during chewing.

Mortality was not observed for the goldfish. However, exposed fish showed histological changes in the proximal and the distal intestine despite fast excretion of the fibers (Jabeen et al., 2018). Structural changes of varying severity, such as breakage or detachment of the epithelium, erosion of villi, and rarely (12.5% of all exposed fish) signs of inflammation via infiltration of leucocytes were detected in the fish. Concurrently, fiber-exposed goldfish had lower weight and lower body condition compared to control animals. In some livers (<10%) of fiber-exposed goldfish, histological alterations, such as sinusoid dilations or inflammatory response, were detected. Stress due to ingestion of fibers was suggested to induce the observed liver alterations (Jabeen et al., 2018).

In general, fish can ingest fibers directly from the water column, and associated with their food. Most uptaken fibers are efficiently egested but can trigger structural alterations in the intestinal system and when encountered at the gills. Constraints on reproduction, energy metabolism and oxidative stress were detected in some fish. Since fish were affected with varying severity, subsequential alterations in food web structures will depend on the vulnerability of fish species in the respective habitats.

3.3. Transfer of microplastic fibers into body tissues

Most incorporated microplastic fibers were detected in the gastrointestinal system of animals, from which they can be excreted with the feces. However, some studies also observed translocation of fibers to other tissues. With small microplastic spheres (<10 μm), translocation from the gut to the hemolymph and into hemocytes was observed in mussels (Browne et al., 2008).

Fiber-exposed blue mussels (*Mytilus edulis*) ingested microplastic fibers (mainly >100 μm) from the water column in intestines, but also incorporated them in foot tissue (Kolandhasamy et al., 2018). Since fibers >100 μm are suspected to be too large to translocate into the circulatory system, Kolandhasamy et al. (2018) proposed a novel uptake route of microplastic fibers into foot tissue of blue mussels. Microplastic fibers might adhere to tissues during the process of production, relocation and excretion of pseudofeces through the mantle and foot (Kolandhasamy et al., 2018).

Another pathway for the incorporation of fibers was observed in sea cucumber (*Apostichopus japonicas*) exposed to suspended polyester fibers (1–5 mm length) for 72 h (Mohsen et al., 2020). Microplastic fibers got stuck in the branches of the respiratory trees during aspiration when their expulsion with the water was attempted. Some adhered fibers translocated to the coelomic fluid subsequently, raising the question of potential harm after internalization. Minor alterations in increased lysozyme activities (as immune defense index) were observed in exposed sea cucumbers. Given that, impaired fitness in addition to physical harm of the respiratory system might happen when microplastic fibers accumulate in the coelomic fluid over the lifetime (Mohsen et al., 2020).

In higher taxa, uptake of microplastic fibers is expected to happen mainly via oral ingestion and many field studies analyze solely the gastrointestinal tract of fish for microplastic fibers (Bessa et al., 2018; Campbell et al., 2017; Hastuti et al., 2019; Hermsen et al., 2017; Jabeen et al., 2017). Microplastic fibers can also attach to the gills of fish, as observed for fish sampled in Chinese waters (Koongolla et al., 2020; Su et al., 2019).

Translocation of microplastic fibers into tissues of fish is predicted to be rather unlikely due to their overall size and elongated dimension (Garrido Gamarro et al., 2020; Gouin, 2020; Su et al., 2019). Already for spherical microplastics in a size range of 10–300 μm, translocation was not observed after dietary administration to adult rainbow trout (*Oncorhynchus mykiss*) (Kim et al., 2020). Though, microplastic fibers

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were found in muscle tissue of fish collected from the Persian Gulf (given that control for contamination was applied) (Akhbarizadeh et al., 2019). The authors hypothesized, that elongated microplastic items, which cannot cross the intestinal barrier via absorption by enterocytes, instead pass between them in a paracellular manner (Akhbarizadeh et al., 2019).

Translocation of microplastic fibers was not reported from laboratory exposure experiments conducted so far. Small microplastic spheres (1–5 μm) were observed to translocate in the enterocyte cytoplasm and goblet cells in zebrafish provided a diet with microplastics (De Sales-Ribeiro et al., 2020). However, in the same study larger plastic fragments (120–220 μm) and fibers (1.5 mm) provided with the diet, were detected exclusively in the intestinal lumen (De Sales-Ribeiro et al., 2020). Follow-up research could look at smaller fibers (e.g. <100 μm and <10 μm) and compare their translocation potential to tissues with that of small spheres.

3.4. Ecological relevance of exposure experiments with microplastic fibers

The experimental studies this review refers to, have used a wide range of concentrations of microplastic fibers and often relatively short exposure times. Significant effects mostly occurred at concentrations above the concentrations currently observed in the wild. Accordingly, these studies have to be interpreted with caution with respect to conclusions relevant for microplastic fiber pollution in nature.

In general, lower trophic levels are at greater risk to suffer from overall microplastic contamination (Walkinshaw et al., 2020). Accordingly, with respect to fibers, compiled results of exposure studies indicate that primary consumers and organisms at lower trophic levels are also more susceptible than higher taxa (Fig. 2). Small planktonic organisms can suffer from mechanical impact of microplastic fibers and show signs of toxicity and even mortality upon exposures. Even though such results were obtained with microplastic fiber concentrations higher than those found in nature so far, it is considerable that fibers can have such strong effects on organismal lives. Higher taxa such as snails and fish seem to be less heavily afflicted by exposure to microplastic

fibers. Egestion and complete excretion of microplastic fibers seems possible for several species. However, mechanical impact on gill and gut structures, metabolic disturbances, and even alterations of fecundity were observed. Most studies have used relatively short exposure times (hours - days) and only limited information about chronic exposure to microplastic fibers is available. Some studies observed translocation of microplastic fibers in body cavities and tissues, even if this does not cause acute toxicity, chronic responses have to be expected which may coincide with negative fitness effects.

Extrapolation from the laboratory experiments towards situations with microplastic fiber contamination in the wild is difficult to date. Nevertheless, the higher susceptibility of lower taxa to microplastic fiber exposure suggests that the basis of the food chain is more severely at risk by microplastic pollution. Even if this is not a pronounced effect at concentrations currently abundant in the wild, it will likely be at rising concentrations as have to be expected in the future (Geyer et al., 2017). Those alterations at the lower end of the food chain might lead to indirect fitness impacts of higher trophic level organisms. Knowledge on acute toxicity thresholds for lower taxa can be useful to set the margins for political decisions acting against microplastic fiber pollution and to classify the severity of local contamination events. Yet, studies with prolonged exposure periods provide a more appropriate basis for environmental impact assessments and should be prioritized in future research.

3.5. Effects of additives associated with microplastic fibers

While some studies tested the detrimental effect of leachates derived from microplastic fragments and beads on aquatic organisms (Beiras et al., 2020; Pérez-Albaladejo et al., 2020; Rendell-Bhatti et al., 2020), exposure studies with microplastic fibers often did not distinguish between effects of fibers and their additives (Horn et al., 2019; Hu et al., 2020; Jabeen et al., 2018; Philips et al., 2020).

The manifold additives of microplastic fibers can add a detrimental impact on aquatic organisms and must be considered as part of the microplastic fiber pollution problem. Co-exposure to dyes and chemicals leaching from plastic fibers is a common phenomenon (Wang et al.,
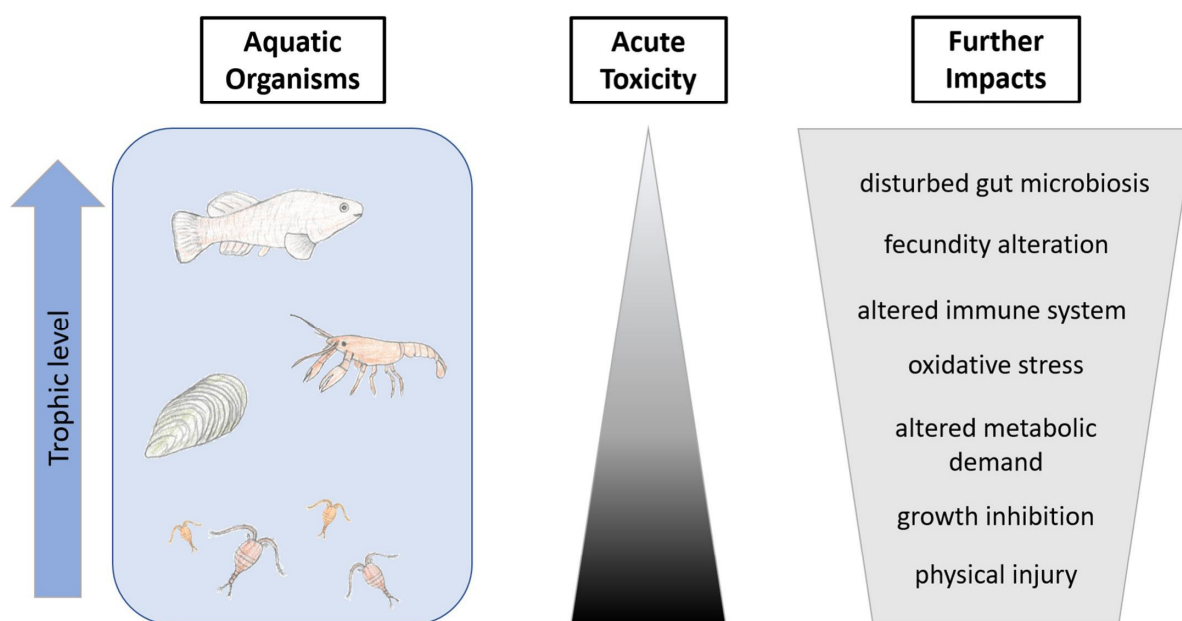

Fig. 2. Summary of effects of microplastic fibers on aquatic organisms observed in exposure studies.

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2017b). Several dyes and chemicals used in the production process of textiles have already been shown to be acutely toxic (Athira and Jaya, 2018; Selvaraj et al., 2015). For example, endocrine disruption was observed as a result of very low doses of plasticizers and other additives of synthetic materials in humans (Vandenberg et al., 2012). Furthermore, chemical additives were detected in aqueous leachates of virgin and aged (photodegraded) microplastic fibers (Sait et al., 2020). However, for persistent, bioaccumulative, and toxic substances sorbed by microplastics from the aquatic environment, models demonstrate a relatively minor role as vector (Bakir et al., 2016; Gouin et al., 2011; Koelmans et al., 2016; Ziccardi et al., 2016). Other exposure pathways, such as the water column and food, are more important than microplastics for uptake into biological organisms. For leachates of plastic additives, the substance composition contained in the polymers is mostly unknown and thus the quantification is hampered. One study demonstrated low amounts of additives (<5% of initial concentration in plastics) in aqueous leachates of different plastic polymer fragments that were submerged in water for 2–3 months (Suhrhoff and Scholz-Bottcher, 2016). Yet, lower trophic levels such as zooplankton and bivalves were already affected at low concentrations of plasticizers in the water, which coincide with environmental levels at some locations (Oehlmann et al., 2009; Zimmermann et al., 2020). Furthermore, gastric and intestinal fluids might enhance leaching of additive chemical substances despite mostly low residence times of fibers within the organisms, as was demonstrated for polyethylene fragments with simulated gastric and intestinal fluids (Chen et al., 2021; Luo et al., 2020).Therefore, we recommend to examine and quantify the leaching potential of additives within organisms once microplastic fibers are ingested. Furthermore, future exposure studies need to address distinct effects of colorants and other additives in fibers that potentially account for or add to microplastic fiber impacts on organisms.

3.6. Effects of organismic processing on microplastic fibers

Ingestion of microplastic fibers and their processing via the digestive system may result in modifications of the fibers in some organisms. Shore crabs (*C. maenas*) use a gastric mill in the cardiac stomach to grind carapace shells and plant tissues before passage to the gut. When polypropylene fibers were added to their diet, smaller overall size and length of fibers was a result of the grinding process in the gastric mill (Watts et al., 2015).

In medaka fish, grooves were detected on the surface of ingested polypropylene fibers extracted from the hindgut. Such grooves were not present in pristine fibers placed into the water column or fibers collected from the foregut (Hu et al., 2020). Peristalsis of the gut wall might have increased the contact between microplastic fibers and adjacent material leading to formation of the grooves (Hu et al., 2020). No significant microplastic fiber breakage occurred during the passage, and risk of increased toxicity due to enhanced release of fiber additives was assessed to be minor in Japanese medaka (Hu et al., 2020), but might happen in other organisms. Overall, processing of microplastic fibers in animal digestive systems will accelerate the rate in which environmental plastic is fragmented and its additives are released.

4. Conclusions

Microplastic fibers represent a substantial, if not the largest, part of microplastic pollution in aquatic environments. The prominence of this microplastic type and high abundance in certain areas makes it essential to consider microplastic fibers and their toxicity in microplastic pollution risk assessments.

Just the physical contact to microplastic fibers in the water column can lead to external damage of zooplankton and other small organisms. The actual uptake of microplastic fibers depends crucially on the concentration and bioavailability of fibers in the environment, their state of degradation, and the feeding mode and assimilation efficiency of the consumers.

Impacts on individual fitness appeared particularly at lower trophic levels, which were more directly affected than higher trophic levels. Negative effects occurred mostly at experimental microplastic fiber concentrations higher than currently measured in nature. Accordingly, we expect higher risk for all aquatic organisms in local pollution events, e.g. from textile production and waste disposal, and with increasing concentration of fibers due to ongoing release into the environment. Moreover, accumulation of microplastic fibers can happen in certain areas due to sedimentation, currents, and structure of the aquatic landscapes.

Exposure to microplastic fibers may disrupt individuals' fitness and can lead to defective community and population development. Moreover, mortality of lower trophic levels might entail a shift of trophic food webs and have large-scale implications on whole ecosystems. Altered species abundance and – in worst case – (local) extinction of certain species, which are more vulnerable to microplastic pollution or rely on affected species as food source, might occur.

5. Future perspectives

To evaluate the exposure risk of aquatic organisms and infer on aquatic ecosystem functioning in a holistic view, future research should investigate the extent and effect of microplastic fibers present in the environment in more detail. Therefore, we suggest:

- To consider microplastic fibers already in the stage of planning of environmental monitoring studies to ensure the selection of appropriate sampling and analysis methods.
- To use consistent protocols for sampling, extracting, analyzing, and reporting microplastic (fiber) occurrence in monitoring studies.
- To determine absolute microplastic fiber concentrations, along with their spatial variation, for different aquatic systems and increase the number of studies which analyze the uptake of microplastic fibers in organisms.
- To conduct future exposure studies in the laboratory with exposure conditions reported from field surveys regarding polymer type, size classes and concentration of the microplastic fibers.
- To investigate potential chronic effects of microplastic fibers by the use of prolonged exposure periods (several weeks to months).
- To analyze the leaching potential of microplastic fiber additives and their (distinct) impact on organisms.
- To investigate interactive effects of microplastic fiber presence together with other environmental factors, such as higher temperatures, food depletion or rising salinity/ chemical hazard pollution.
- To determine threshold concentrations for microplastic fiber impact on important biological functions, such as oxidative stress, metabolic shifts, disruption of the natural gut microbiota community and changes in the immune system.
- To identify impact thresholds for different life stages of organisms, including more sensitive early life stages.
- To analyze a broad range of taxa and species for fitness impairments by microplastic fibers, and subsequently extrapolate possible hazardous effects on ecosystem level and predict ecological implications.

Whether recovery from or even acclimation to microplastic fiber pollution could happen in aquatic organisms is a completely unexplored field so far, but worth testing due to the persistence of microplastic fibers in the environment. Potential acclimation to microplastic fibers of some species could modify ecosystem functioning on a broad scale. Overall, neglected microplastic fibers must play a substantial role in future microplastic research.

CRediT authorship contribution statement

Anja Rebelein: Conceptualization, Writing - original draft, Writing - review & editing, Visualization. **Ivo Int-Veen:** Writing - review &

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Chapter II. Exposure to microplastic fibers does not change fish early life stage development of three-spined sticklebacks (*Gasterosteus aculeatus*)

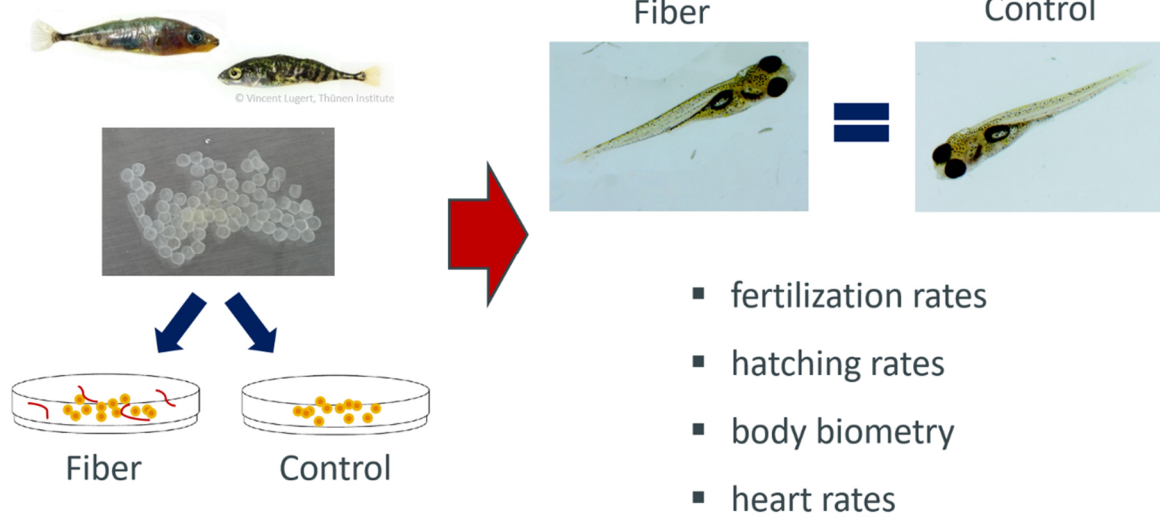Anja Rebelein*, Ulrike Kammann, Jörn Peter Scharsack

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Short Report & Supplementary

Graphical abstract

SHORT REPORT   **Open Access**

Exposure to microplastic fibers does not change fish early life stage development of three-spined sticklebacks (*Gasterosteus aculeatus*)

Anja Bunge (née Rebelein)[*] iD, Ulrike Kammann and Jörn Peter Scharsack

Abstract

Microplastic fibers are frequent contaminants of aquatic ecosystems. Early life stages of aquatic organisms are predicted to be especially vulnerable to microplastic pollution. We hypothesized that microplastic fibers in the water column might interfere with fertilization and embryonic development of fish. We tested this with an in vitro fertilization system with three-spined sticklebacks. Six egg clutches were divided and one half was fertilized and bread out in water with polyester fibers (PET fibers; mean diameter 9.7 ± 2.3 µm; mean length 245.6 ± 163.1 µm) at a concentration of 1×10^4 fibers/L while the other half served as control without fibers.

Observation with a dissection microscope revealed that some polyester fibers stuck to the outside of the eggs in the fiber treatments. Yet, overall $67.4 \pm 12.9\%$ eggs were fertilized from which $97.2 \pm 4.2\%$ larvae hatched without any significant difference between treatments. Mortality and abnormal development of larvae was low and was not changed by microplastic fibers, as was the heart rate of developing embryos five days post fertilization.

The present study illustrates that polyester fibers, even at concentrations three to four orders above levels reported from the environment, do not impair fertilization success, embryonic and early larval development of sticklebacks. Accordingly, concentrations of microplastic fibers currently observed in aquatic habitats do not appear to be harmful to early live stages of fish.

Highlights

First use of fish egg in vitro fertilization assay for microplastic fiber exposure
Fertilization and hatching success of fish was not altered by microplastic fibers
Fish early life stage development was unaffected by microplastic fiber presence

Keywords: Microplastic exposure, In vitro fertilization, Fish eggs, Early life stages, Polyester fiber, Embryonal development

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Introduction

Recent monitoring studies outline that microplastic fibers are the most prevalent type of microplastic debris in many aquatic habitats [1–4]. Accordingly, microplastic fibers often are the dominant microplastic shape that fish encounter [5–7]. Nevertheless, most effect studies of microplastics on fish were conducted with microplastic spheres and fragments, not with fibers [8]. Furthermore, the majority of exposure studies focused on adult life stages [8] although early life stages of aquatic organisms are generally more vulnerable to toxicants [9, 10]. With the present study, we wanted to test if the presence of microplastic fibers in the water column influences fertilization success and early development of three-spined sticklebacks. We suspected that the potential attachment of microplastic fibers to early life stages of fish affect their development.

Changes in embryonic development such as decreased hatching rates and delayed hatching time [11], and changes in blood circulation were reported for fish embryos exposed to polystyrene (PS) spheres and fragments [11, 12]. For example, in zebrafish (*Danio rerio*) exposed to microplastic fragments via the water column, accelerated blood flow velocities and heart rates were explained by hypoxic conditions in the eggs [12]. The microplastic fragments were not internalized but accumulated on the surface of the chorion. Thereby, externally adhered microplastic fragments covered the chorion pores and might have reduced oxygen availability for the embryos. The hypoxic microenvironment likely induced and established the observed alterations in the circulatory system [12].

Similarly, accelerated blood flow velocities and heart rates, and slightly inhibited hatching rates, were observed in a first study conducted with microplastic fibers (polyethylene terephthalate (PET), 3–5 mm) and zebra-fish embryos [13]. For the present study, we chose smaller microplastic fibers (< 0.3 mm) similar to the fiber size class produced during household washing [14, 15], which enters the environment as laundry effluents [16]. Microplastic fibers < 300 μm slip through neuston nets commonly used for sampling fibers in environmental surveillance [17]. Smaller fibers are thus often neglected in monitoring studies [18] and little is known about their potential environmental impact. We used a concentration of 1×10^4 fibers/L, which is in the range of previous exposure studies conducted with adult life stages and microplastic fibers in the water column [19–22]. However, the concentration chosen for the present study is still higher than the concentrations reported from nature that are in the range of 1–10 fibers/L [17, 23, 24]. Yet, microplastic fiber concentrations used for exposure studies must be a compromise between environmental observations and concentrations that can be maintained

as a reproducible and homogenous dispersion of fibers in the water column under laboratory conditions [20]. Furthermore, concentrations of microplastic fibers above currently reported levels can occur in local fiber contamination events, which might become more frequent in the future with rising plastic pollution [25].

In the present study, we used low concentrations of surfactant to facilitate the challenging issue to keep fibers dispersed in the water column, as described previously [26–28]. Furthermore, we used a setup with square-shaped glass bowls for breeding and constant agitation to promote irregular movement of the water column and thus fiber distribution.

We collected egg clutches from mature three-spined stickleback (*Gasterosteus aculeatus*) females and divided them in halves. One half was exposed to pristine polyester fibers (polyester = fibrous form of PET) from fertilization onwards, while the other half served as control. Biological endpoints were fertilization rates, heart rates of embryos, and hatching success. Furthermore, we investigated abnormal development rates and alterations in morphological features of hatched larvae. We hypothesized that microplastic fibers in the water might block the micropyle and thereby prevent fertilization. In addition, we hypothesized that microplastic fibers (< 0.3 mm), smaller than those tested previously [13], can also adhere to the chorion and possibly impair oxygen exchange, which might delay or disturb fish embryo development and lead to changes in heart rates.

Methods

Experimental design

Effects of microplastic fibers in the water column on early development of sticklebacks were tested with eggs from six breeding pairs of sticklebacks. In brief, each egg clutch ($N = 6$) obtained from mature females was divided in two halves before in vitro fertilization with sperm from one male. Half of the egg clutch (85–217 eggs each, Table S1) was fertilized and bread out in water containing polyester fibers (1×10^4 fibers/L; 200 mL total volume) and surfactant (Tween-80, final concentration 3.8×10^{-6}% (v/v)), while the other half served as control in water with surfactant only. Each egg clutch was subjected to complete water exchange every 48 h, whereby fiber treatments received water with the desired fiber concentration. Exposure lasted until three days post hatching (total experimental time of 12 days), the period for which the current EU animal welfare legislation does not apply for stickleback larvae [29]. Fertilization rates, hatching rates, mortality, and frequencies of abnormal body shapes of larvae were recorded. The heart rates of ten embryos per egg clutch half were determined at day five post fertilization, and three days post hatching 15

larvae from each egg clutch half were imaged to monitor potential differences in morphological development.

Microplastic material and quality control

Microplastic fibers were prepared in clean-room facilities from commercial pink polyester knitting yarn (diameter 9.7 ± 2.3 µm (mean ± standard deviation, $N = 206$), Fig. S1) with autofluorescence (excitation 511–551 nm, emission 573–613 nm). The polyester yarn was washed with water and ethanol and cut manually with scissors into small pieces, as described in Rebelein & Focken [30]. Briefly, to exclude large and very small fibers, cut pieces were washed twice through a 300 µm metal sieve (Retsch, Germany) and collected on a 25 µm metal sieve (Retsch, Germany) with pre-filtered 96% ethanol. Microplastic fibers were dried, and 50 mg/L were suspended in ultrapure water for a stock suspension. The stock suspension contained 0.001% (v/v) Tween-80 surfactant solution (Merck, CAS-Nr. 9005-65-6) to facilitate even dispersal of microplastic fibers [28]. We determined fiber concentration of the polyester fiber stock suspension using a Nikon fluorescence microscope (ECLIPSE, Ts2R-FL, Japan; filter setting: excitation 511–551 nm, emission 573–613 nm) with the software NIS-Elements AR (Nikon, 5.02.00). The fiber suspension (25 µL) was pipetted onto microscope slides ($N = 25$), covered with a petri dish while the water evaporated, and directly thereafter autofluorescent polyester fibers were counted under the microscope on the slide. Fiber size distribution was characterized from images of fibers filtered onto 0.8 µm polycarbonate membrane filters. The average size of the polyester fibers in the stock suspension was 245.6 ± 163.1 µm (mean ± standard deviation, $N = 1446$, Fig. S1) in length.

For the exposure of the egg clutches, we prepared experimental treatment suspensions from the stock suspension (2.63×10^6 fibers/L) to contain 10,000 polyester fibers per liter in pre-filtered, temperature-adjusted tap water (equivalent to a mass concentration of 0.19 mg/L). For control treatments, the same volume (761 µL) of ultrapure water that contains 0.001% (v/v) Tween-80 surfactant only was diluted in pre-filtered, temperature-adjusted tap water.

To prevent contamination, plastic labware was avoided and glass and metal labware used whenever possible. Ethanol and tap water were pre-filtered through a Whatman (Typ 1) cellulose filter to remove potential microplastic fiber impurities. All equipment was thoroughly rinsed with filtered deionized water followed by a rinse with filtered 96% ethanol to exclude microplastic contamination. Every workspace was wiped with filtered 96% ethanol before work and utensils were kept covered until use.

Furthermore, blank glass fiber filters (GF/C, Whatman) were placed in the experimental area and exposed to the ambient air for 48 h and one week to check for airborne fiber contamination (Fig. S2). Exposure bowls were kept loosely covered to minimize airborne contamination throughout the experimental period (Fig. S3).

Fish collection and in vitro fertilization

Three-spined sticklebacks in breeding condition were caught at the Luneplate estuary (53°28′37.3″N 8°31′08.9″E), Bremerhaven. Fish were transported to the lab and breeding pairs were subjected to in vitro fertilization as described by Barber & Arnott [31]. Briefly, egg clutches of six females were stripped and each of the six egg clutches was split in halves into two glass petri dishes. We used sperm from one male to fertilize both halves of the split egg clutches from a female (six males in total). Therefore, a drop of sperm buffered in Hank's Balanced Salt Solution was pipetted to each petri dish next to the egg clutch. Treatment suspensions with microplastic fibers or with surfactant only (control) were added and petri dishes swirled for mixing eggs and sperm. The clutches were left for 30 min and thereafter washed with pre-filtered tap water and transferred to glass bowls containing 200 mL of the experimental treatment suspensions.

The square-shaped glass bowls (base area 10×10 cm) facilitated homogeneous dispersion of microplastic fibers in suspension when placed in an angle to the movement direction on a shaker (GFL 1083, Germany) with continuous horizontal agitation (Fig. S3). This setup created an irregular movement of the water column and kept fibers dispersed in the water column, while regular stirring or swiveling induced fiber aggregation (tested in previous method tests). Bowls were maintained at an ambient temperature of 16 °C and treatment suspensions (200 mL) were exchanged every 48 h. Eggshells and dead larvae were removed daily (twice daily during hatching) to ensure good water quality. Daily records were taken of dead respectively unfertilized eggs. The amount of fertilized eggs was determined five days after fertilization when eyes of the embryos were visible. From fertilized eggs, hatching rates were determined. Abnormal development of larvae such as spinal deformities, pericardial edema or yolk sac edema were documented for each treatment according to the description of Cong et al. [32].

Morphometric measurements and heart rate determination

Videos of embryos were taken at day five post fertilization using a stereomicroscope (Nikon SMZ745T, Japan) equipped with a BRESSER MikroCam (SP 5.0, Germany) and Bresser MikroCamLabII software

(v3.7.13814, 2019, Germany). The video material was used to count the heart rates (for 60 s) of ten embryos per half egg clutch.

Three days after hatching the survival rate of stickleback larvae was recorded and 15 randomly chosen larvae per half clutch were measured and photographed under the stereomicroscope. Pictures of the larvae were analyzed with ImageJ 1.52r [33]. Total body length, head length, eye diameter, and length of the swim bladder were analyzed as described by Le Bihanic [34] and Ireland [35].

Microfiber treatment concentration

To check the microplastic fiber concentration as supplemented to the treatment bowls, five additional suspensions were prepared from the microplastic fiber stock suspension in glass bottles. As for the exposure treatments, 761 µL of fiber stock suspension were added to 200 mL pre-filtered, temperature-adjusted tap water in each glass bottle. The bottles were inverted ten times to homogenize the fiber suspension directly before two 50 mL subsamples were taken from each bottle. Subsamples were filtered onto 0.8 µm polycarbonate membrane filters. Filters were imaged under the fluorescence microscope as described for the microplastic fiber stock suspension and fibers counted using the software ImageJ 1.52r [33]. Furthermore, the amount of fibers in suspension was investigated in supplementary glass bowls without egg clutches with 200 mL pre-filtered tap water and either control or fiber experimental treatment suspensions as specified above. The fiber concentration was determined directly after preparation, after 24 h on the shaker, and after 48 h on the shaker from three bowls per control and fiber treatment respectively (18 bowls in total). We filtered two subsamples (50 mL) per bowl on membrane filters and counted the fibers under the microscope as specified above.

Statistics

Statistical tests were performed with RStudio v1.1.463 [36]. Normality distribution and homogeneity of variances of the data were checked with Shapiro-Wilk's test and Levene's test, respectively. Developmental rates were normalized to the total amount of eggs or embryos hatched (Table S1). Potential differences between treatments were analyzed with a Wilcoxon signed-rank test. Biometric and heart rate data were analyzed with a two-way ANOVA (factors treatment and egg clutch) followed by a post-hoc Tukey test, when data were normally distributed. With non-normally distributed data, non-parametric Kruskal-Wallis tests were performed for factor treatment and factor egg clutch with a subsequent post-hoc Wilcoxon rank sum test. A $p < 0.05$ was considered statistically significant.

Results

Exposure with microplastic fibers

We exposed stickleback eggs and larvae to microplastic fibers at a nominal concentration of 1×10^4 fibers/L. Counting of microplastic fibers in additional prepared suspensions ($N = 5$, measured in duplicates) revealed concentrations of 9236 ± 552.7 (mean ± standard deviation) polyester fibers per liter. In the additional square-shaped glass bowls prepared with treatment suspensions but no fish eggs, we quantified $10,924 \pm 1701.8$ fibers (mean ± standard deviation, $N = 3$, measured in duplicates) directly after preparation and found no polyester fibers on the control filters. After 24 h, on average 36.4% of the polyester fibers were still dispersed in the water column (4297.4 ± 1376.2, mean ± standard deviation, $N = 3$, measured in duplicates), which was similar to fiber counts after 48 h (34.4%, 3761.4 ± 1321.5, mean ± standard deviation, $N = 3$, measured in duplicates). We detected only one PES fiber on one subsample filter from the controls at 48 h, which presumably resulted from handling during the filtering procedure. On the other control filters and additional blank filters exposed to the ambient air for one week, we detected only fibers that had a clearly distinguishable appearance in color, shape, or fluorescence intensity to the PES fibers used in the experiment (Fig. S2; maximum of four other fibers per filter compared to > 150 PES fibers on fiber treatment filters). Thus, the level of fiber contamination was low. As fibers tended to aggregate as soon as any irregular shapes, such as (broken) eggshells, dead larvae, or protein aggregates were present, such debris was removed daily and treatment suspensions were exchanged every other day. The treatment bowls were placed on a shaker and agitated throughout the experiment. Together these measures ensured a consistent exposure of egg clutches and larvae to floating microplastic fibers during the experiment. We did not observe microplastic fiber aggregates on the water surface or walls of the treatment bowls. The individual fibers were floating in the water column and we noticed a small proportion moving on the ground of the exposure bowls due to the irregular movement of the water column during agitation. Under the microscope, we observed some fibers that attached to the chorion of the eggs. Yet, fibers tended to attach rather on unfertilized or damaged eggs than fertilized healthy ones (Fig. S4).

Egg & Larval survival and development

Fertilization rates and hatching rates in fiber treatments were not significantly different from control treatments (Table S1, Fig. S5). The mean (± standard deviation) egg fertilization rate was $71.8 \pm 11.4\%$ for fiber treatments and $63.7 \pm 12.3\%$ for control treatments and the hatching rate was $96.5 \pm 5.0\%$ and $96.7 \pm 6.8\%$ for fiber and control

treatments, respectively. Mortality (range 0–5.6%) and abnormal development (range 0–2.4%) of embryos and larvae were generally low (Table S1, Fig. S5) except for one clutch that showed higher mortality (17.1 and 12.6%) and higher abnormal development rates (4.8 and 9.6%) in control and fiber treatment halves of the clutch, respectively. Overall, mortality and abnormal development did not significantly differ between treatments.

Morphological parameters
The morphological parameters measured (body length, head length, eye diameter, swim bladder length, and head-to-body length ratio), did not differ significantly between larvae exposed to polyester fibers and control animals (Table S2). Yet, morphological parameters (except head-to-body-ratio) differed between egg clutches ($p < 0.05$), which demonstrates a greater natural variability between egg clutches of different breeding pairs than between treatments with and without microplastic fibers. The length of the larvae ranged between 6.16 mm and 7.69 mm and the head length ranged between 1.23 mm and 1.81 mm (Table S2).

Heart rates
The heart rates at day five post fertilization did not differ significantly between fiber-exposed and control embryos (Fig. 1). However, the heart rates between egg clutches differed significantly ($p = 0.0117$). The mean heart rate per egg clutch ranged from 86.4 ± 4.2 to 96.1 ± 4.8 beats per minute.

Discussion
The present study addressed possible effects of microplastic fibers in the water column on fertilization of eggs and early development of embryos and larvae of three-spined sticklebacks. We assessed fertilization and hatching success, heart rates of embryos, and morphological features of three-day-old larvae during a laboratory exposure experiment with microplastic fibers. Exposure in square-shaped bowls with slight and irregular movement of the water column, together with frequent water exchanges and fiber replacement, was applied to facilitate that fibers were kept in suspension throughout the experiment. We did not observe significant effects of the microplastic fibers on the vitality parameters investigated here and natural variation between offspring of different adult breeding pairs was higher than treatment effects. This suggests that relatively small microplastic fibers, even at three to four orders higher concentrations than currently observed in the wild, are not harmful to fertilization success and early development of fish larvae.

Environmental relevance of the used microplastic fibers
We chose polyester fibers for the present study, since they are predominantly used in the global textile production in fabrics for apparel, garments, and other finished textiles [37]. Accordingly, polyester fibers are the most common fiber polymer polluting natural water systems [1, 38–40]. We used red-pink polyester fibers, which also showed strong autofluorescence with red filter settings (excitation 511–551 nm, emission 573–613 nm), since they are easy to distinguish in color and shape from other natural or worn fibers that might occur as

Fig. 1 Heart rates of stickleback embryos at day five post fertilization in beats per minute (bpm). Data show mean values (± standard deviation) of 10 embryos per half egg clutch. Different letters indicate statistical differences between egg clutches (ANOVA with post-hoc Tukey test ($p < 0.05$))
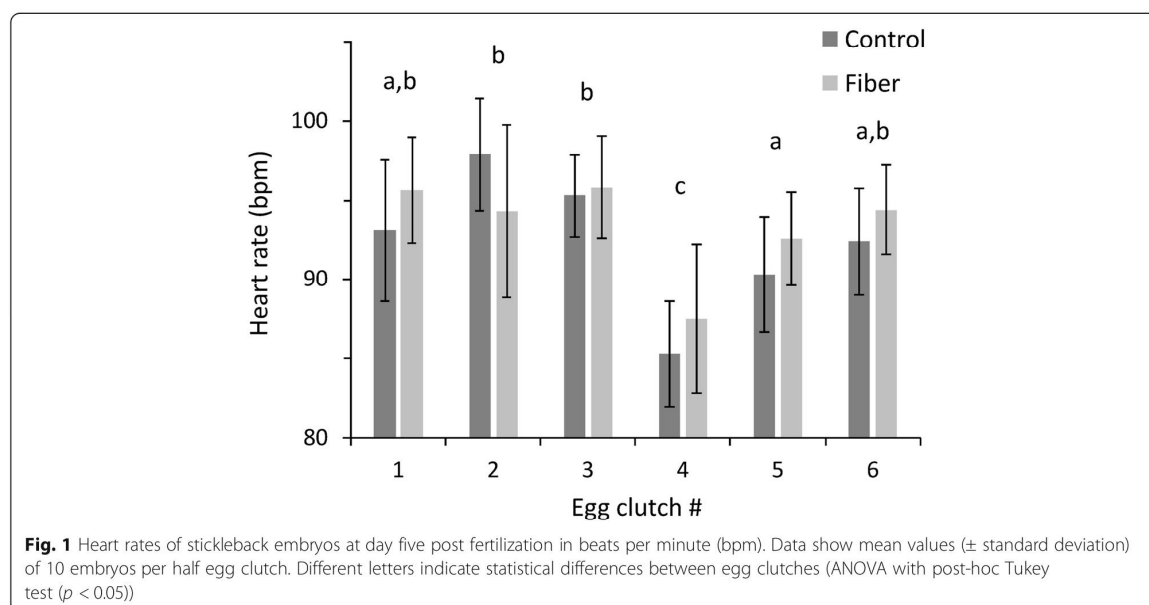
contaminants in the laboratory. For the present study, we filtered fibers through a < 300 μm sieve to resemble the fiber size class that is released during household washing, which can reach the environment as laundry effluent (93% of the released fibers were below 500 μm in length [14]).

The nominal concentration of 1×10^4 fibers/L, as used in the present study, is about three to four orders above values reported from the wild, which were collected with small mesh sizes (0.7 μm and 20 μm) [17, 23, 24]. Yet, higher concentrations might occur in local events of microplastic accumulation or contamination, and globally with expected increases of plastic pollution in the environment [41].

Furthermore, the European Marine Strategy Framework Directive (MSFD) [42] is aiming to reach the good environmental status (GES) in European seas. MSFD covers microplastic as environmental indicator for GES and demands that „The amount of litter and micro-litter ingested by marine animals is at a level that does not adversely affect the health of the species concerned". To reach this goal the MSFD Commission Decision on Methodological Standards [43] demands the development of threshold values for possible adverse effects of microplastic on marine animals. To our knowledge, such threshold values do not exist for microplastic beads, fragments, and fibers in fish yet. Effect studies that cover concentration levels that might occur in nature in the future, like the present one, are crucial to develop such threshold values for microplastic fibers.

Experimental handling of microplastic fibers in the lab

A major concern for aquatic exposure studies with microplastics is to achieve a rather homogeneous distribution within the water column, which is often supported by the use of surfactant [44]. Previous exposure studies with microplastic fragments and spheres used higher surfactant concentrations than the present study and did not observe impacts on the development of zooplankton or fish and sea urchin embryos [26, 27, 45]. Continuous movement of the water in the experimental tanks can also promote homogeneous distribution of microplastics. This was previously achieved in exposure studies with adults and microplastic fibers by mixing the water in the experimental tanks by aeration, thus keeping the fibers in suspension [19, 21]. Yet, strong aeration, which also whirls around the egg clutches and yolk-sac larvae, is not ideal for sensitive embryonic and larval stages. In the present study, we therefore used a setup with square-shaped glass bowls, which were placed diagonal on a horizontal shaker. The slight but irregular movement of the water column kept fibers in motion while not disturbing the egg clutches and hatched larvae.

In laboratory exposure studies, microplastic fibers tend to aggregate, settle to the bottom and adhere to the exposure vessels, and very little fibers stay suspended in the water column at low concentrations [20]. These difficulties often lead to the use of high concentrations of microplastics in exposure studies. For example, a previous study with PET fibers, exposed zebrafish embryos to fibers 3–5 mm in length at a concentration of 20 mg/L [13]. For the present study, we chose shorter fibers (< 0.3 mm) and a much lower concentration of 0.19 mg/L. Methodological tests showed that after 48 h more than a third of the polyester fibers were still dispersed in the water column (equal to more than 600 fibers in the 200 mL exposure volume). The other fibers presumably attached as individual fibers to the bottom and walls of the exposure bowls, since no fiber aggregates were visible. We could not observe fiber aggregates in the exposure bowls when additional obstacles such as the egg clutches were present. Overall, a considerable amount of fibers stayed dispersed in the water column in the present study, even at 100 times lower concentration than used in previous exposure studies.

Effects of microplastic fibers on early life stages

In the present study, individual polyester fibers attached to the chorion of eggs in the fiber treatments. We did not observe internalization of microplastic fibers into eggs. Similar observations of an efficient barrier function of the chorion were made with fish embryos exposed to microplastic and nanoplastics fragments and spheres in the water column [12, 34, 46]. In the present study, we observed that more fibers got stuck to broken eggshells and debris than to intact eggs (Fig. S4). The question is if this observation means that the fibers have caused egg damage or if fibers are simply more adhesive to egg shells and eggs that were damaged for other reasons. Given the absence of difference between treatments with and without fibers, it is unlikely that the fibers had damaged the eggs. Thus, we propose that fibers predominantly stick to broken eggshells and irregular shaped material, and healthy egg clutches with smooth egg surfaces are less susceptible to fiber attachment.

Our results demonstrate that in vitro fertilization rates did not differ between control and fiber treatments. The data indicate that polyester fibers in the water column at the concentration used here do not hinder sperms to reach an egg and enter in through the micropyle. Similarly, in zebrafish in vivo fertilization rates did not change in the presence of small PS spheres (diameter of 1 μm) at concentrations of 1.82×10^7 spheres/L and higher [47]. The present study illustrates that also larger-sized microplastic fibers in the water column do not impair (in vitro) fertilization rates of eggs. Yet, with adult Japanese medaka (*O. latipes*) that were exposed to

polyester fibers in the water column in vivo, slightly increased fertilization rates were seen after two weeks of exposure [19]. Leaching additives that interfere with the endocrine system were suggested as explanation, but not further tested [19]. Thus, in nature chronic exposure of parental life stages with microplastic fibers and/or their additive leachates might affect fertilization rates. However, the present study suggests that the fertilization process itself is not altered by the presence of microplastic fibers in the water column.

Previous studies that used higher microplastic concentrations than the present study reported delayed hatching time, decreased hatching rates, and also altered heart rates of medaka and zebrafish embryos exposed to PS and PET microplastics [11, 13, 48]. This was presumably caused by hypoxic conditions in the eggs due to aggregation of microplastics on the egg surface that hindered the gas exchange [12, 13]. However, significant effects were detected only in treatment groups exposed to relatively high concentrations of 1×10^6 particles/L to 1×10^9 particles/L, which is at least five orders higher than currently observed in nature [17, 23, 24]. In general, toxicity of microplastics seems to increase with rising numbers of particles in the water [49]. Additionally, the present study used shorter microplastic fibers than a previous study [13], which might also have less impact on fish embryos in terms of surface area and adherence to the eggs, and consequential physiological implications to the embryo. Zhao et al. [50] recently demonstrated that intestinal toxicity was more severe when zebrafish were exposed to 200 μm long microplastic fibers than shorter fibers (50 μm) and suggested the aspect ratio of fibers to influence fiber toxicity. A limitation of our study in this respect is that we used only one type and size class of (pristine) fibers at only one concentration to investigate microplastic fiber toxicity on early life stages of fish. In nature, embryos encounter a mix of microplastic fiber polymers, sizes, with and without additive components. Microplastic fibers also interact with the environment and processes such as weathering and biofouling change their characteristics and thereby potentially their impact on organisms.

With the present study, we demonstrated that pristine polyester fibers are not toxic to early life stages of sticklebacks and do not inhibit their development, even at concentrations three to four orders higher than reported from nature. Furthermore, we observed that differences in heart rates of embryos and morphological features of larvae were higher between clutches from different breeding pairs than between half clutches if one half was exposed to microplastic fibers. Our results suggest that natural variability in early life stage development of sticklebacks is bigger than the effect of microplastic fibers in the water column.

Abbreviations
EU: European Union; GES: good environmental status; MSFD: Marine Strategy Framework Directive; PET: polyethylene terephthalate; PS: polystyrene

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s43591-021-00015-x.

Additional file 1 Table S1. Total egg number per half clutch, rates of fertilization and hatching success of egg clutches, and development and mortality of early life stages up to day three post hatching. Fertilization rate refers to fertilized eggs of the total egg number, hatching rate refers to hatched eggs of fertilized eggs, abnormal development refers to the number of abnormal developed embryos and larvae of all fertilized eggs that did survive, and mortality refers to the number of dead embryos and larvae of all fertilized eggs. **Table S2.** Morphometric parameters of stickleback larvae three days post hatching (mean ± standard deviation). Different letters indicate significant differences between egg clutches of the same breeding pair (Kruskal-Wallis test with post-hoc pairwise t-test, $p < 0.05$). **Fig. S1.** Polyester fiber length ($N = 1446$) (**A**) and width ($N = 206$) (**B**) distribution of manual cut pieces after sieving. **Fig. S2.** Polyester fibers with autofluorescence that we used in the study (left) are clearly distinguishable from fibers detected on the filters exposed to the ambient air in the experimental area for one week (right). Size bar marks 500 μm. **Fig. S3.** Experimental setup with square-shaped glass bowls placed in an angle towards the movement direction on the shaker to facilitate irregular movement of the water column (left). Bowls were kept loosely covered with lids, which were previously washed with filtered water and ethanol to prevent air-borne contamination during the experiment (right). **Fig. S4.** Fibers stuck to broken eggshells of sticklebacks (left) and occasionally to the chorion of embryos (right). Pictures were taken at day five post fertilization. Scale bar = 0.5 mm. **Fig. S5.** Fertilization (**A**) and hatching rate (**B**) of stickleback eggs, abnormal development (**C**) and mortality (**D**) of stickleback early life stages up to day three post hatching. Coordinates on the abscissa show the percentage values observed in the control half of the egg clutches and the coordinate on the ordinate gives the percentage observed in the respective fiber treatment half egg clutch.

Declarations

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Supplementary for Chapter II

Bunge (née Rebelein), A., Kammann, U., & Scharsack, J. P. (2021). Exposure to microplastic fibers does not change fish early life stage development of three-spined sticklebacks (*Gasterosteus aculeatus*). *Microplastics and Nanoplastics*, 1:15.

Table S1. Total egg number per half clutch, rates of fertilization and hatching success of egg clutches, and development and mortality of early life stages up to day three post hatching. Fertilization rate refers to fertilized eggs of the total egg number, hatching rate refers to hatched eggs of fertilized eggs, abnormal development refers to the number of abnormal developed embryos and larvae of all fertilized eggs that did survive, and mortality refers to the number of dead embryos and larvae of all fertilized eggs.

egg clutch	treatment	egg number	fertilization rate (%)	hatching rate (%)	abnormal development (%)	mortality (%)
1	Control	91	73.6	98.5	0.0	1.5
	Fiber	107	69.2	97.3	0.0	2.7
2	Control	158	48.1	82.9	4.8	17.1
	Fiber	217	65.9	87.4	9.6	12.6
3	Control	117	47.9	100	0.0	0.0
	Fiber	89	74.2	100	1.5	0.0
4	Control	114	73.7	98.8	2.4	1.2
	Fiber	127	84.3	100	0.0	0.0
5	Control	122	68.0	100	0.0	0.0
	Fiber	102	53.9	100	0.0	0.0
6	Control	85	70.6	100	0.0	0.0
	Fiber	107	83.2	94.4	2.4	5.6

Table S2. Morphometric parameters of stickleback larvae three days post hatching (mean ± standard deviation). Different letters indicate significant differences between egg clutches of the same breeding pair (Kruskal-Wallis test with post-hoc pairwise t-test, p<0.05).

| egg clutch	treatment	length body (mm)	length head (mm)	ratio head/body	diameter eye (mm)	length swim bladder (mm)
1	Control	6.59 ± 0.09 [a]	1.45 ± 0.09 [a]	0.22 ± 0.01	0.62 ± 0.02 [a]	0.62 ± 0.07 [a]
	Fiber	6.58 ± 0.17 [a]	1.45 ± 0.09 [a]	0.22 ± 0.02	0.64 ± 0.02 [a]	0.67 ± 0.04 [a]
2	Control	6.91 ± 0.20 [b]	1.54 ± 0.11 [b]	0.22 ± 0.02	0.64 ± 0.02 [a,b,c]	0.68 ± 0.05 [a,b]
	Fiber	6.82 ± 0.22 [b]	1.58 ± 0.06 [b]	0.23 ± 0.01	0.65 ± 0.03 [a,b,c]	0.64 ± 0.07 [a,b]
3	Control	7.06 ± 0.09 [b]	1.62 ± 0.09 [b]	0.23 ± 0.01	0.65 ± 0.02 [b,c]	0.77 ± 0.06 [c,d]
	Fiber	6.90 ± 0.16 [b]	1.59 ± 0.09 [b]	0.23 ± 0.01	0.65 ± 0.02 [b,c]	0.68 ± 0.06 [c,d]
4	Control	6.89 ± 0.15 [b]	1.56 ± 0.10 [b]	0.23 ± 0.02	0.63 ± 0.02 [a,c]	0.72 ± 0.06 [c,d]
	Fiber	6.87 ± 0.14 [b]	1.52 ± 0.11 [b]	0.22 ± 0.02	0.64 ± 0.03 [a,c]	0.71 ± 0.04 [c,d]
5	Control	7.02 ± 0.77 [c]	1.57 ± 0.20 [b]	0.22 ± 0.01	0.65 ± 0.07 [b]	0.69 ± 0.14 [c]
	Fiber	7.29 ± 0.17 [c]	1.61 ± 0.08 [b]	0.22 ± 0.01	0.66 ± 0.02 [b]	0.80 ± 0.07 [c]
6	Control	7.01 ± 0.16 [b]	1.57 ± 0.06 [b]	0.22 ± 0.01	0.65 ± 0.02 [a,b,c]	0.70 ± 0.04 [a,b,d]
	Fiber	6.83 ± 0.27 [b]	1.54 ± 0.09 [b]	0.23 ± 0.01	0.63 ± 0.03 [a,b,c]	0.67 ± 0.07 [a,b,d]
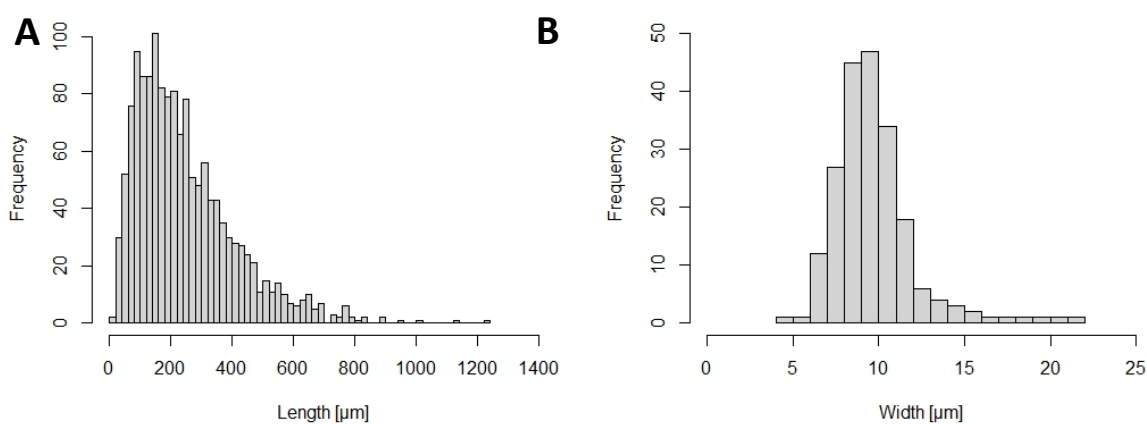
Figure S1. Polyester fiber length (N=1446) (A) and width (N=206) (B) distribution of manual cut pieces after sieving.
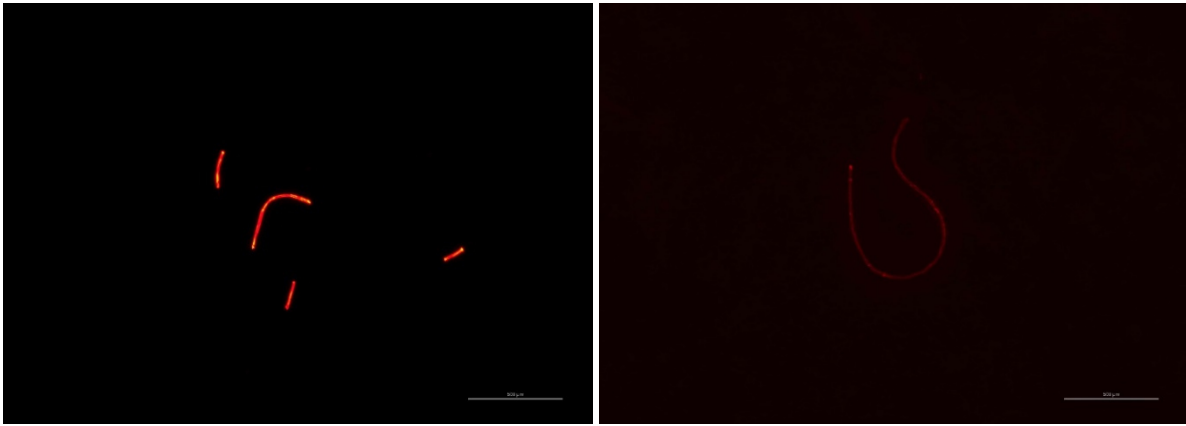
Figure S2. Polyester fibers with autofluorescence that we used in the study (left) are clearly distinguishable from fibers detected on the filters exposed to the ambient air in the experimental area for one week (right). Size bar marks 500 µm.

Figure S3. Experimental setup with square-shaped glass bowls placed in an angle towards the movement direction on the shaker to facilitate irregular movement of the water column (left). Bowls were kept loosely covered with lids, which were previously washed with filtered water and ethanol to prevent air-borne contamination during the experiment (right).
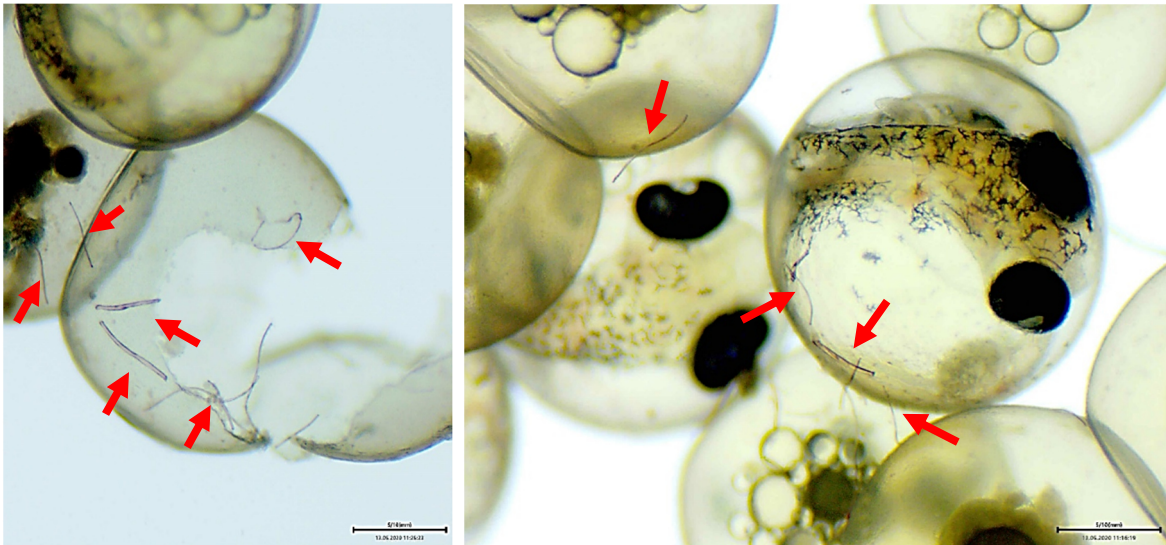
Figure S4. Fibers stuck to broken eggshells of sticklebacks (left) and occasionally to the chorion of embryos (right). Pictures were taken at day five post fertilization. Scale bar = 0.5 mm.
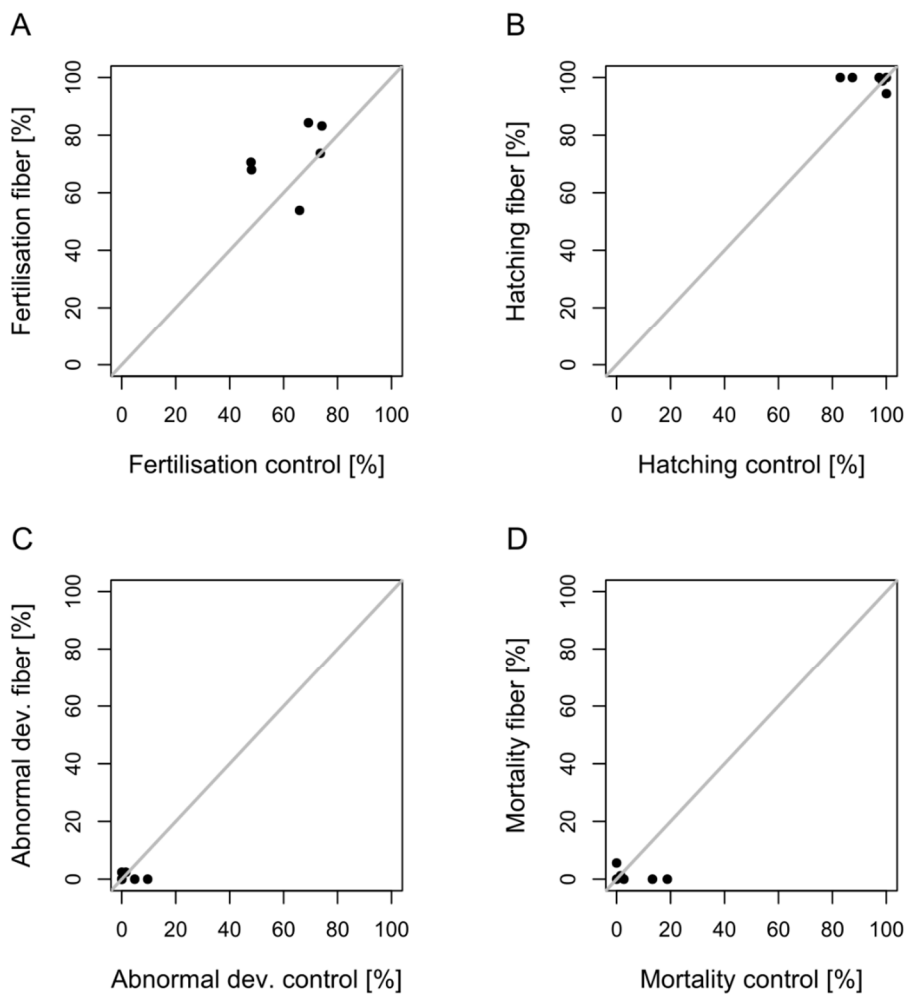

Figure S5. Fertilization (A) and hatching rate (B) of stickleback eggs, abnormal development (C) and mortality (D) of stickleback early life stages up to day three post hatching. Coordinates on the abscissa show the percentage values observed in the control half of the egg clutches and the coordinate on the ordinate gives the percentage observed in the respective fiber treatment half egg clutch.

Chapter III. Microplastic fiber diet—Fiber-supplemented pellets for small fish
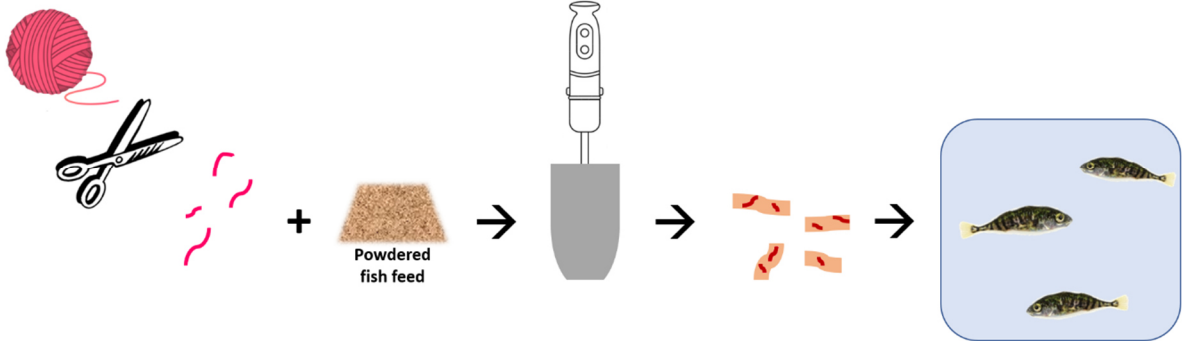
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Method article & Supplementary

Graphical abstract

Method Article

Microplastic fiber diet—Fiber-supplemented pellets for small fish

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ABSTRACT

Ingestion of microplastic particles and fibers is frequently reported for aquatic organisms collected in the field. At the same time, only few studies investigate potential effects of ingestion of microplastic fibers due to handling issues in the laboratory. Exposure studies, which provide organisms with microplastic fibers via the diet, are a necessary step to analyze impact thresholds of vital and fitness parameters of aquatic organisms. Based on the limited number of studies providing fish with fiber-supplemented pellets, the following protocol presents a way to prepare a diet for fish that is supplemented with homogeneous distributed microplastic fibers for exposure studies. Produced pellets are suitable for small experimental fish, such as sticklebacks (2–5 cm), and can be manufactured up to amounts of several hundred grams and even few kilograms. The method can be adapted to different commercial fish feeds and microplastic fiber types due to manual preparation.

- Low-cost, manual preparation of microplastic fibers
- Preparation of a pelleted fish diet with uniformly distributed fibers
- Adaptable to different commercial fish feeds and microplastic fiber types.

Specifications table

Subject Area:	Environmental Science
More specific subject area:	Microplastic fiber exposure via dietary pellets
Method name:	Manual preparation of fish pellets containing microplastic fibers
Name and reference of original method:	n.a.
Resource availability:	n.a.

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Method details

Background

Microplastic items (< 5 mm) are part of anthropogenic litter that are now ubiquitous in marine, freshwater and terrestrial ecosystems, and turned into an issue of global concern. Within the last decade increasing numbers of studies that investigate potential adverse effects of microplastic items on organisms were published. Provencher et al. [12] have recently called for more standardization in order to achieve repeatable methodologies, Barcelo [1] has stressed the importance of standardized analytical methods, and Barletta et al. [2] have presented a detailed sampling design to study microplastics in coastal and estuarine systems. However, in open systems such as estuaries, multiple stressors are present, and laboratory studies are necessary to identify their individual effects and the potential interaction between them. In aquatic environments the actual microplastic fiber contamination is suspected to be much higher than that of microplastic particles [3,4,7]. Microplastic fibers are even more difficult to handle than microplastic particles due to omni-present contamination issue, the entanglement and aggregation potential of fibers, and the lack of reference material – and were thus often neglected. The main challenges to overcome when handling microplastic fibers in the laboratory are exclusion of other airborne fibers, difficulties in weighing and counting the thin and irregular shaped fibers, and production of a homogeneous distribution of fibers in water and other matrices. Only a small number of studies were published so far that conducted exposure experiments with microplastic fibers provided via the diet [6,8,9,11,13]. While for gastropods and crustaceans inclusion of fibers in a biofilm or gelatinous matrix is possible, dietary pellets that contain microplastic fibers are more suitable for fish. Yet, manual insertion of fibers in fish pellets [8,11] is elaborate and only feasible for small number of pellets. Longterm-feeding of small fish (<3 g) already requires several gram feeds per fish (1 g equals 400–600 pellets), which cannot be manufactured by hand. Here, a protocol for the production of a fish diet (pellets), which contains an adjustable content of microplastic fibers in amounts that allow long-term feeding experiments with small fish, is presented. The described method was developed for a long-term exposure of juvenile stickleback (4–5 months old) with microplastic fibers via the diet. Pellets are prepared from a commercial fish feed that resembles natural food sources for sticklebacks. The pellets are supplemented with polyester fibers, which are a common fiber type in the textile industry [4] and in aquatic environments [7].

Quality control

Glass and metal lab ware were used whenever possible. All equipment was thoroughly rinsed with ultra-pure water or filtered deionized water followed by a rinse with 96% ethanol to exclude microplastic contamination. Work was conducted under a laminar flow hood and every workspace was cleaned with ethanol before work. A cotton lab coat and green disposable gloves were worn at all times and it was refrained from wearing accessories. The ethanol was pre-filtered through a Whatman (Typ 1) cellulose filter to remove potential microplastic fiber impurities. Utensils and products were kept covered whenever possible and tools observed for contamination prior to operations.

Microplastic fiber preparation

Material

- Microplastic fiber wool (e.g. autofluorescent polyester)
- Fine scissors
- Glass petri dish
- Metal/ ceramic bowls
- Test sieves made from stainless steel (e.g. 25 μm and 300 μm)
- Glass tubes with aluminum screw cap
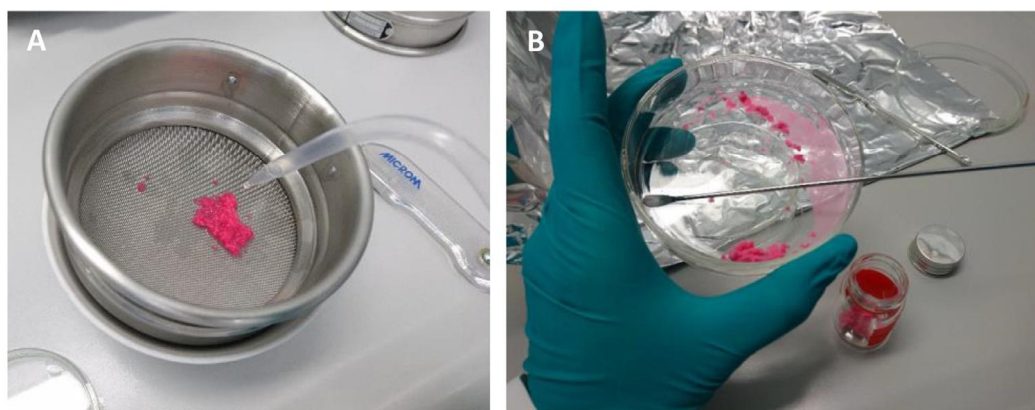- Metal spatula and tweezers
- Aluminum foil

Fig. 1. Preparation of microplastic fibers from commercial garment: sieving of cut fiber pieces through metal sieve (A) and collection of dried fibers into a glass tube (B). © Thünen-Institut/ Anja Rebelein.

- 96% Ethanol (filtered, in a spray bottle)
- Ultrapure water
- Fluorescence microscope (e.g. Nikon ECLIPSE, Ts2R-FL)
- Software NIS-Elements AR (Nikon, 5.02.00)

Procedure

Microplastic fibers were prepared in cleanroom facilities. Pink-red commercial polyester wool (Kuschelgarn, JES Collection, Germany) was used as raw material for the fibers. The garment was washed with water and ethanol to remove potential external dirt. Afterwards the threads were cut manually with scissors into small pieces. The process involves extensive cutting of folded wool sections for 45–60 min each. A high number of small-sized fiber pieces results from repeated chopping of the wool material. Cutting was done over a petri dish filled with some filtered ethanol to prevent that cut fiber pieces spread and distribute in the laminar flow hood due to electrostatic charging and instead stick to the petri dish after dropping. Moisten the garment with ethanol prior to cutting also helps to minimize fiber spreading. Extensive cutting of wool garment (mean 29.8 cm/ 292.2 mg per section) for 45–60 min releases about 159.5 mg small fiber pieces. Cut pieces were sieved twice through a 300 μm metal sieve and collected with a 25 μm metal sieve (Retsch, Germany) using pre-filtered ethanol (Fig. 1A) to narrow the fiber size spectrum of manual cut pieces. The respective filter mesh sizes were selected to create fibers that resemble the size range of polyester fibers released from textiles during washing (majority between 100 and 800 μm) [10]. However, this can be modified, and other mesh sizes facilitate the collection of different size fractions of fibers.

Collected polyester fibers were flushed with ethanol from the sieve into glass petri dishes. Petri dishes were left under the fume hood to evaporate the ethanol overnight. A loose cover with aluminum foil restricted potential airborne contamination. Dried fibers were collected with metal spatulas and stored in (aluminum) screw capped glass tubes (schuett-biotec, Germany) (Fig. 1B). Fibers were kept dry for storage to prevent aggregation as happens in aqueous suspensions.

Diet preparation

Material

- Cut microplastic fibers (e.g. polyester)
- Commercial fish feed, fine powdered (e.g. Essence (0.2–0.3 mm), Alltech Coppens)
- Commercial blender with metal whisks
- Metal press with disc insert for 1 mm diameter diet

Table 1

Ingredients for 50 g fish diet supplemented with 0.2 mg and 2 mg microplastic fibers per gram commercial feed (Essence).

Ingredients	Polyester Fibers	Essence feed	Deionized water
0.2 mg/g diet	0.01 g	50 g	21.5 ml
2 mg/g diet	0.1 g	50 g	21.5 ml

- Metal mixing beaker
- Glass beaker
- Glass pipette
- Sieve (0.6 mm mesh)
- Aluminum foil
- Metal tweezers
- Chopping knife
- Deionized Water
- 96% Ethanol (filtered, in a spray bottle)
- Analytical balance (e.g. Sartorius)
- Fluorescence microscope (e.g. Nikon ECLIPSE, Ts2R-FL)
- Software NIS-Elements AR (Nikon, 5.02.00)

Procedure

The fish diet was prepared by adding commercial fish feed to cut microplastic fiber pieces. Fine powdered fish feed (Essence, Alltech Coppens, Netherlands (S1)) with 0.2–0.3 mm grain size was used, as this feed is designed for recirculating systems and features an Artemia alternative that supplies the nutrient demand of sticklebacks.

Test diets were prepared with 0.2 mg and 2 mg fibers per g of fish feed (Table 1). Plastic fiber amounts (dry cut pieces) for about 50 g of feed were weighed into glass vials using an analytical balance (Sartorius, Germany) and suspended in pre-filtered ethanol. Microplastic fibers are easier to suspend and keep separate in ethanol than water, and aqueous suspension with surfactant should be avoided for fish diets. The fiber suspension was transferred to a metal mixing beaker (WMF, Germany) and the vial was rinsed twice with ethanol to transfer the total fiber amount. The ethanol suspension facilitates an equal distribution of fibers, which form a thin layer on the bottom of the mixing beaker. Fiber spreading with as little overlay as possible is necessary to ensure that fibers do not clump during mixing and distribute homogeneously within the diet. Depending on the size of the mixing container and the targeted final fiber concentration, only certain amounts of fibers can be processed at once. With the described setup (mixing beaker with 63.6 cm^2 bottom surface), a maximum of about 150 mg fibers or 50–100 g of feed (depending on the fiber concentration) can be produced in one step.

The fiber suspension was left to dry in the fume hood loosely covered with aluminum foil over night to evaporate the ethanol completely (Fig. 2A). Commercial fish feed (1 g per 0.2/ 2 mg fibers weighed) was added on top of the spread-out fibers and the dry components mixed thoroughly with a commercial blender (Fig. 2B). The well-mixed dry mass was supplemented with deionized water to form a homogeneous dough that could be pressed into feed strings (Fig. 2C, Table 1). A water content of 43% (v/w), which results in a just formable diet dough, was determined as optimum for the Essence feed and used setup. A higher water content would lead to a smoother dough, but results in more dense and harder pellets after drying, which are difficult to ingest for the fish. The feed strings were produced with a mechanical press ("Sugar paste extruder", LIHAO, China) and had a diameter of 1 mm (Fig. 2D). The feed was left loosely covered with aluminum foil to dry in the fume hood overnight. Dried feed was crushed manually and with a chopping knife into pellets which can be easily ingested by the fish (1–5 mm in length). The pellet mix was sieved (0.6 mm mesh) to exclude powder and broken pieces smaller than 0.6 mm when feeding experimental sticklebacks.
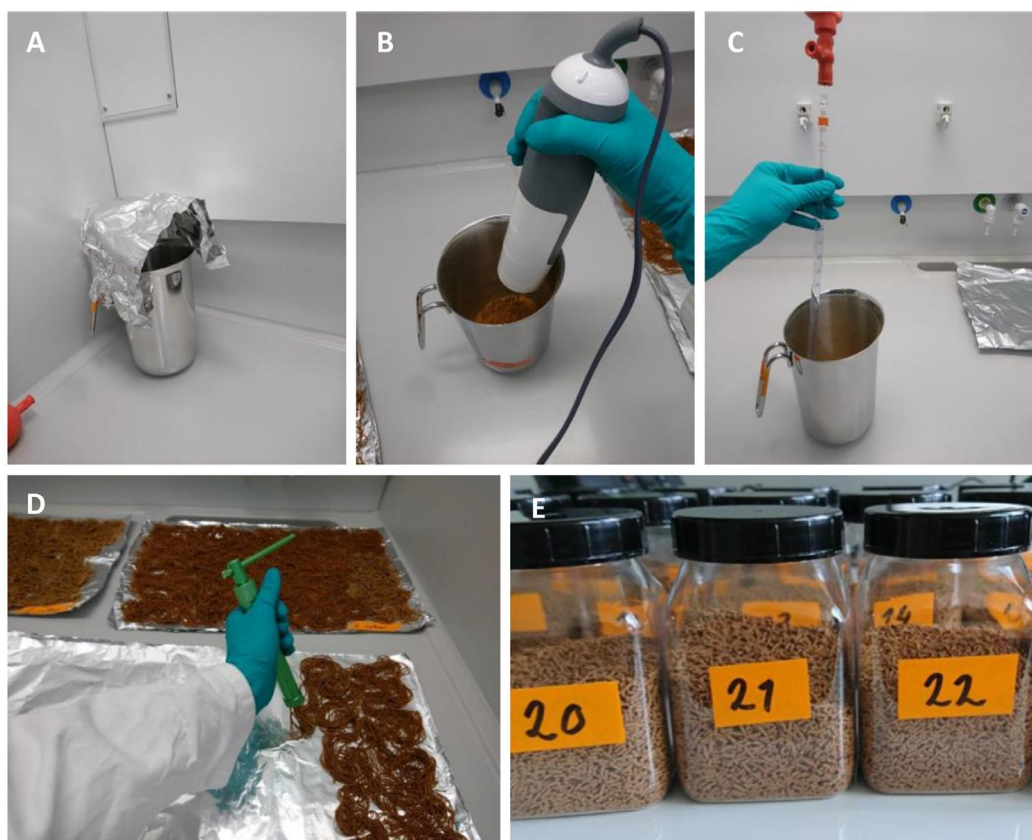
Fig. 2. Preparation of the fish diet: evaporation of ethanol (A), mixing of dry components (B), addition of deionized water (C) and production of diet strings with a mechanical press (D). Dried feed pellets with and without fibers (E). © Thünen-Institut/ Anja Rebelein.

Characteristics of prepared fibers and diet

Characterization of the fibers

Characterization of the polyester fibers was conducted using a Nikon fluorescent microscope (ECLIPSE, Ts2R-FL, Japan). The garment showed strong auto fluorescence in the red fluorescence filter (excitation 511–551 nm; emission 573–613 nm), while less auto fluorescence is visible with the green filter (excitation 490–510 nm; emission 520–550 nm) and no auto fluorescence could be detected with the DAPI filter (excitation 382–392 nm; emission 430–480 nm) (S2). Other fibers, such as fibers from paper towels used in the laboratory, did not fluorescent as intense in the red filter but did show strong fluorescence in the DAPI filter and were thus clearly distinguishable.

For analysis, a fiber suspension was prepared with dried fibers (0.05 mg/mL) in ultrapure water with 0.001% (v/v) Tween-80 surfactant solution (Merck, CAS-Nr. 9005–65–6) to ensure even dispersal of microplastic fibers without fiber aggregation. Low concentrations of the surfactant prevent the development of foam during homogenization of the solution when the glass vial is inverted multiple times, which would lead to uneven distribution of fibers.

The suspension was pipetted onto microscope slides and analyzed using the software NIS-Elements AR (Nikon, 5.02.00). Mean polyester fiber length was 245.6 ± 163.5 µm ($N = 1446$) and fiber widths was 9.7 ± 2.3 µm ($N = 206$). Sieving narrows down the fiber size spectrum (length) to a certain extent (Fig. 3). Yet, the fiber diameter is well below 300 µm and some longer fibers slip through the meshes.
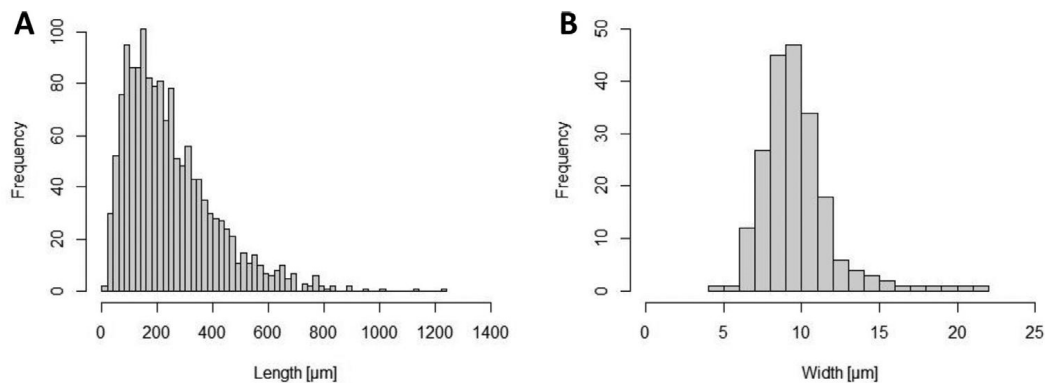
Fig. 3. Polyester fiber length ($N = 1446$) (A) and width ($N = 206$) (B) distribution of manual cut pieces after sieving.
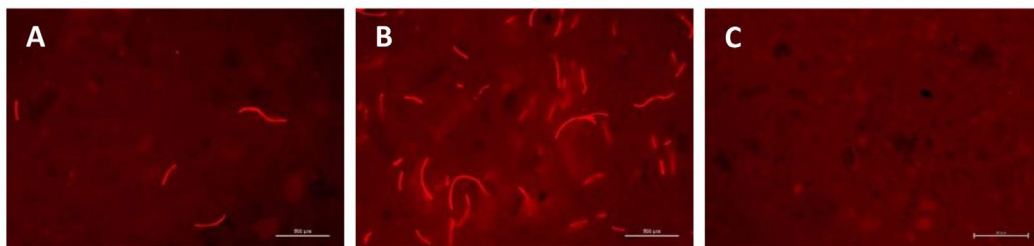

Fig. 4. Homogenous spread of polyester fibers in produced pellets with 0.2 mg (A) and 2 mg (B) fibers per gram feed. Control pellets without fibers produced with pure Essence diet powder (C). © Thünen-Institut/ Anja Rebelein.

Pre-experimental trials revealed manual cutting to be the best comminution technique for the used polyester fibers as these fibers are soft and flexible. Mechanical cutting as e.g. described by Cole [5] might be used as well, but relies on the availability of specific lab equipment. Furthermore, the use of grinders and mechanical blades work better for more stiff plastic fiber materials than polyester, such as nylon. Depending on the fiber material used, mechanical cutting could facilitate a more homogeneous size distribution if that is desired for the experimental design.

Verification of diet homogeneity and diet variations

Homogeneity of the diets was verified by fluorescence microscopy of moistened and flattened pellets. Fibers are clearly visible within the diet (Fig. 4A and B) and thorough mixing ensures a homogeneous spread of the fibers. Control of repeated production lots demonstrated a homogeneous distribution of the fibers in all batches. Once inserted in the tanks, the diet sinks to the bottom of the tanks (5.3 cm/s mean sinking velocity) and sticklebacks feed on it in the water column and on the bottom. Submersed pellets soak some water but remain pelletized for more than an hour (S3), which is similar for feed with or without fibers.

Other commercial feeds can be used as basis as well if different nutrient requirements need to be supplied. Potential commercial feeds should be checked prior to the diet preparation to have low auto fluorescence in the red filter in order to easily identify and check the homogeneity of added fibers (Fig. 4C). Commercial feeds can be powdered or grain feed, but bigger-sized grains should be grinded prior to diet preparation to obtain a fine powder that ensures a homogeneous distribution of fibers. The amount of water necessary to form a smooth dough that is optimal for extrusion might vary depending on the nutritional composition and must be determined for each commercial feed separately. As outlined above, should the consistency of the prepared dough be just wet enough to be pressed with a mechanical press. Other types of microplastic fibers might be used as well provided that they show (at least low) fluorescent signals to confirm uniform distribution within the pellets.

Declaration of Competing Interest

The Authors confirm that there are no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.101204.

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Supplementary for Chapter III

Rebelein, A., & Focken, U. (2020). Microplastic fiber diet–Fiber-supplemented pellets for small fish. MethodsX, 101204.

Table S1. Composition and Energy content of the Essence Feed, as stated by Alltech Coppens.

compound	amount
Protein	45 %
Fat	11 %
Crude Fiber	1.3 %
Ash	7.2 %
Total Phosphor	2.06 %
Vitamin A	14 000 IE/kg

energy content	
Gross energy	16.5 MJ/ kg
Digestible Energy	14.8 MJ/ kg
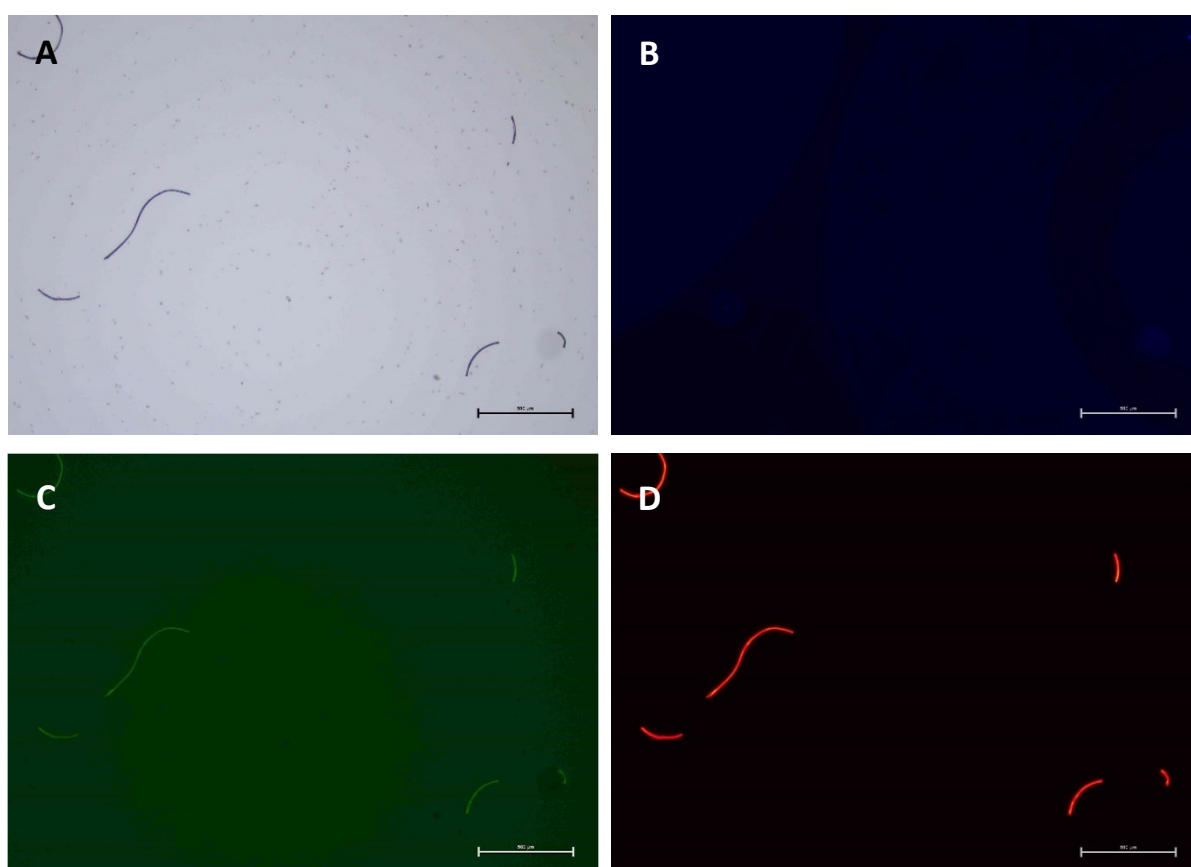
Figure S1. Polyester fibers under a microscope, viewed with transmitting light (A), with a DAPI (B), green (C) and red (D) fluorescence filter captured with 500 ms illumination time (scale bar = 500 µm). © Thünen-Institut/ Anja Rebelein
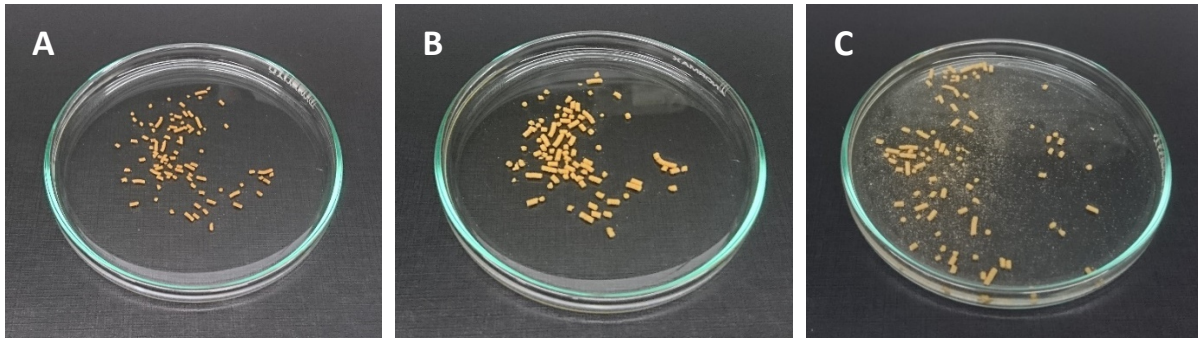
Figure S2. Produced diet placed in water (A), after 60 minutes (B) and after 90 minutes and gentle shaking (C). Petri dishes are 9 cm in diameter. © Thünen-Institut/ Anja Rebelein

Chapter IV. Less impact than suspected: Dietary exposure of three-spined sticklebacks to microplastic fibers does not affect their body condition and immune parameters

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Less impact than suspected: Dietary exposure of three-spined sticklebacks to microplastic fibers does not affect their body condition and immune parameters

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HIGHLIGHTS

- Dietary exposure to polyester fibers does not affect the body condition of fish.
- Polyester fiber amounts higher than encountered in nature do not affect fish growth.
- Immune parameters are sensitive enough to measure gender differences.
- Ingestion of fibers does not induce alterations in immune parameters.
- Absence of impacts on fish similar for ingested polyester and natural fibers.

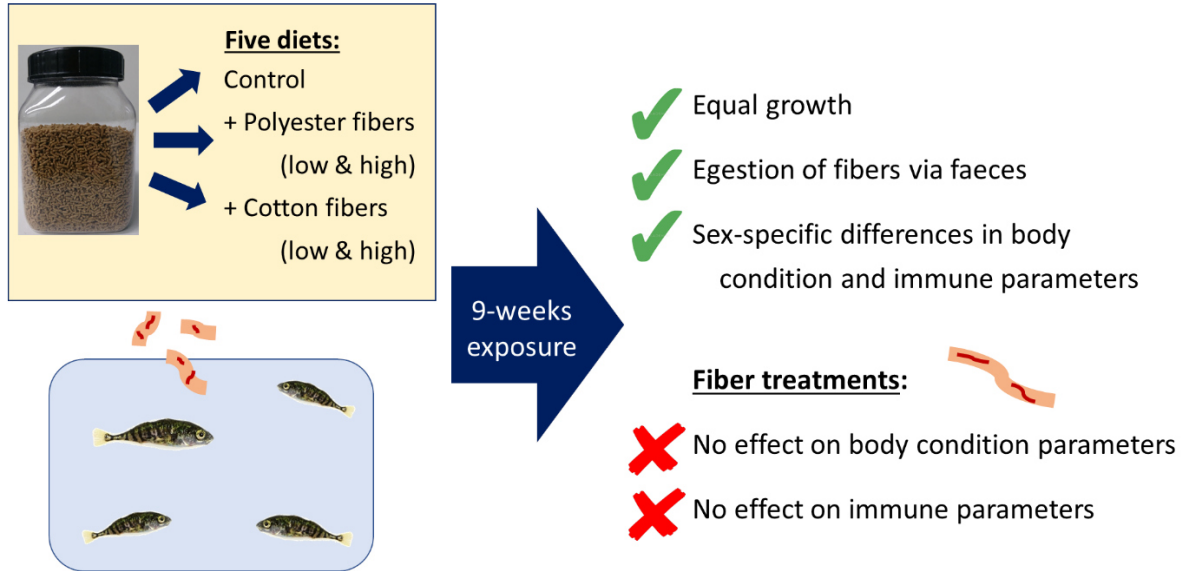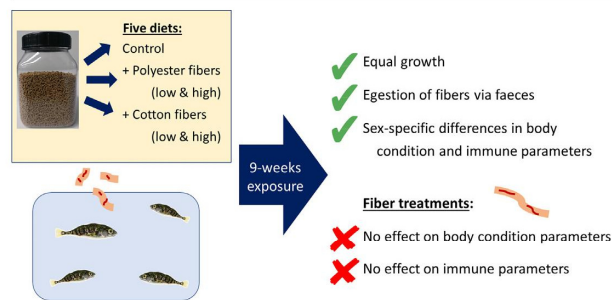## GRAPHICAL ABSTRACT

ARTICLE INFO

ABSTRACT

Microplastic fibers are frequent anthropogenic contaminants in most aquatic environments and have consequently been detected in the digestive tract of many fish species. Upon ingestion, microplastic fibers pose risks of interference with nutrient uptake, impaired intestinal health, and as a consequence may alter growth performance and fitness. In addition, foreign particles such as fibers might cause tissue irritations and stress, and thus interfere with immune parameters. In nature, fish regularly encounter microplastic fibers as well as fiber debris from natural sources and materials. Thus, we wanted to test the potential impact of microplastic fibers on growth, organosomatic indices, and immune parameters of subadult fish and compare these to possible effects caused by natural fibers. We administered sticklebacks diets, which were supplemented with either polyester or cotton fibers (each at concentrations of 0.2 mg/g and 2 mg/g feed) or a control diet without fiber supplementation for nine weeks. Mortalities did not occur and sticklebacks grew equally well across treatments. Neither organosomatic indices nor immune parameters revealed significant differences between treatments. While natural differences between males and females were observed for some parameters, no treatment-related gender-specific effects were detected. Our results suggest that the dietary uptake of polyester fibers does not affect growth, body condition, gonad development, and immunity of sticklebacks – even at fiber concentrations higher than what can be encountered in the wild. Furthermore, virgin microplastic fibers do not seem to affect fish differently than fibers from natural origin. The present study implies that at least some species are resilient towards pollution with (virgin) microplastic fibers even at high concentrations.

1. Introduction

Microplastics are ubiquitous in freshwater, marine, and terrestrial environments (Andrady, 2011; Barnes et al., 2009) and pose a global environmental threat (Galgani et al., 2015). Intended and unintended microplastic ingestion by fish, birds, reptiles, and mammals is widespread and expected to increase with the abundance of plastics in the environment (Savoca et al., 2021). The ingestion of microplastics is suspected to be harmful to organisms. For instance, some studies reported lower body conditions along with a higher concentration of ingested microplastics in wild fish (Mizraji et al., 2017; Sbrana et al., 2020). However, it remained unclear if higher ingestion of microplastics leads to lower body condition or if individuals with lower body condition are more prone to microplastic ingestion (Mizraji et al., 2017; Sbrana et al., 2020). Overall, it is difficult to detect and quantify potential negative effects caused by microplastics in the wild and consequently, experimental studies are conducted.

Fish that were experimentally exposed to microplastics in the water and their food showed a wide range of responses of different severity, such as effects on growth, body parameters, blood components, dietary enzymes, intestinal permeability, oxidative stress, and their microbiome, as reviewed by Jacob et al. (2020). However, the meta-analysis by Jacob et al. (2020) revealed that only 32% of the 782 endpoints investigated in effect studies were significantly affected by virgin microplastics. Whether or not an effect is detected and the severity of it can be influenced by the microplastic dose, morphology, polymer type, and size, but clear correlations do not exist up to date (Bucci et al., 2020). Exposure studies conducted so far vary markedly in used microplastic characteristics (polymer, size, shape), species investigated, exposure pathway (via the water, via food, with prey), and other exposure characteristics (time of exposure, concentration) (Jacob et al., 2020), which makes it difficult to provide general microplastic toxicity statements. This forces exposure studies to pay closer attention to the (environmental) relevance of exposure criteria and how to interpret reported results (Phuong et al., 2016).

In many aquatic environments, microplastic fibers (MPFs) are the most prevalent microplastic component (Barrows et al., 2018; Gago et al., 2018; Li et al., 2021) and are concurrently the most common microplastic component detected in fish (Bessa et al., 2018; Collard et al., 2018; Horton et al., 2018; Jabeen et al., 2017; Parker et al., 2020). However, the effects of fiber ingestion on fish are barely investigated, since most experimental effect studies were conducted with microplastic spheres and fragments, and not fibers (Jacob et al., 2020). The studies that did expose fish to different microplastic shapes suggested that the toxicity induced by microplastics is form-dependent, with worse effects induced by microplastic fibers than fragments and spheres (Cheng et al., 2020; Qiao et al., 2019).

Most studies on potential impacts on fish that were conducted with microplastic fibers, exposed the fish via the water column (Hu et al., 2020; Qiao et al., 2019; Zhao et al., 2021). The reported effects of microplastic fibers on fish vary depending on the microplastic fiber characteristics and species used. Hu et al. (2020) did not observe changes in body condition, gonadosomatic- or hepatosomatic indices in Japanese medaka (*Oryzias latipes*) after three weeks of exposure to waterborne polyester (PES = fibrous polyethylene terephthalate (PET), mean length of 350 μm) and polypropylene (PP, mean length of 380 μm) fibers at concentrations of 10,000 fibers per liter. In contrast, Qiao et al. (2019) reported lower body weights and body conditions in zebrafish (*Danio rerio*) exposed to PP fibers (mean length of 25 μm, concentration of 10 μg/L (~680 fibers per liter)) via the water column for three weeks compared to control animals. This was explained by mucosal damage and inflammation in the intestinal tract upon ingestion of the microplastic fibers, which presumably caused metabolic disruption and subsequently growth inhibition (Qiao et al., 2019). Furthermore, fiber exposure was reported to increase the permeability of the intestines and induce gut microbiota dysbiosis (Qiao et al., 2019). Intestinal damage and inflammation were also observed in zebrafish exposed to PP fibers of different size classes (50 ± 26 μm and 200 ± 90 μm in length) in the water, whereby higher concentrations and longer sized fibers led to more severe impacts, which were attributed to a potentially

longer retention time within the fish (Zhao et al., 2021). Together, those observations show the complexity of the microplastic pollution problem due to the heterogeneity of microplastics. Yet, previous studies point to the fact that oral uptake of microplastic fibers is a major issue when determining the potential impacts of microplastic fibers on fish.

Fish encounter microplastics not only in the water column but also within and attached to their food items and prey (Cole et al., 2013). Organisms at lower trophic levels were more frequently observed to be contaminated by microplastic pollution than apex predators, and microplastics do not appear to accumulate in the food chain (Gouin, 2020; Walkinshaw et al., 2020). Nevertheless, in nature fish will take up microplastics including fibers with their food items from lower trophic levels, in addition to active and passive uptake from the water column (Roch et al., 2020). Furthermore, aquaculture species might ingest microplastics unintentionally with their feed since recent analyses detected microplastics in fish meals from ten different countries (Wang et al., 2021).

Only one previous study investigated the effects of direct fiber ingestion in goldfish with microplastic fibers inserted in food pellets (Jabeen et al., 2018). Fiber-exposed goldfish had lower body weights and body condition parameters and showed signs of intestinal inflammation (Jabeen et al., 2018). However, the fibers were manually inserted in the individual pellets (2 mg per pellet), which resulted in high amounts of 18 mg of fibers provided to each fish per week. In addition, the study used long (0.7–5 mm) ethylene vinyl acetate fibers, a polymer type that is not frequently found in nature (Gago et al., 2018). The dominant fiber types reported in waters are polyester (PES = fibrous polyethylene terephthalate (PET)) as a synthetic material and cotton as natural material (Deng et al., 2020; Miller et al., 2017; Stanton et al., 2019; Suaria et al., 2020), which correlates with the most commonly used materials in textiles (Carr, 2017). The potential impact on the gastrointestinal system and fish health by direct ingestion of those fiber polymers has not been studied so far.

Globally, microplastic and natural fibers occur in different ratios, depending on the analyzed water body, but both types are pervasive (Barrows et al., 2018; Lahens et al., 2018; Suaria et al., 2020). Accordingly, aquatic organisms encounter considerable concentrations of suspended natural fibers mixed in with microplastic fibers (Avio et al., 2020). Therefore, we included both, polyester and cotton fibers, in the present study and used fiber sizes similar to the size class (mainly <800 μm) released during washing (Cai et al., 2020; Galvao et al., 2020; Hernandez et al., 2017). We used two different concentrations of fibers included in the feed, to investigate if the factor concentration matters when fish ingest those fiber types common in nature. We were interested in whether fiber ingestion can affect nutrient uptake and growth and other important biological functions such as the immune system, gonadal development, and possibly reproduction success in fish.

The immune system of fish is known to be highly sensitive and can therefore serve as an indicator of responses to environmental stressors (Tort, 2011). It is composed of innate and acquired mechanisms of defense, which counteract exogenous and endogenous disturbing factors (Biller and Takahashi, 2018). Changes in immune parameters can function as an early warning sign for stress in individuals exposed to pollutants. Zebrafish and gilthead seabream (*Sparus aurata*) exposed to microplastic spheres or fragments showed alterations in the transcription of immune genes and the leucocyte respiratory burst activity while no effect was observed on growth performance during short-term exposure (< 1 month) (Espinosa et al., 2017; Jin et al., 2018; Limonta et al., 2021). Whether microplastic fibers have the potential to alter fish immune parameters is unclear.

In the present study, we used the three-spined stickleback (*Gasterosteus aculeatus*) as model organism, a small fish that inhabits marine, estuarine, and freshwater environments. Its wide distribution in the Northern hemisphere and well-documented biology make the stickleback a useful sentinel species for water quality assessments and studies on environmental pollutants (Katsiadaki et al., 2007). The present study aimed at analyzing the potential effects of microplastic and natural fibers administered with the diet over nine weeks. Mortality, growth performance, body condition factor, and organosomatic indices were recorded. In addition, we analyzed cellular

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immune parameters in the head kidneys, the major lymphatic organ of fish (Bjørgen and Koppang, 2021). We assessed total leucocyte counts, frequency of granulocytes, and respiratory burst activity in head kidney leucocytes (HKL).

Ingestion of added fibers might lead to false satiation, irritated intestinal walls, disturbed gut microbiota, or altered energy metabolism. We thus hypothesized that microplastic fiber ingestion lowers growth performance, changes the body condition and organosomatic indices, and possibly the reproduction capacity (e.g. delayed sexual maturation via slowed growth and development of the gonads) of exposed fish. Furthermore, we hypothesized that ingested fibers might cause stress to intestinal tissues and directly or indirectly stimulate the immune system, which induces changes in head kidney leucocyte numbers and activity. We assumed that effects might be more severe with higher concentrations of fibers and potentially as well with polyester fibers compared to cotton fibers. Overall, the study contributes to assessing the potential risk that polyester fibers pose to wild aquatic animals.

2. Methods

2.1. Experimental animals

The present study was carried out following the regional law on animal welfare under a permit of the senator for health, women, and consumer protection, Bremen, Germany (animal experiment No. 159, 500–427–103-7/ 2019-1-11). The experiment was conducted with three-spined sticklebacks (*Gasterosteus aculeatus*) that were raised in the lab after in vitro fertilization of adult sticklebacks captured at the Luneplate estuary (53°28′37.3"N 8°31′ 08.9″E), Bremerhaven, Germany. Fish were kept in freshwater in small aquaria during the first weeks and fed with live Artemia nauplii. After four weeks equal numbers of offspring deriving from nine adult breeding pairs (75 individuals each) were pooled. Fish from that pool were transferred to tanks (180 individuals per 60 L tank) connected to a recirculation system (about 1500 L total volume, freshwater, 14 °C, 12/ 12 h light/ dark cycles) and fed commercial fish feed (Essence, Alltech Coppens, Netherlands).

2.2. Fiber material & diet preparation

In the present exposure study, sticklebacks were fed with pelleted commercial fish feed (Essence, Alltech Coppens, Netherlands) supplemented with polyester fibers 0.2 mg per g feed ("0.2 PES"), polyester fibers 2 mg per g feed ("2 PES"), cotton fibers 0.2 mg per g feed ("0.2 Cotton"), cotton fibers 2 mg per g feed ("2 Cotton") and without fiber supplementation ("Control"). The fiber feeds were prepared as described in Rebelein and Focken (2021). Briefly, microplastic fibers were prepared from pink-red commercial polyester thread (Kuschelgarn, JES Collection, Germany; FTIR spectrum Fig. S1). Pink commercial cotton thread (Topflappengarn, Max Gründl, Germany; FTIR spectrum Fig. S1) was used as a natural source of fiber. The garments were washed and cut into pieces. To exclude large and very small fibers, cut pieces were flushed twice through a 300 µm metal sieve (Retsch, Germany) and collected on a 25 µm metal sieve (Retsch, Germany) with pre-filtered 96% ethanol. Fibers were dried before storage. Mean polyester fiber length was 245.6 ± 163.1 µm ($N = 1446$, Fig. S2) and fiber widths were 9.7 ± 2.3 µm ($N = 206$). Cotton fibers were 197.1 ± 148.9 µm in length ($N = 1574$, Fig. S3) and 13.9 ± 3.9 µm ($N = 118$) in widths.

Fibers were suspended in pre-filtered ethanol to prevent aggregation and ensure equal distribution before they were spread out in a metal mixing beaker and the ethanol was allowed to evaporate. Commercial fish feed was added on top of dried fibers and blended thoroughly. The blended dry mass was supplemented with deionized water to form a homogeneous dough that was pressed into feed strings. Dried feed strings were crushed into pellets that had a diameter of 1 mm and were 1–5 mm in length. Broken pellet pieces smaller than 0.6 mm were excluded by sieving. Homogeneity of fiber distribution was verified by fluorescence microscopy of moistened and

flattened pellets (Fig. S4). The control diet was prepared using the same procedure without the addition of fibers.

2.3. Experimental design and sampling

The experiment was conducted in a flow-through freshwater aquarium system with 24 tanks (20.8 L each) at 13.1 ± 0.4 °C and 12/ 12 h light/ dark cycles. The temperature was recorded daily and water quality was measured twice a week (ammonia: <0.06 mg/ L, nitrate: <4.4 mg/ L, nitrite: <0.07 mg/ L, pH: 7.8 ± 0.1, oxygen: 9.85 ± 0.63 mg/ L (mean ± standard deviation)). At the start of the experiment, sticklebacks were five months old, and accordingly subadult and sexually immature. By exposing sticklebacks during this phase of live to microplastic fibers, we intended to measure possible effects on their body growth and sexual maturation (gonad growth). Sexual maturation of the experimental animals was not completed within the exposure period (September–December) to avoid any bias on potential effects introduced by breeding activity. Sticklebacks were randomly taken from a pool of offspring derived from nine breeding pairs. Fish were weighed (to the nearest mg) and length measured (to the nearest 0.1 cm) before 20 individuals of comparable size were allocated to each tank to achieve a similar mean weight and weight distribution in each tank (Table S1). Overall mean weight (± standard deviation) was 238 ± 73 mg across all tanks, with a minimum mean per tank of 231 ± 68 mg and a maximum of 243 ± 70 mg per tank. All fish were left to acclimate to the new tank system for at least 18 days, during which the control diet was fed ad libitum three times a week.

The experimental treatments (low-, and high-concentration polyester, low-, and high-concentration cotton, control) were assigned randomly to the tanks (five replicates per treatment and four replicates for the low cotton treatment (for technical reasons)). Fish were fed ad libitum three days a week (Monday, Wednesday, Friday) for nine weeks, whereby the operator was blind for the food types supplied. Leftover feed was removed from the tanks 1 h after feeding. In addition, tanks were cleaned from feces and other debris twice a week (about 24 h after feeding). Water samples that were taken directly after feeding and 4 h later revealed that almost all fibers released from the feed and feces into the water column during/after the feeding were flushed out of the tanks after 4 h (data not shown).

At the end of the experiment, fish from one tank after the other were harvested according to the initial random assignment. The wet weight (to the nearest mg) and standard length (to the nearest 0.1 cm) of each fish were recorded. Fish were anesthetized and sacrificed by cutting the vertebral column. Ten randomly selected fish per tank were dissected and their sex determined. After decapitation, the body cavity was opened by two lateral cuts, the head kidney was excised for immune analyses and kept on ice until further processing. Liver and gonads were extracted and weighed. Gastrointestinal tissue, liver tissue, and gonad tissue samples were taken and stored at −80 °C for further analyses of metabolism-related endpoints in case significant differences would have been detected in growth and body condition parameters (data not presented in this manuscript).

2.4. Growth and body condition

Absolute growth rates (AGR) of the fish were calculated as wet weight gain per day fed (mg d^{-1}) for each individual:

$$AGR = (\text{final weight} - \text{initial weight})/\text{days fed}$$

With initial weight as the average initial weight (mg) per tank and final weight as the individual weight of specimen (mg) sampled after the exposure period.

The Fulton's condition factor (Nash et al., 2006), hepatosomatic index (HSI), and gonadosomatic index (GSI) were calculated as follows:

$$\text{Condition factor} = (\text{final weight}/10)/(\text{standard length})^3$$

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$$HSI = \text{liver weight/final weight} \times 100$$

$$GSI = \text{gonadal weight/final weight} \times 100$$

With final weight as the individual body weight (mg), the standard length of the individuals (cm), liver weight as the wet weight of the liver (mg), and gonadal weight as the wet weight of ovaries or testes (mg).

2.5. Immune parameters

Immunological assays were performed with leucocytes isolated from the head kidney of sticklebacks as described in Scharsack et al. (2007). All steps for leucocyte preparation were performed on ice and refrigerated media were used. Cell suspensions from head kidneys were prepared by forcing the tissues through a 40 μm nylon screen (Falcon, Corning, USA). Isolated head kidney leucocytes (HKL) were centrifuged (5 min at 500 g) and washed twice with RPMI-1640 medium (with glutamine and with HEPES, 9086.1, Carl Roth) diluted with 10% (v/v) distilled water (R-90). The cell pellet was resuspended in a final volume of 500 μL R-90.

The total cell number of HKL isolates was determined with a dynamic imaging particle analyzer (FlowCam 8400, Fluid Imaging Technologies Inc., USA). Suspension of HKL (165 μL) was analyzed with a Field of View 100 flow cell (FC100FV) and a $10\times$ objective lens. Samples were run at a flow rate of 0.15 mL/min and 22 frames per second and pictures of imaged particles were analyzed with the software Visual Spreadsheet 4.16.7 (Fluid Imaging Technologies Inc., USA). Cellular debris was excluded from further evaluation with the software to determine the leucocyte concentrations. The coefficient of variation in cell count measurements was between 0.76% and 2.35% ($N = 7$) in prior tests. Total cell counts were needed to adjust individual cell suspensions to 1×10^6 cells per mL for subsequent activity assays. In addition, the proportion of small (lymphocytes) and big (granulocytes) leucocytes in the samples (Scharsack et al., 2004) was determined from imaged HKL's.

The generation of reactive oxygen species (ROS) by HKL serves as a functional estimate of the cell-mediated innate immune activity. ROS activity (= oxidative burst activity) was measured with a lucigenin-enhanced chemoluminescence assay, modified after Kurtz et al. (2004). 100 μL of cell suspension (1×10^6 cells per mL) were added to 50 μL of R-90 and 25 μL of lucigenin solution (2.5 mg/mL phosphate-buffered saline (PBS)) in white, flat-bottomed microtiter plates. Plates were left covered dark for 30 min at room temperature to allow uptake of lucigenin by the cells. Phagocytosis and production of reactive oxygen species were initiated with 25 μL zymosan suspension (7.5 mg/mL PBS), while a second sample was measured non-activated with 25 μL R-90. Chemoluminescence was measured with a microplate luminometer (CLARIOstar Plus plate reader, BMG LABTECH GmbH, Germany) at 23.2 ± 0.2 °C for 3 h.

To control for background luminescence, R-90 medium and lucigenin solution, with or without zymosan were measured without cells. Measured values without cells were averaged and subtracted from all samples. The addition of hydrogen peroxide to the used assay solutions in different concentrations resulted in a concentration-dependent increase in chemoluminescence (Fig. S5), which was similar when measured with or without zymosan ($R^2 = 0.979$ and $R^2 = 0.981$, respectively). The coefficient of variation of chemoluminescence measurements was determined in prior measurements and ranged between 3.28% and 5.20% (N = 6) for cell suspensions of the same fish while the coefficient of variation was 26.4% (N = 6) for the different fish analyzed.

Oxidative burst activity was determined in relative luminescence units (RLU) for each sample using the CLARIOstar data analysis software MARS (version 3.42R3, BMG LABTECH, Germany). The obtained RLUarea resembles the integration of the activity curve recorded during the 3 h measurement and represents ROS activity. Reported values refer to the RLUarea of zymosan-activated samples. Non-activated samples without zymosan suspension generally yielded low relative luminescence values (mean ± standard deviation: $0.45 \pm 0.39 \times 10^6$ RLU ($N = 237$) compared to $5.98 \pm 3.61 \times 10^6$ RLU (N = 237) with zymosan stimulation). A stimulation index was calculated as RLUarea stimulated / RLUarea unstimulated.

2.6. Data analysis

Statistical tests were performed with RStudio v1.1.463 (RStudio Team, 2020). Investigated parameters were analyzed with linear mixed models (LMM) (Schielzeth and Nakagawa, 2013). Sex was included as a factor in the analysis of all data. Treatment and sex as well as their interaction were set as fixed factors, while the tank was used as a random factor. Model residuals were tested with a dispersion test, an outlier test, and a Kolmogorov-Smirnov test for normality. Wherever necessary, data were square-root or log-transformed to account for non-normally distributed residuals in the model. Significant differences of fixed factors were determined by an ANOVA (Type III, with Kenward-Roger approximation) and a posthoc Tukey test, with $p < 0.05$ being considered statistically significant.

3. Results

3.1. Fiber uptake

All experimental groups showed similar feeding behavior and fish readily consumed the different diets during the feeding trial. Microplastic fibers and cotton fibers were detected in the feces of the sticklebacks exposed to fiber treatments, confirming uptake and egestion (Fig. 1). No experimental fibers were observed in the feces of control animals.

3.2. Fish condition and growth performance

Mortality did not occur in the experiment. The growth performance did not differ significantly between fiber treatments and the control treatment (Table S2). The mean absolute growth rate was 8.8 ± 2.6 mg per day and was similar for all fish irrespective of treatment and sex (Fig. 2, Table 1). Condition factor, hepatosomatic index, and gonadosomatic index did not differ significantly between treatments, but between sexes (Fig. 2, Table S2). The overall mean condition factor for females was 1.24 ± 0.14 and for males 1.30 ± 0.15 (LMM, $p = 0.0026$), the hepatosomatic index 7.38 ± 2.50 for females and 6.26 ± 1.52 for males (LMM, $p < 0.001$), and the gonadosomatic index 8.98 ± 6.01 for females and 1.29 ± 1.46 for males (LMM, p < 0.001) respectively. However, no significant differences were observed between treatments within sexes (Table S2).

3.3. Head kidney immune parameters

Immune parameters were analyzed in extracted head kidney leucocytes of exposed sticklebacks. The oxidative burst activity of HKL was determined as it indicates the activation of phagocytic leucocytes. Total leucocyte count per mg fish, the granulocyte frequency, and the oxidative burst activity of extracted leucocytes were not significantly different in fish fed diets with fibers compared to control fish, and in between fiber treatments (Table S2, Table S3). The overall mean HKL count was $2.70 \pm 1.32 \times 10^3$ cells per mg fish. Only minor oxidative burst activity was observed with HKL without additional stimulation by zymosan and the stimulation index did not differ significantly between any treatments. Granulocyte frequency (LMM, $p < 0.001$) and oxidative burst activity of activated HKL's (LMM, p < 0.001) differed significantly between sexes with higher absolute values observed in females than in males (Fig. 2). Overall mean granulocyte frequency was 55.5 ± 10.4% for females and 50.1 ± 9.7% for males and oxidative burst activity was 637 ± 378 RLU/ s for females and 467 ± 254 RLU/ s for males. None of the analyzed parameters showed a significant interaction between sex and treatment (Table S2).
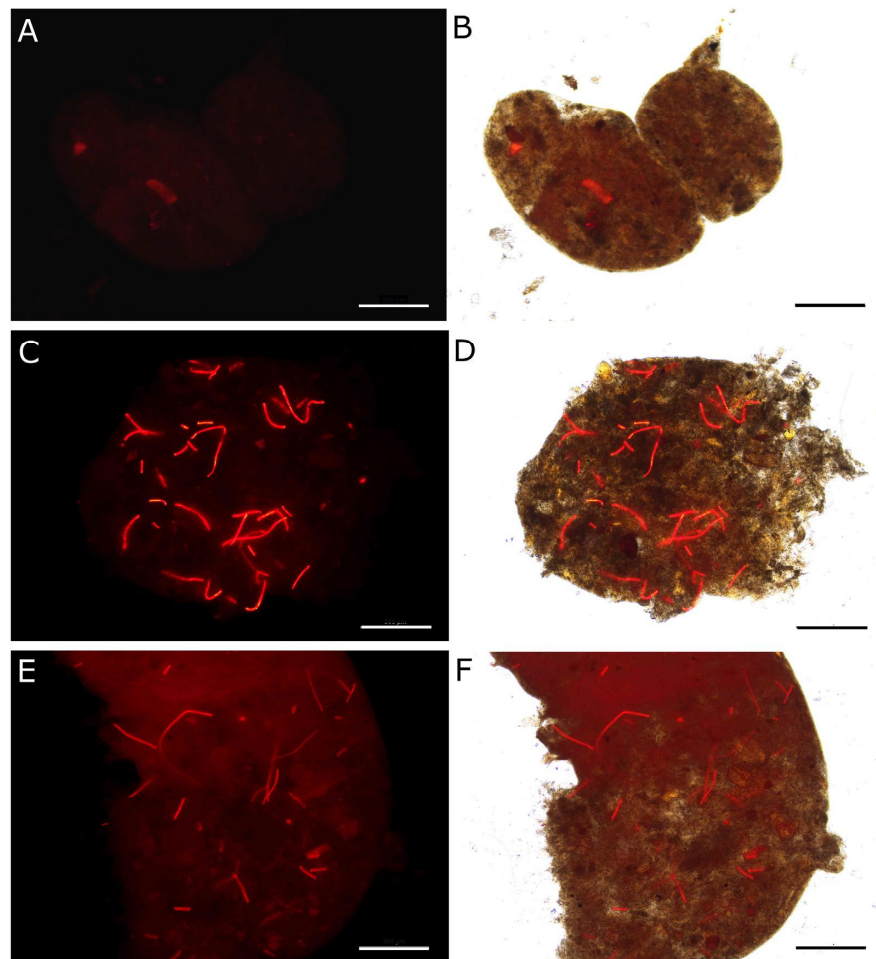
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Fig. 1. Feces of fish fed the experimental diets. Images were taken with a fluorescence microscope and the red fluorescence filter (excitation 511–551 nm; emission 573–613 nm) (left) and were merged with images taken with bright field settings (right). Feces of fish fed the control diet with insignificantly fluorescing organic material (A, B), the high polyester (2 PES) diet with strong fluorescing fibers (C, D), and the high cotton (2 Cotton) diet with less intense fluorescing fibers (E, F). Scale bar = 500 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The present study addresses the potential effects of oral ingestion of microplastic fibers and their passage through the digestive tract on body condition and immune parameters of three-spined sticklebacks. We included treatments with cotton fibers to compare the effects of microplastic fibers on fish with natural-based fibers that fish also encounter in nature. We fed the sticklebacks for nine weeks diets supplemented with polyester fibers or cotton fibers (low/high fiber concentrations each), or a control diet without fiber supplement. Exposed sticklebacks did not develop significant differences in body condition parameters and tested immune traits between the different dietary treatments. No differences were observed between the distinct fiber types and concentrations added to the feed. Variation between sexes was higher than any possible differences due to fiber exposure, but interactions between sex and fiber exposure were not observed. The present study was robust and sensitive enough to detect differences in body condition and immune traits between sexes, suggesting that potential treatment effects would have been detectable. Our results indicate that ingestion of polyester fibers does not cause changes in body condition

parameters, gonad development, and immune parameters of sticklebacks, even at levels much higher than these fish currently encounter in nature.

4.1. Fiber ingestion effects on body condition and immune parameters

In the present study, mortality did not occur, and experimental sticklebacks gained length and weight during the experimental period. Overall, growth and body condition data (Table 1) are in the range of values reported for subadult three-spined sticklebacks under laboratory conditions in other studies (Hani et al., 2019; Hani et al., 2018). Our results show that ingested fibers were excreted via feces and none of the dietary treatments administered hampered growth performance and reproductive capacity of the sticklebacks. Similarly, other studies observed efficient egestion of ingested microplastic fibers in Japanese medaka (*O. latipes*) and sticklebacks when exposed to microplastics via the water column (Bour et al., 2020; Hu et al., 2020). Microscopic examinations of exposed medaka revealed that most ingested fibers were encased in food, mucus, and waste material within the lumen and excreted rapidly without further damage (Hu et al., 2020). We did not observe differences in ingestion and

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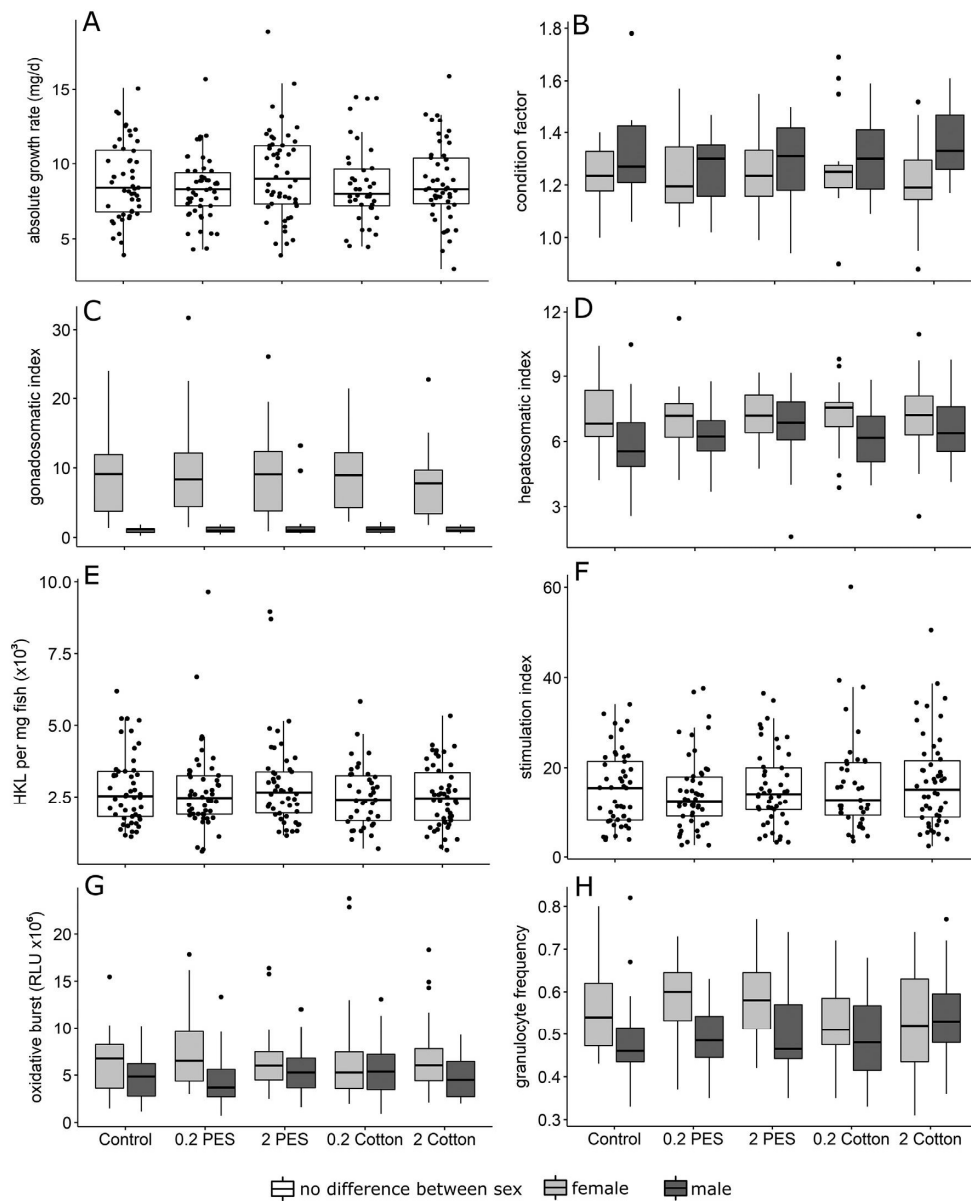
Fig. 2. Body condition (A-D) and immune parameters (*E*-H) of sticklebacks exposed to control and the different fiber treatment diets (0.2 PES, 2 PES = diets with 0.2 and 2 mg polyester fibers per g feed, and 0.2 Cotton, 2 Cotton = diets with 0.2 and 2 mg cotton fibers per g feed). Absolute growth rates in mg per day (A), body condition factor (B), gonadosomatic index (C), hepatosomatic index (D), head kidney leucocyte (HKL) count (E), stimulation index (RLUarea stimulated / RLUarea unstimulated) (F), oxidative burst activity in relative luminescence units (RLU) (G), and granulocyte frequency (H). Data of all dissected fish are shown combined if they did not differ significantly between treatments or sexes and for females and males separately if there was no significant difference between treatments, but between sexes (determined by Type III ANOVA in linear mixed-effect models).

egestion between polyester and cotton fibers. In previous studies, the internal dietary crude fiber fraction of pelleted feed (primarily plant origin) was used as an indigestible marker when testing fish feeds, as they resemble characteristics of commonly used markers such as chromic oxide (Tacon and Rodrigues, 1984). Ideal markers should be indigestible, do not affect the metabolism, and pass evenly through the gastrointestinal tract with the food (Austreng et al., 2000). Our results indicate that cotton and polyester textile fibers are similarly indigestible and effectively transported with

the food and excreted with the feces by fish as observed for internal dietary fibers.

Our findings deviate from previous observations of structural and mucosal damage in the gastrointestinal system of goldfish caused by pellets with ethylene vinyl acetate fibers (0.7–5 mm long), which led to reduced body weight and lower body condition (Jabeen et al., 2018). The shorter polyester fibers used in the present study might be more easily passed through the intestinal system and excreted without damaging the gastrointestinal tract.

Table 1
Body condition parameters of sticklebacks exposed to control and fiber supplemented diets, grouped by sex. Values are mean ± standard deviation (HSI = hepatosomatic index, GSI = gonadosomatic index).

Treatment	Sex	N	Absolute growth rate (mg/d)	Length (cm)	Weight (mg)	Condition factor	HSI	GSI
Control	F	26	9.6 ± 2.8	4.4 ± 0.3	1095 ± 274	1.23 ± 0.11	7.27 ± 1.52	9.47 ± 6.38
Control	M	23	8.0 ± 2.0	4.2 ± 0.3	945 ± 207	1.30 ± 0.15	5.87 ± 1.83	1.04 ± 0.44
0.2 PES	F	22	8.9 ± 2.1	4.4 ± 0.3	1050 ± 216	1.24 ± 0.15	8.10 ± 5.05	9.92 ± 7.41
0.2 PES	M	28	8.0 ± 2.0	4.2 ± 0.2	954 ± 197	1.26 ± 0.13	6.11 ± 1.13	1.11 ± 0.43
2 PES	F	28	9.4 ± 3.3	4.4 ± 0.3	1075 ± 318	1.25 ± 0.12	7.21 ± 1.25	9.26 ± 6.19
2 PES	M	22	9.2 ± 2.5	4.3 ± 0.2	1061 ± 234	1.28 ± 0.16	6.66 ± 1.69	2.02 ± 3.11
0.2 Cotton	F	19	8.3 ± 2.4	4.2 ± 0.3	980 ± 239	1.27 ± 0.18	7.19 ± 1.56	9.20 ± 5.35
0.2 Cotton	M	20	8.8 ± 2.9	4.2 ± 0.3	1015 ± 278	1.30 ± 0.15	6.21 ± 1.40	1.21 ± 0.50
2 Cotton	F	31	8.8 ± 3.0	4.4 ± 0.3	1041 ± 299	1.22 ± 0.14	7.21 ± 1.69	7.50 ± 4.80
2 Cotton	M	19	8.8 ± 1.8	4.3 ± 0.2	1057 ± 169	1.36 ± 0.15	6.54 ± 1.53	1.10 ± 0.42

However, exposure characteristics such as fiber concentrations could play an even bigger role. Jabeen et al. (2018) provided each goldfish with 18 mg of fibers per week, whereas sticklebacks in our study received about 0.3 mg fibers per fish per week with the high polyester fiber feed. A high fiber concentration in the feed is more likely to cause adverse effects, as a high bulk mass of foreign materials will unavoidably scratch and thereby damage the intestinal walls during its passage through the gastrointestinal system. Yet, adverse impacts observed at extremely high threshold concentrations can be irrelevant in an environmental context (Burns and Boxall, 2018). With our study, we demonstrate that polyester fiber ingestion does not affect the growth performance and health of subadult sticklebacks – even when ingested in concentrations of up to 2 mg polyester fibers per gram feed. The amount of the polyester fibers used in our study in the feed correlates to more than 15,500 fibers ingested per fish per week, while a global synthesis of microplastic ingestion by fish reported an average microplastic load of 3.5 ± 0.8 (mean ± standard deviation) microplastic items (all shapes) per fish at the time of sampling (Wootton et al., 2021).

Our findings correlate with the observation of tolerance to virgin microplastic fragments by Jovanovic et al. (2018), who fed gilt-head seabream (*Sparus aurata*) pellets supplemented with microplastics of six different polymers at a concentration of 3.33 mg per gram feed. The dietary exposure did not induce stress, alter growth rates, and cause pathology, which was explained by the fast and effective elimination of ingested microplastics from the gastrointestinal tract (Jovanović et al., 2018).

We observed progressing development of the gonads in all experimental fish, which indicates that sexual maturation of exposed fish was not affected. Moreover, we noticed differences between sexes for condition index, organosomatic indices, and oxidative burst activity of head kidney leukocytes, and granulocyte frequency in the experimental sticklebacks, which demonstrates high sensitivity of the analyzed parameters. However, none of the parameters was affected by fiber uptake through the diets. The absence of significant interaction between treatment and sex effects suggests that sticklebacks show no gender-specific susceptibility towards ingested microplastic fibers.

4.2. Mechanisms and characteristics that shape fiber effects on fish

Additives incorporated in microplastic fibers were previously suggested as potential agents causing fiber toxicity (Horn et al., 2019; Mishra et al., 2019). In the present study, we did not analyze the additive content of the used commercial polyester and cotton fibers. Yet, their coloration and potential other additives included did not affect the health of the exposed sticklebacks. Overall, we expect additives to play a minor role for the fibers used in our study since no (additive) compounds were detected in 14-day aqueous leachates of virgin PET fibers (Sait et al., 2021) and considerable leaching does likely not happen within fish when fibers are excreted rapidly.

Several environmental factors, such as aquatic pollutants, cause metabolic reorganization and oxidative stress of exposed organisms (Biller and Takahashi, 2018; Tort, 2011). Oxidative stress stimulates ROS production

which, when produced excessively, can cause exhaustion of the immune system and damage of cells and tissues (Biller and Takahashi, 2018). Concurrently, oxidative stress was suggested to be one of the mechanisms through which microplastics can adversely affect marine organisms (Espinosa et al., 2019). In addition, the immune system can be regarded as part of a network across body functions, and it is likely that stress in distinct functional units, such as the intestine, would have been detectable in the immune system too.

In the present study, measurements of the oxidative burst in HKLs did not reveal any signs of oxidative stress, which suggests that ingestion of microplastic and natural fibers does not lead to successive impairment of the stickleback immune system. In contrast to the present study, European sea bass (*Dicentrarchus labrax*) showed significantly increased respiratory burst activity in head kidney leucocytes, when exposed to PE fragments or polyvinylchloride fragments (PVC) (size range 40–150 μm) in a similar concentration range (0.1 and 0.5 mg/ g feed) as in the present study, via their diet (Espinosa et al., 2019). The effects in the head kidney cellular immune system were related to histological alterations observed in the gut epithelium and mucus secretion (Espinosa et al., 2019). Mechanical disruption of the gastrointestinal system and subsequent dysbiosis of the gut microbiome were suggested as missing links between microplastic ingestion and host health (Fackelmann and Sommer, 2019; Varó et al., 2021), which then can have negative effects on fish health. The probably limited or absent mechanical damage of fibers towards the intestinal tract in the present study could thus explain the absence of stress responses and adverse effects on the immune system of exposed sticklebacks. In principle, microplastic fibers might cause less mechanical damage than hard and irregular-shaped microplastic fragments, and thus induce less impact on the immune system of fish. Yet, different polymer types are also suggested to affect organisms differentially (Bucci et al., 2020), which might also explain the differences observed in the microplastic exposure studies. Our results indicate that ingested polyester fibers in the size range 25–800 μm do not affect growth performance, body condition and immune parameters, even after frequent and prolonged ingestion. Therefore, the microbiome of exposed fish was likely not affected by ingested (virgin) polyester fibers.

Small particles (< ~5–10 μm) can pass the intestinal epithelium barrier via endocytosis (Browne et al., 2008; Zeytin et al., 2020). Generally, microplastic fibers are expected to be too big to cross the gastrointestinal barrier (Rebelein et al., 2021). Translocation of microplastics to fish tissues was observed in laboratory exposure studies only when microplastic spheres (1–5 μm in diameter) were used in the water column (Ribeiro et al., 2020; Zeytin et al., 2020), but not for dietary exposure to microplastic spheres in the size class 10–300 μm (Kim et al., 2020). Very small fibers (<5 μm) might thus potentially pass the intestinal epithelium barrier and subsequently interfere with the metabolism and immune cells of fish. Greven et al. (2016) demonstrated that in vitro exposure of plasma neutrophils (differentiated leucocytes) to PS nanoplastics or polycarbonate nanoplastics (<1 μm) caused significant increases in degranulation of primary granules and neutrophil extracellular trap release compared to a non-treated control. Furthermore, in vitro developing immune cells from

the anterior kidney of rainbow trout (*Oncorhynchus mykiss*) took up PS microplastic spheres (0.83–3.1 μm) and then showed a decreased abundance of non-phagocytic developing B cells (Zwollo et al., 2021). Thus, small microplastic and nano-sized fibers might pose a greater health risk on fish than the relatively larger microplastic fibers used in the present study.

Experimental studies illustrated that absence or presence of adverse effects of microplastic fibers (in varying severity) on fish depends on specific fiber characteristics (e.g. size, polymer type) and exposure characteristics (e.g. concentration, pathway of exposure) (Bucci et al., 2020; Jacob et al., 2020). The transition of such experimental findings towards situations in the environment is difficult, in particular since many experimental studies used fiber exposure characteristics barely observed in the wild (Rozman and Kalčikova, 2021). The experimental results presented here, suggest that common polyester fibers, in the size class released in laundry processes, do not induce gut damage and subsequent impairment in growth and body condition of fish.

5. Conclusion

The absence of effects of dietary microplastic exposure on growth, body condition parameters, and immune system-related variables observed in the present study, suggests that current microplastic fiber concentrations in natural habitats pose no threat to sticklebacks. While mostly lower trophic levels and vulnerable species are affected by microplastic and natural fibers in terms of gastrointestinal damage and mortality, sticklebacks in particular – and presumably fish in general – seem to be less prone to fiber pollution probably due to the effective excretion of ingested fibers. Even high amounts of fibers (plastic and natural) included in the diet were egested efficiently and did not affect growth performance, body condition, and immunity. Yet in nature, fibers are accompanied by various other factors that can influence fish and ecosystem health, such as rising temperature and other pollutants of anthropogenic origin (e.g. heavy metals, antibiotics, pesticides). Therefore, the potential of fibers to aggravate impacts of other stressors and pollutants on fish should be taken into account when evaluating the overarching potential of a species to cope with fiber pollution. In summary, our results suggest that for mere fiber pollution fish are unaffected by ingested polyester fibers up to relatively high amounts.

CRediT authorship contribution statement

Anja Bunge: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Visualization. **Vincent Lugert:** Data curation, Writing - review & editing. **Melissa McClure:** Investigation, Writing - review & editing. **Ulrike Kammann:** Writing - review & editing, Project administration, Funding acquisition. **Reinhold Hanel:** Conceptualization, Funding acquisition, Writing - review & editing. **Jörn Scharsack:** Conceptualization, Writing - review & editing, Supervision.

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Declaration of competing interest

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

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Appendix A. Supplementary data

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Supplementary for Chapter IV

Bunge, A., Lugert, V., McClure, M., Kammann, U., Hanel, R., Scharsack, J.P. (2022). Less impact than suspected: Dietary exposure of three-spined sticklebacks to microplastic fibers does not affect their body condition and immune parameters. *Science of The Total Environment*, 153077. doi: 10.1016/j.scitotenv.2022.153077.
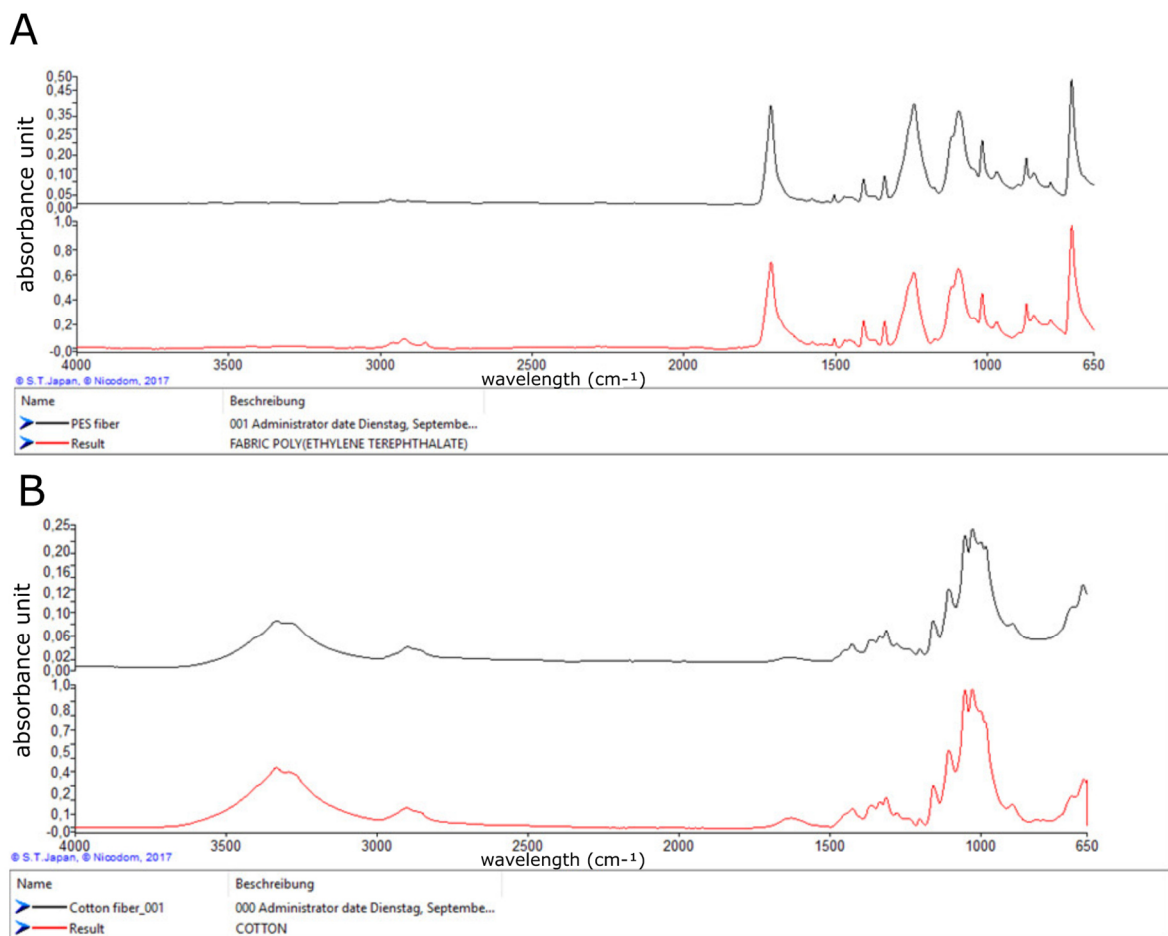
Figure S1. Fourier-transform infrared spectrum (single bounce) of the used fiber materials (in black) and spectrum of reference material in database (red) from polyester (=fibrous form of polyethylene terephthalate, A) and cotton (B). © Thünen-Institute/ Ivo Int-Veen.
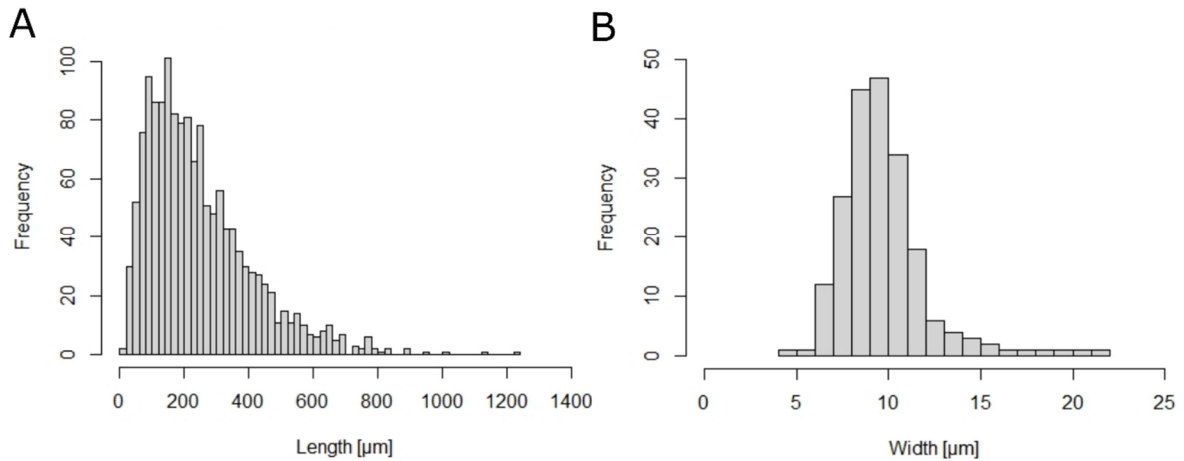
Figure S2: Polyester fiber length (N=1446) (A) and width (N=206) (B) distribution of manual cut pieces after sieving. Reprinted, under CC BY 4.0 licence, from "Microplastic fiber diet—Fiber-supplemented pellets for small fish." by Rebelein, A., & Focken, U. (2021). MethodsX, 8, 101204.
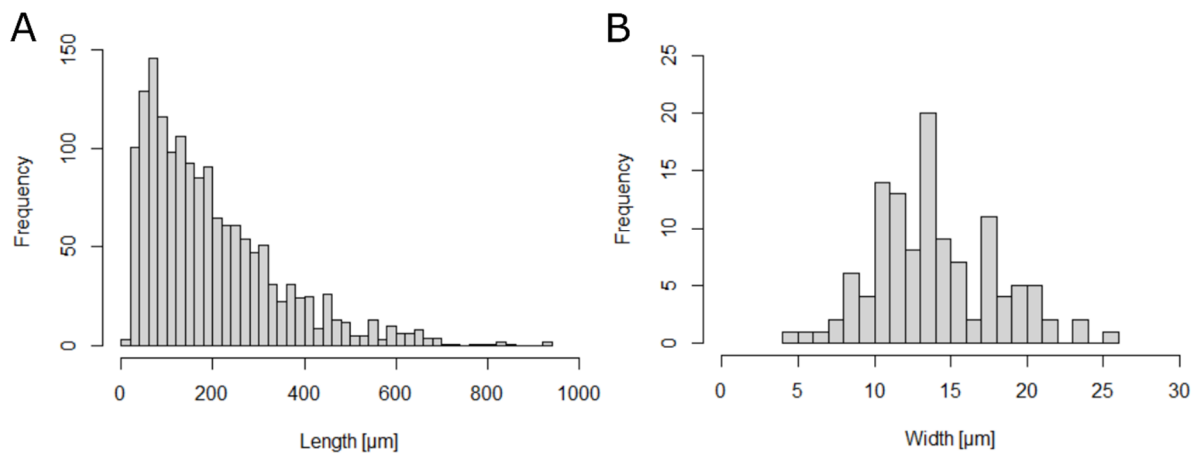
Figure S3. Cotton fiber length (N=1574) (A) and width (N=118) (B) distribution of manual cut pieces after sieving.
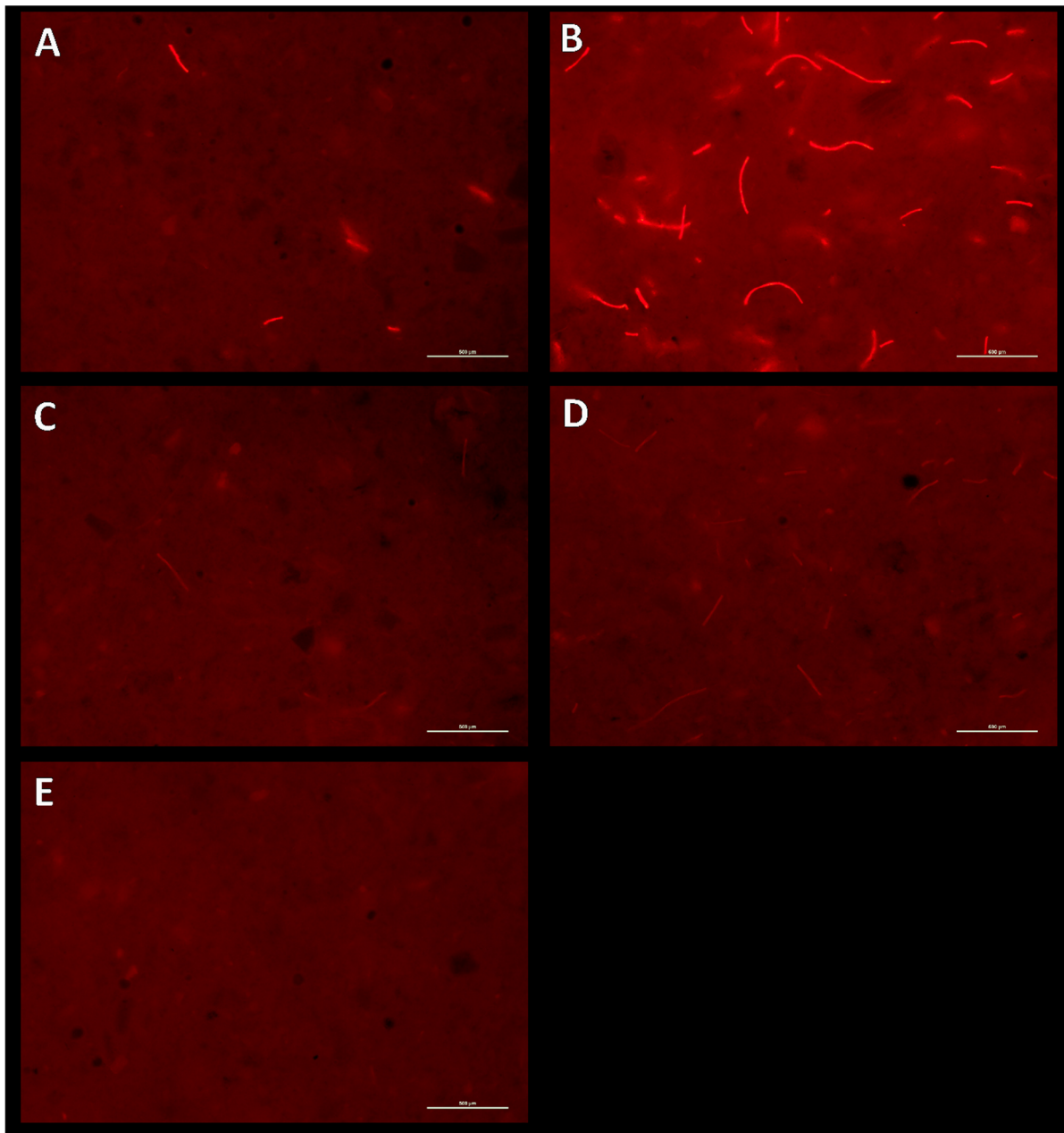
Figure S4. Homogenous spread of fibers in produced pellets with 0.2 mg (A) and 2 mg (B) polyester fibers per gram feed and 0.2 mg (C) and 2 mg D) cotton fibers per gram feed. Control pellets without fibers produced with pure Essence diet powder (E). Scale bar = 500 µm.
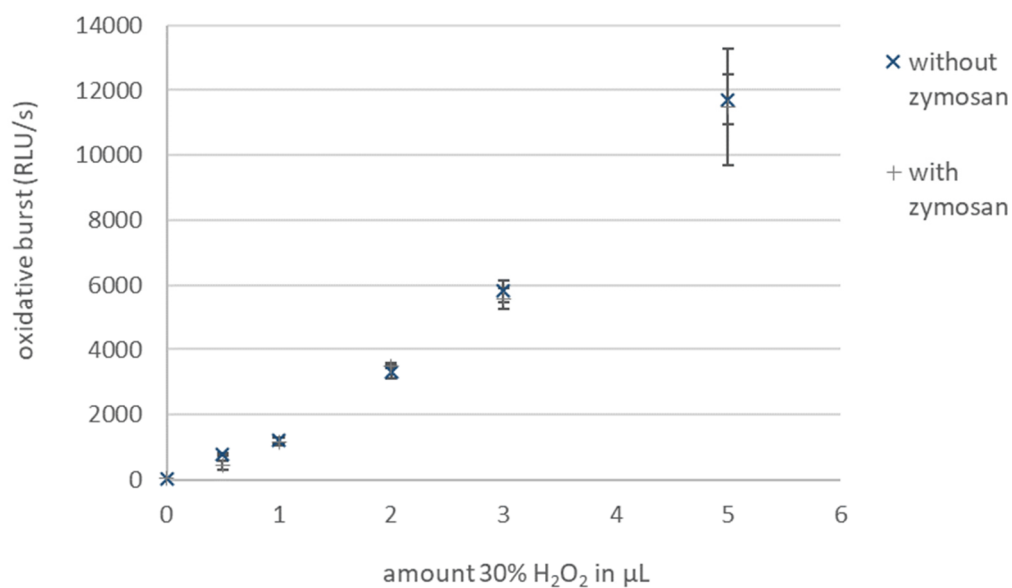
Figure S5. Test of assay reagents used in the chemoluminescence assay without cells. Relative luminescence values increased with an increase in hydrogen peroxide used in the assay (instead of cells that produce oxygen radicals when stimulated). Each dot represents the mean relative luminescence unit per second (RLU/ s) of three measurements and error bars represent the standard deviation.

Table S1. Initial fish condition parameters measured during random distribution of experimental fish to the tanks before the acclimation phase. Values are given as mean ± standard deviation per tank (stocked with 20 fish each).

tank	weight (mg)	length (cm)
1	238 ± 84	2.7 ± 0.3
2	240 ± 82	2.7 ± 0.3
3	241 ± 85	2.7 ± 0.2
4	237 ± 62	2.7 ± 0.2
5	238 ± 51	2.7 ± 0.2
6	243 ± 70	2.7 ± 0.2
7	235 ± 84	2.6 ± 0.3
8	234 ± 67	2.7 ± 0.3
9	240 ± 58	2.7 ± 0.2
10	240 ± 81	2.7 ± 0.3
11	239 ± 59	2.7 ± 0.2
12	235 ± 69	2.7 ± 0.3
13	243 ± 78	2.7 ± 0.3
14	238 ± 61	2.7 ± 0.2
15	241 ± 72	2.7 ± 0.2
16	236 ± 96	2.6 ± 0.3
17	240 ± 81	2.7 ± 0.3
18	236 ± 62	2.7 ± 0.2
19	231 ± 68	2.6 ± 0.3
20	238 ± 82	2.7 ± 0.3
21	241 ± 79	2.7 ± 0.3
22	237 ± 80	2.7 ± 0.3
23	232 ± 93	2.7 ± 0.3
24	241 ± 66	2.7 ± 0.2
overall mean	**238 ± 73**	**2.7 ± 0.3**

Table S2. Growth and immune parameters investigated with linear mixed-effects models, with treatment, sex, and interaction as fixed factors. Tank was used as random effect in the model to account for variation nested in treatments. Pr (>F)-values from ANOVA with Kenward-Roger approximation on model residuals (Pr <0.05).

	ANOVA – Pr (>F)-value			
parameter	treat-ment	sex	treatment:sex	*remark*
condition factor	0.8516	< 0.001	0.2751	
HSI	0.8921	< 0.001	0.6354	*data were square root-transformed to achieve normal distribution*
GSI	0.7040	< 0.001	0.7051	*data were log-transformed to achieve normal distribution*
absolute growth rate	0.8392	0.1413	0.3178	
HKL per mg fish	0.8154	0.7046	0.5986	
granulocyte frequency	0.6172	< 0.001	0.0770	
stimulation index (RLU stimulated/unstimulated)	0.9261	0.3013	0.5243	*data were square root-transformed to achieve normal distribution*
oxidative burst	0.9292	< 0.001	0.3217	*data were square root-transformed to achieve normal distribution*

Table S3. Immune parameters of head kidney leucocytes of sticklebacks exposed to the different fiber treatment diets and control diet, grouped by sex (F = female, M = male). Values for head kidney total leucocyte count per mg fish, granulocyte frequency, stimulation index, and oxidative burst in relative luminescence units (RLU) are given as mean ± standard deviation.

treatment	sex	N	head kidney cells per mg fish (×10³)			granulocyte frequency			stimulation index			ROS activity (×10⁶) (RLU)		
Control	F	26	2.93	±	1.30	0.56	±	0.09	15.6	±	8.8	6.36	±	3.07
Control	M	23	2.56	±	1.20	0.49	±	0.10	14.7	±	7.8	4.70	±	2.57
0.2 PES	F	22	2.62	±	1.81	0.58	±	0.09	16.5	±	8.8	7.46	±	4.06
0.2 PES	M	28	2.80	±	1.19	0.49	±	0.08	12.6	±	7.6	4.43	±	2.69
2 PES	F	28	3.01	±	1.52	0.58	±	0.10	14.4	±	7.8	6.50	±	3.35
2 PES	M	22	2.82	±	1.68	0.50	±	0.09	16.7	±	9.2	5.68	±	2.66
0.2 Cotton	F	19	2.34	±	1.01	0.53	±	0.11	17.2	±	13.5	7.34	±	6.34
0.2 Cotton	M	20	2.71	±	1.17	0.49	±	0.10	15.5	±	9.2	5.82	±	3.33
2 Cotton	F	31	2.62	±	1.15	0.53	±	0.12	18.0	±	11.7	6.80	±	3.76
2 Cotton	M	19	2.34	±	1.02	0.55	±	0.11	14.7	±	7.5	4.64	±	2.26

General discussion

The intention of the present thesis was to address the potential effects of microplastics that are environmentally relevant on fish. The focus was placed on fibers that account for a major share of microplastic components in the environment. The present thesis outlines that despite their prevalence in the environment, microplastic fibers are the least studied microplastic component in effect studies (Chapter I). Given their elongated shape, fibers might entangle with appendages, gill filaments, and within the gastrointestinal system of organisms. Yet, methodological challenges in detection and handling of fibers led to their negligence in previous environmental and exposure studies. Within the present thesis, methods were developed to utilize fibrous microplastics in laboratory experiments and disperse fibers homogeneously in water and feed (Chapter II & III). Polyester fibers were used since this polymer type is very common in textiles (Carr, 2017) and is frequently shed to the environment (Galvao et al., 2020). The fibers were cut to a size class similar to fibers released during household washing, which can reach the environment as laundry effluent (Galvao et al., 2020). The prepared fibers were used in exposure studies with different life stages of fish to investigate potential impacts on fertilization, early life stages, and sub-adult life stages of sticklebacks (Chapter II & IV). The following research questions were addressed within the present thesis:

Potential effects of microplastic fibers in the water on early life stages of fish

The present results are the first to demonstrate that fiber presence in the water already during fertilization did not affect *in vitro* fertilization rates of fish eggs – even at concentrations two orders of magnitude higher than maximum reported environmental concentrations (Lahens et al., 2018). The conducted experiment could not confirm concerns about microplastic fibers that accumulate on the surface of the egg and potentially block the micropyle for sperm cells at concentrations up to 1×10^4 polyester fibers per liter. While some individual fibers attached to the egg surface (chorion) in the fiber treatments, they did not impair fertilization, embryo development, and hatching rates. Furthermore, attached polyester fibers were not internalized. Overall, the chorion of fish eggs seems to be an efficient barrier for microplastic fibers, similar to observations with other types of microplastics and even nanoplastics (Cheng et al., 2020; Duan et al., 2020; Le Bihanic et al., 2020). Given that more fibers were observed attached to broken eggshells and debris during exposure (Chapter II, Figure S4), this suggests that fibers are more likely to attach to irregular shaped materials than to smooth surfaces of intact fish eggs. This would imply that in nature, where commonly lots of debris and broken particles are around, healthy fish

eggs are less susceptible to microplastic attachment than in laboratory settings and are mostly unaffected by microplastic fiber presence.

Potential adverse effects due to additives seem rather unlikely, since no negative impacts on the early fish development were observed. Furthermore, no chemicals were identified in 14-day leachates of very high concentrations of PET (= polyester) fibers (10 mg/ mL) in seawater and in freshwater (Sait et al., 2021).

The differences in heart rates of embryos and in morphological features of larvae (three days post hatching) were higher between egg clutches from different breeding pairs than between split half-clutches that were used for the polyester fiber and the control treatment. Thus, natural variability in early life stage development of sticklebacks was higher than any effects of microplastic fiber encounter in the water.

Overall, the results obtained in the present thesis could therefore not confirm the hypothesis that the very early life stages are particularly vulnerable to (fibrous) microplastics. Though fiber attachment was visible to a small extent, it did not cause negative effects on fertilization and early fish development.

Potential effects of ingestion of microplastic fibers by sub-adult fish

Later life stages of fish frequently ingest microplastic fibers and oral uptake is likely the most important interaction between fibers and fish. In principal, fish ingest most fibers in the water unintentionally – and at higher frequencies when food is present (Li et al., 2021). The use of a commercial fish diet, which was supplemented with microplastic fibers in the laboratory, allowed to study the oral uptake as important exposure path of fish to microplastic fibers.

In the present thesis, later life stages of sticklebacks egested microplastic (polyester) and natural (cotton) fibers included in their diet via feces, which corresponds to the efficient excretion of other microplastic fibers encased in food, mucus, and waste material observed in adult medaka and goldfish (Grigorakis et al., 2017; Hu et al., 2020). The supplemented polyester and cotton fibers used in the present study are presumably as indigestible and evenly egested with the food as the crude fiber fraction (mostly plant material) of fish diets that was repeatedly applied as indigestibility marker when testing fish feeds (Krontveit et al., 2014; Tacon & Rodrigues, 1984).

Growth performance, which is a well measurable marker in fast-growing subadult fish stages, was similar for all experimental fish. Therefore, it is unlikely that the used polyester fibers entangled and accumulated in the gastrointestinal tract, which would have caused deficient nutrient uptake and growth. Furthermore, the absence of adverse effects on growth performance, gonad maturation, and the immune system, indicate that ingested fibers did not cause internal damage or microbiome disruption in the gastrointestinal tract,

which were suggested to cause microplastic toxicity (Espinosa et al., 2019; Fackelmann & Sommer, 2019; Jabeen et al., 2018; Varó et al., 2021).

(Natural) differences were observed in condition index, organosomatic indices, and some immune parameters (e.g. oxidative burst activity) of head kidney leucocytes between sexes of subadult sticklebacks fed different fiber supplemented and control diets. Yet, none of the parameters was affected by fiber ingestion and no significant interactive effects between treatment and sex were observed. The results suggest that sticklebacks show no gender-specific susceptibility towards ingested fibers and resemble the observation of higher natural variation in investigated parameters than any effects due to encounter of microplastic fibers, as seen in fiber-exposed early life stages.

When gilt-head seabream (*Sparus aurata*) were fed diets supplemented with different irregular-shaped microplastic fragments, the microplastics were also fast and effectively eliminated from the gastrointestinal tract and did not induce stress, altered growth rates, or caused pathology (Jovanović et al., 2018). Accordingly, fish seem to egest ingested fibrous microplastics as efficiently and fibers have insignificant effects on their health. This presumably applies not only to the tested polyester and cotton fibers in the size class released during washing but also to microplastic fibers in general. Overall, the results of the present thesis indicate negligible effects of fiber uptake on fish at currently reported low numbers of microplastic fibers ingested per fish in nature (Chapter I, Table 3).

Handling microplastic fibers in laboratory exposure settings

The majority of exposure studies is still conducted with the easier manageable spheres due to challenges in extracting, analyzing, and handling fibers in experimental settings. The present thesis, therefore, established methods to work with microplastics fibers in laboratory exposure studies that can be used in future experimental studies with fibers.

In general, the handling of microplastics is challenging due to their small size, electrostatic properties, and potential contamination by other ambient microplastics. Fiber contamination, in particular, is a major problem when analyzing and characterizing microplastics due to their ubiquity in the environment. Preparation of the experimental fibers and their stock solutions was thus conducted in cleanroom facilities to minimize potential airborne fiber contamination. In addition, all equipment was thoroughly rinsed with ultra-pure water or filtered deionized water followed by a rinse with 96% ethanol to exclude microplastic contamination. Utensils and products were kept covered whenever possible. Those are requirements proposed by many reviews on quality in sampling and characterization of microplastics in the environment (Brander et al., 2020; Hermsen et al.,

2018; Koelmans et al., 2019), but should also be applied in all laboratory exposure studies that use microplastics (de Ruijter et al., 2020).

Specific characteristics, such as fluorescence, can facilitate microplastic detection, identification, and characterization when using them in experimental settings. Many exposure studies used fluorescent-labeled microplastic beads to record microplastic uptake and accumulation in aquatic organisms (Ding et al., 2018; Ribeiro et al., 2020; Scherer et al., 2017). The polyester fibers used in the present thesis showed strong autofluorescence in the red fluorescence filter, which facilitated their detection, counting, and differentiation from other (ambient) fibers. Fluorescent microscopy was used for size determination, counting, and verification of homogeneous spread in the stock solution and feed of the experimental fibers. Though automated analysis could not be executed due to characteristics such as twisted and overlaying fibers, the autofluorescence of used fibers allowed an unambiguous and faster detection and characterization compared to light microscopy. Overall, autofluorescing fibers provide a convenient tool to conduct microplastic fiber exposure studies in terms of identification and characterization, which are highly relevant quality criteria in microplastic research.

The second major concern when conducting microplastic exposure studies besides contamination, is to establish and maintain microplastics as homogenously distributed as possible. This relates to stock suspensions, as well as exposure media such as feed and water. The best way to prevent fiber aggregation in aqueous suspensions that was determined in the present thesis, was the use of surfactants (e.g. Tween 80). This is also recommended for microplastic suspensions by suppliers of commercial reference spheres (Connors et al., 2017). The use of surfactant along with gentle mixing before subsamples were taken from stock suspensions ensured the extraction of a consistent fiber concentration, which was confirmed with fluorescence microscopy of multiple subsamples. In the experimental setting with early life stages of fish, very low concentrations of surfactant were used along with constant agitation of the bowls to prevent fiber aggregation in the water. Thereby, surfactant concentrations were kept as minimal as possible to avoid disturbance of exposed eggs and embryos. To rule out toxicity effects due to used surfactants (de Ruijter et al., 2020), the experimental setup was designed to include surfactant in the control and fiber treatments.

More challenging than the creation of a homogeneous fiber suspension was the production of a diet with homogeneously dispersed microplastic fibers since thorough blending of commercial powdered feed with microplastics added on top caused entanglement and aggregation of the fibers. Therefore, a diet preparation method was developed that used an intermediate step (Chapter III). The experimental fibers were first

dispersed homogeneously in ethanol. A suspension of a defined mass of dried polyester fibers in ethanol was used to spread out the fibers evenly in the mixing bowl. Then, the ethanol was left to evaporate completely before powdered feed was added and the mixture blended thoroughly. Fluorescent microscopy confirmed that supplemented fibers were placed separate and spread out in the produced fiber pellets (Chapter III, Figure 4).

Overall, the use of surfactant and ethanol are supportive measures to challenge microplastic fiber entanglement and aggregation and provide experimental fish and embryos with media that contain dispersed microplastic fibers. Previously, little experience was published how to cope with challenges in microplastic fiber characterization and handling in laboratory settings. The described methods developed and implemented in this thesis can serve as guidance how to approach methodological difficulties in handling fibers in the future.

Assessment of the risk posed by microplastics in the aquatic environment

The risk of microplastics in the environment can be assessed based on knowledge of the toxicity (concentration) of a compound and the anticipated exposure of an organism to this compound. The anticipated exposure can be approximated by the environmental microplastic concentrations and the actual encounter and uptake rates of organisms. Yet, comprehensive data that were collected with standardized methods do currently not exist for microplastic encounter rates and effect concentrations of environmental relevant microplastics. Therefore, classic risk assessment calculations, as exist for chemical pollutants, cannot be set up for (environmentally relevant) microplastics up to date. However, indications on the risk of microplastics on fish and in the environment can be provided according to the current scientific knowledge.

Ecotoxicity approaches that concentrated on known lethal effects and impaired individual fitness revealed that calculated effect concentrations were a lot higher than measured environmental microplastic concentrations and thus demonstrate an overall low risk of microplastics to various taxonomic groups at present (Burns & Boxall, 2018; Foley et al., 2018). Though those meta-analyses included only low numbers of effect studies conducted with fibers, results from exposure studies conducted in the present thesis support the currently limited risk of microplastics to fish also for textile fibers. While Chapter I outlines the environmental abundance and widespread distribution of microplastic fibers, exposure studies conducted within the following chapters demonstrated the concurrent absence of biological impacts of fibers on fish in laboratory settings. Microplastic fibers can be excreted efficiently and do not seem to be more

detrimental than other microplastic shapes to fish. This suggests a negligible risk of wild fish towards microplastics in the environment, which are frequently fibers.

In the present thesis the focus was placed on microplastic fiber attachment to early life stages and ingestion of fibers since organisms in the environment encounter microplastics mainly in the water column and during foraging (amongst or attached to food items). Previous exposure studies demonstrated that adult fish (*Oryzias latipes*) that were exposed to microplastic fibers via the water did not show entangled or attached fibers at the gills, even when fiber containing fluid was flushed through the mouth cavity (Hu et al., 2020). Attachment to outer surfaces and oral ingestion are thus the main interactions between fish and microplastics and most important when considering the risk of microplastic fibers.

The microplastic fiber concentrations used in exposure studies are a compromise between environmental observations and concentrations that can be established and maintained as a reproducible and homogenous dispersion of fibers in the water column and in the feed. Environmental fiber concentrations were reported in the order of 1-10 fibers per liter up to a maximum concentration in the order of 10^2 fibers per liter in a highly polluted river (Lahens et al., 2018; Luo et al., 2019; Ryan et al., 2020; Song et al., 2015). Higher fiber concentrations might occur in local events of microplastic accumulation or contamination, and are predicted with rising global plastic pollution in the future (Everaert et al., 2020). Thus, a nominal concentration of 1×10^4 fibers per liter was used when exposing early life stages of fish to fibers in the water, which is two orders of magnitude above current reports of maximum fiber concentrations (Lahens et al., 2018), but lower than most previous exposure studies conducted with microplastics (Burns & Boxall, 2018; Jacob et al., 2020).

Results obtained in the present thesis deviate from previous observations of adverse effects of microplastics on fish determined in laboratory studies. While in the present thesis polyester fibers in the water did not impair embryo development, hatching success, or did alter the heart rate, those parameters were impacted in medaka and zebrafish embryos exposed to PS and PET microplastics of different shapes (Chen et al., 2020; Cheng et al., 2020; Qiang & Cheng, 2019). Yet, the adverse effects in medaka and zebrafish embryos were observed only for treatment groups exposed to microplastic concentrations between 1×10^6 particles per liter and 1×10^9 particles per liter (Chen et al., 2020; Cheng et al., 2020; Qiang & Cheng, 2019), which is manifold orders above current concentrations in the environment. When lower concentrations were tested as well, no significant impact on hatching time and hatching rate were observed for concentrations of 1×10^2 particles per liter and 1×10^6 particles per liter (Chen et al., 2020; Qiang & Cheng, 2019). Nevertheless,

study results were presented as deleterious impacts of microplastics on early life stages of fish.

Commonly, toxicity of microplastics increases with rising numbers of microplastics in the water (Burns & Boxall, 2018). Conceivably, a higher bulk mass of microplastics does more easily attach to the outer surfaces of fish eggs and thus has a higher potential to block surface pores, which can induce hypoxic conditions in the egg and cause physiological alterations. In contrast, no negative effects could be detected at concentrations up to 1×10^4 fibers per liter in the present study with early life stages of sticklebacks, which is still four orders of magnitude higher than average fiber concentrations reported in marine systems (Luo et al., 2019; Ryan et al., 2020; Song et al., 2015).

Similarly, contrary results compared to results obtained in the present thesis were reported in previous exposure studies that provided fish with microplastics via their diet. Goldfish that fed on pellets packed with microplastic fibers (each fish received 18 mg fibers per week in contrast to 0.3 mg fibers per week in the present thesis) showed structural and mucosal damage in the gastrointestinal tract (Jabeen et al., 2018). Yet, the number of fibers that the experimental sticklebacks in the present study received when they fed on the high-amount polyester fiber diet (~>15500 fibers per fish per week), is already far higher than environmental realistic ingestion rates. The average global microplastic load detected in wild fish at the time of sampling was 3.5 ± 0.8 microplastic items per fish (mean ± standard deviation) (Wootton et al., 2021).

Commonly, many exposure studies that report deleterious effects of microplastics on fish use extremely high concentrations. For instance, gilt-head seabream (*Sparus aurata*) that were exposed to PE-particles through food (pre-exposed *Artemia salina*) showed higher mortality, increased abundance of several brain and liver primary metabolites, and hepatic and intestinal histological defects compared to the control fish (Jacob et al., 2021). However, the estimated daily microplastic dose that was provided to the experimental fish was about 48000 ± 10000 microplastics (mean ± standard deviation) per fish. The daily dose is such at least four orders of magnitude more than the average global microplastic load reported in fish (Wootton et al., 2021). An excessive bulk mass of microplastics ingested by fish poses a high chance to scratch and damage tissues such as intestinal walls during the passage through the gastrointestinal tract. Therefore, the microplastic dose is likely the most important factor determining the presence or absence of effects on organisms and should be chosen thoughtfully. While it is useful to establish toxicity threshold concentrations, the tested concentrations should not exceed extreme values without any relation to (future) microplastic concentrations in the environment.

Adverse effects observed at extremely high concentrations might point to potential mechanisms of plastic toxicity but are irrelevant in an environmental context at presence

and in the closer future. A meta-analysis showed that only a minor amount of effect studies (17%) tested environmentally realistic concentrations of microplastics (Bucci et al., 2020). Testing somewhat higher concentrations that might occur in the future or in local contamination events is relevant and necessary for environmental risk assessments. However, effect studies that use microplastic concentrations five or more orders of magnitude higher than reported from the environment, have little value in terms of environmental relevance and interpretations should not interpolate on environmental risk of tested microplastics. In addition, little is known about mechanisms and duration of recovery processes in aquatic organisms, which might happen when fluctuating concentrations of microplastics are encountered in nature. Filter-feeding silver carp (*Hypophthalmichthys molitrix*) exposed to low concentrations of microplastic fragments via the water column did not show damage in the intestinal tract and were able to recover from oxidative stress induced in the gastrointestinal tissues within a 48-hour period without microplastics (Zhang et al., 2021).

Another aspect to consider is that micro-sized particles of natural origin have been in the environment long before microplastics were introduced – and exist in much higher concentration than microplastics in the environment (Koelmans et al., 2022). Natural particles can have similar impacts on organisms (e.g. food dilution, physical damage, oxidative stress) compared to microplastics once ingested (Koelmans et al., 2022). However, organisms were able to cope with the encounter and ingestion of natural particles long before microplastics were introduced into the environment until now. This perception is supported by experimental results of the present thesis, which show that ingestion of natural as well as microplastic fibers did not have adverse effects on experimental sticklebacks. Recently, a concept was proposed to assess the risk of particles in the environment – including the contemporary subcategory of microplastics – more as a continuum of characteristics rather than a categorical phenomenon (Koelmans et al., 2022). Thereby, microplastics would become only one of many foreign particles that can be encountered by fish and the potential harm would be determined mainly by their concentration. In this regard, negative effects of microplastics observed at extremely high concentrations in laboratory studies are negligible in an environmentally relevant context. Caution must be taken to infer from negative effects observed in laboratory fish exposed to reference microplastic spheres to similar effects of bigger-sized microplastics with an irregular shape on fish in natural environments. Overall, the results from the present thesis suggest that the major concerns of microplastic pollution are – at current microplastic concentrations – likely less reasonable for fish than suggested in the past years.

While microplastics play a minor role for fish in nature, little is known about aquaculture species so far. Aquaculture fish frequently encounter microplastic fibers within their diets since fibers get unintentionally incorporated during the production process of fish meal and fish diets (Wang et al., 2022). Furthermore, fish that are cultivated in recirculating aquaculture systems are surrounded by plastic material (e.g. tanks, pipes, food container) and likely more plastic particles can be found in surrounding waters than in the open ocean when ageing materials shed plastic particles. Yet, up to date only few studies addressed whether cultured fish take up more microplastics compared to wild fish, and whether this is comparable to wild fish living in areas with high microplastic concentrations, such as Asian rivers. To this end, cultured species will become the focus of future investigations on the microplastic load and potential health impact on fish. Based on results obtained from the present thesis, alternative materials and methods for culturing fish in plastic-reduced conditions are likely not necessary in terms of potential microplastic shedding from culturing equipment and uptake from the water column. Cultured fish will likely cope with microplastic ingestion by efficient egestion up to high concentration of microplastics in the water. However, quality controls on feed ingredients such as fish meal and the feed production processes could be a major improvement to control for additional inputs of microplastics via direct dietary intake and reduce the overall microplastic exposure of cultivated fish.

In terms of human health, microplastic intake by fish consumption should not be of concern since humans eat mostly gutted fish without the gastrointestinal tract. A major exposure pathway to microplastics for humans was estimated to be the intake of atmospheric microplastics through inhalation, which clearly outnumbers exposure to microplastics by seafood consumption (Zhang et al., 2020).

In aquatic environments, microplastics pose a greater risk to organisms at lower trophic levels (Foley et al., 2018; Walkinshaw et al., 2020). Smaller organisms such as zooplankton and filter-feeding organisms such as bivalves likely have more difficulties to egest or excrete microplastics taken up orally and during filtration compared to the efficient elimination of microplastics by fish. While filter-feeding bivalves filter primarily for organic, nutritious particles, it was demonstrated that bivalves can lose their selective capability and accidentally ingest inorganic components when particles are present in high concentrations in the surrounding water (Jørgensen, 1996). In a laboratory setup, synthetic PVC particles emulated the effects of natural suspended solids (red clay) on byssus production and respiration rate of mussels (Yap et al., 2020). Yet, the observed overall (little) effect of exposure to suspended particles was seen as indication for an enormous robustness of the mussels toward high particle loads (Yap et al., 2020). Comparably, experimental exposure

of adult mussels from five different geographic regions (temperate to tropical) to natural and microplastic particles elicited small effects on byssus production, respiration rate, and condition index – but mainly as acute responses rather persistent carry-over effects (Hamm et al., 2022). Similar to observations with particles, filter-feeding organisms likely show comparable filtration and elimination rates for microplastic fibers and fibers from natural origin. At present, bivalves presumably can cope well with the fiber concentrations in the environment – comparable to fish. However, mussels have the ability to selectively accumulate and eliminate particles, including microplastics, in the different tissues, which can results in tissue-specific depuration rates (Li et al., 2021). Thus, uncertainties remain whether fibrous items are retained for longer periods in other tissues than the digestive system, which is, contrary to fish, not the mere organ exposed to ingested microplastics in filter-feeding organisms. The potential body retention of fibrous items might make filter-feeding bivalves more vulnerable than fish to fiber intake and should be investigated for a more detailed risk assessment of fibers that considers all aquatic taxa. While bivalves are likely not affected at present fiber concentrations in aquatic systems, environmental threshold concentrations, at which filter-feeding organisms in particular are negatively affected by suspended particles, should be determined considering the concentration of microplastic as well as natural particles and fibers combined.

The sticklebacks used in the present thesis can serve as model organism to demonstrate that large and small-sized fish and likely other organisms in the size class 2-5 cm can egest ingested fibrous microplastics very efficiently. Care must be taken to transfer this knowledge to even smaller organisms such as zooplankton, for which the size relation of the intestinal tract to ingested fibers is smaller. Yet, also for lobster larvae (7-15 mm) that ingested microplastic fibers, survival and oxygen consumption rates were only affected at high concentrations and not at a fiber concentration of 1×10^3 fibers per liter (Woods et al., 2020). Furthermore, inorganic suspended particles are, among algae, an integral part of seston, which is frequently ingested by zooplanktonic species (Major et al., 2017; Müller-Solger et al., 2002). When microplastics are seen as foreign particles similar to natural inorganic particles as proposed by Koelmans et al (2022), zooplankton might thus be similarly capable to cope with ingestion of microplastic and inorganic other particles up to certain concentrations. Adverse effects would be expected when the overall concentration of indigestible particles in the size class similar to their prey and food items are higher than the organisms can cope with. Therefore, future studies are necessary to look at the overall presence of indigestible particles, including fibers, in the environment and determine whether threshold concentrations, at which smaller organisms could be affected, are reached in the future. Thereby regions with higher natural suspended solids might be of higher risk when microplastic concentrations increase.

Ultimately, a higher sensitivity of lower trophic levels to microplastics could entail an increased feeding pressure of higher trophic levels, which demonstrates the need to pay more attention to lower trophic levels in terms of microplastic toxicity in the environment. Yet, the current environmental concentrations of solely microplastics are orders of magnitude below the lowest concentrations that elicit adverse effects in aquatic organisms (Beiras & Schönemann, 2020; Burns & Boxall, 2018; Duis & Coors, 2016).

Perception shift in microplastic research

During the last years, some researchers demonstrated that not as much microplastics are actually ingested by fish than expected and that microplastics in the environment might not be as harmful to organisms as anticipated (Beiras & Schönemann, 2020; Müller, 2021). Though the number of published studies demonstrating no or limited impact is rising, many effect studies still seem to search in particular for negative impacts of microplastics on organisms, which were proposed in the early phase of microplastic research. Results from the present thesis endorse a broader acceptance of effect studies demonstrating little to no effect of potential pollutants, in particular when conducted in environmentally relevant settings.

In general, bigger plastics seem to be more detrimental to aquatic organisms due to entanglement and gut blockage compared to the smaller microplastics (Blettler & Wantzen, 2019; Thiel et al., 2018). Furthermore, the very small plastic fragmentation products, so-called nanoplastics, are under debate to pose a greater risk in the environment than microplastics (Gaylarde et al., 2020). Currently, no suitable standard methods for determining the concentrations and characteristics of nanoplastics in aquatic systems exist. Yet, their potential to pass through membrane barriers and potential to be retained within cells and tissues after ingestion or inhalation by organisms raise the concern to pose an environmental threat. Their high surface to volume ratio and conceivable retention within organisms might make nanoplastics more important in terms of potential vectors for chemicals and microbes compared to microplastics. Research expertise gained with microplastics in nature and in the laboratory can be adapted in the future to focus more on the very small microplastic and nanoplastic size classes regarding their environmental abundance and potential toxicity to aquatic organisms.

Furthermore, microplastic pollution is not occurring as an individual factor in the environment but rather co-exists with a variety of other pollutants. Synergistic adverse

effects on the immune system have been reported in marine bivalves (*Mytilus coruscus*), which were co-exposed to microplastic beads and veterinary antibiotics (Han et al., 2021), and in terrestrial crustaceans (*Porcellio scaber*), which were exposed to microplastic fibers and insecticides simultaneously (Dolar et al., 2021). Moreover, aquatic organisms already weakened by other environmental factors such as higher temperatures or food depletion might be more susceptible to oxidative stress and other physiological impacts already at lower concentrations of microplastics. For that reason, it must be considered to test the stress-on-stress effect for selected environmental relevant factors (e.g. rising temperatures or local pollutant inflows) together with microplastics. Modelling approaches could then be used to analyze and consider potential synergistic effects in risk assessments for different settings, globally and locally. This would facilitate to assess the factors that are most relevant to address when we want to achieve and maintain a 'good environmental status' in aquatic environments, as demanded by the European MSFD.

In summary, previous effect studies demonstrated that some microplastics can cause adverse effects on fish and other aquatic organisms, which raised concerns of microplastics in aquatic environments. However, adverse impacts were mostly observed at very high concentrations and with spherical microplastics that account for a minor proportion of microplastics detected in the environment. In contrast, microplastic fibers – in particular polyester fibers – are commonly used in textiles and found in aquatic environments but were rarely investigated in exposure studies so far. Results obtained in the present thesis demonstrate that polyester fibers present in the water or in feed do not affect different life stages of fish, including sensitive early life stages, and even at higher concentrations than are currently encountered in nature. Thereby, the present thesis marks a shift in microplastic effect research from detecting effects in unrealistic scenarios by measuring no effects in more environmentally relevant scenarios. This implies that results obtained in laboratory effect studies should be carefully interpreted regarding concentration, types of microplastics used, and exposure conditions, when inferring on the environmental impact of microplastics. Caution should be taken to not exaggerate effects of microplastics observed in unrealistic scenarios in laboratory studies. The studies included in this thesis contribute largely to the shift in perception of microplastics as part of foreign particles commonly encountered, ingested, and also egested by fish similar to other debris around – without negative impacts on their health. Overall, the present results show that (fibrous) microplastics in the environment likely do not pose an acute harm to fish at present levels.

Perspective – Life with (micro-)plastics

Plastics play a pivotal role for modern society. We are surrounded by plastics and rely on them in many sectors such as food safety, as disposable medical equipment, as packaging material, in the construction industry, and in the agricultural industry. Though events like the COVID-19 pandemic demonstrate the necessity of plastic products as economic and hygienic material that is required for the preservation of public health, negative consequences of plastic usage, such as plastic waste mismanagement must be minimized. Discarded disposable face masks can shed thousands of fibers in the size of micro- and nanoplastics due to mechanical stress in the environment (Liang et al., 2021; Morgana et al., 2021; Wu et al., 2021).

On the one hand, the present thesis demonstrated that at current concentrations, microplastics in the environment are presumably not as harmful to fish as suspected in earlier microplastic research. Therefore, the 'fear of microplastics' perceived by the public should not be intensified by speculative proposals of deleterious impacts of microplastics on organisms in the future. On the other hand, the probably low impact of current microplastic concentrations on aquatic organisms does not imply humankind can carry on with its excessive use and waste of plastics, which will continue to accumulate in nature given their long persistence. Next to the more aesthetic aspect of plastic waste piling up in the oceans, the ingestion of bigger plastics does harm sea birds, turtles, marine mammals, and other organisms (Kühn & van Franeker, 2020; Wilcox et al., 2016). Furthermore, plastic items will slowly fragment and disintegrate into smaller plastic pieces over the next hundreds and thousands of years. The amount of micro- and nanoplastics in the oceans will merely increase over time up to higher concentrations, which might cause more harm than current concentrations. Yet, humans can influence at which rate concentrations increase by reducing the overall plastics and microplastics use and their dumping.

The annual plastic waste generation is projected to increase from 215 million tons in 2016 to more than 419 million tons in 2040, if the use of plastics is continued unchanged (Lau et al., 2020). Modelling approaches revealed that if every measure known to reliably restrict plastic waste production (recycle plastic, switch to systems of reuse, and adopt alternative materials) would be implemented in 2020, the annual terrestrial and aquatic plastic pollution could be reduced by 78% relative to expected pollution in 2040 without any restrictions (Lau et al., 2020). Thereby, measures and progress are needed on all levels, from political actions to technological innovations, and an increase in public awareness to not aggravate the overall plastic pollution problem. We must consider the entire lifecycle of plastics – from design and production to end-of-life options (Patrício Silva et al., 2020; Prata

et al., 2019). In addition, efforts are required worldwide, whereby multiple countries could benefit from resource and knowledge sharing of more experienced countries in terms of plastic innovations and waste management.

The global operation of wastewater treatment plants (WWTPs) would be an achievable approach to tackle in particular inflows of microplastic fibers into aquatic environments. WWTPs can remove (micro-) plastics from household and industrial sewage waters and other inflows such as rainwater runoffs. The retention efficiency of the plastic load of treatment plants was determined to be between 95 and 99% and can reach even beyond 99% (Talvitie, 2018; Waldschläger et al., 2020). However, on a global scale only about 20% of the industrial and municipal wastewater is cleaned before it gets discharged in the environment (United Nations, 2021). Those facts demonstrate the major potential to improve the reduction of microplastic input into global aquatic systems by the implementation of feasible measures.

Humans will not be able to cast off the versatile and useful plastic materials in the near future, yet we can decide how we deal with this resource in a considerate manner to preserve our environment.

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Contribution of the authors

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3. **Rebelein, A., & Focken, U. (2020). Microplastic fiber diet–Fiber-supplemented pellets for small fish.** *MethodsX***, 101204. https://doi.org/10.1016/j.mex.2020.101204**

   Authors Contribution: Anja Bunge and Ulfert Focken discussed the nutritional requirements of fish and different methods to produce pelleted fish feed. Anja Bunge designed, prepared, and characterized the feed, and wrote the manuscript. Ulfert Focken contributed with helpful comments before the submission of the manuscript.

4. **Bunge, A., Lugert, V., McClure, M., Kammann, U., Hanel, R., Scharsack, J.P. (2021). Less impact than suspected: dietary exposure of three-spined sticklebacks to microplastic fibers does not affect their body condition and immune parameters.**

Authors Contribution: Anja Bunge and Jörn Scharsack discussed and designed the study together. Reinhold Hanel contributed with helpful remarks concerning the study design. Anja Bunge performed the experiment. Jörn Scharsack and Melissa McClure helped with the final sampling of the experiment. Anja Bunge carried out sample analyses, processed all data, performed the statistical analysis, and wrote the manuscript. Vincent Lugert provided helpful comments for the statistical analysis. Jörn Scharsack, Reinhold Hanel, and Ulrike Kammann provided critical feedback and helped shaping the analysis and manuscript. All authors contributed with helpful comments before the submission of the manuscript.

Eidesstattliche Erklärung

Hiermit erkläre ich, dass die vorliegende Dissertation, abgesehen von der Beratung durch meinen Betreuer Prof. Dr. Reinhold Hanel, nach Inhalt und Form eine eigenständige Arbeit ist und nur mit den angegebenen Hilfsmitteln verfasst wurde. Diese Arbeit wurde weder ganz noch zum Teil an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegt, veröffentlicht oder zur Veröffentlichung eingereicht. Die Dissertation ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden. Des Weiteren versichere ich, dass mir nie ein akademischer Grad entzogen wurde.

Kiel, den 30.04.2022

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Anja Bunge