

EXAMINATION OF THE MICROPLASTIC BURDEN IN FRESHWATER FISHES AND PHYSICAL EFFECTS TO ITS EXPOSURE

**Examination of the microplastic burden in freshwater
fishes and physical effects to its exposure**

Doctoral thesis for obtaining the academic degree Doctor of
Natural Sciences (Dr. rer. nat.)

By Samuel Roch

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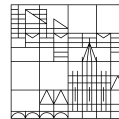
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Summary

Pollution of the environment with plastic waste has long been an ignored issue, but is now considered a major global threat to aquatic systems and their inhabitants. Microplastics, comprising plastic fragments, beads, and fibers smaller than 5 mm, are detected in rivers, lakes and oceans all over the world. Due to their small size, they can be ingested by a wide range of aquatic organisms, including teleost fish. To date, little is known about how severely native freshwater fish species are affected by microplastics. There is also limited knowledge about how the differing gastrointestinal morphologies and foraging strategies of fish affect the uptake mechanisms and the retention time of microplastics. The aim of the thesis was to tackle some of these knowledge gaps in order to better understand the interaction of fish with microplastics in freshwater systems.

First, a new method for the detection of microplastics in fish was developed, which allowed efficient and rapid (<1 h) digestion of the entire fish gastrointestinal tract, and included an optional density separation step to reduce mineral components. ([Manuscript I](#)). This novel method made it possible to reliably and rapidly examine a large number of samples, allowing a large-scale analysis of microplastic burden in fish. This method was then used to investigate the microplastic burden of native fish species across the German state of Baden-Württemberg ([Manuscript II](#)). The overall burden of microplastics was found to be low, with an average prevalence of ~19 % and an intensity of between one and four particles per individual. Several relevant biotic and abiotic factors, such as sampling site and trophic state, were shown to have only a minor influence on microplastic burden. The results also revealed a major limitation with currently available microplastic detection methods: particles <40 µm could not be reliably detected in the gastrointestinal tract of the examined fish. However, by using the dataset acquired in this thesis it was possible to calculate the theoretical total microplastic burden in local fish with a size distribution analysis. It was found that as particle size decreases, particle concentration increases – with a power law growth fit likely indicating that over 95 % of all microplastic particles in fish are currently being excluded from collected data. This means that only a fraction of the potential size spectrum of microplastics can currently be considered in research data.

It is still not fully understood how microplastics are taken up by fish. To gain a more holistic understanding of microplastic uptake pathways, pre-existing and recently developed theories were explored through a number of practical and theoretical approaches ([Manuscript III](#)). Four fish species (rainbow trout

(*Oncorhynchus mykiss*), grayling (*Thymallus thymallus*), common carp (*Cyprinus carpio*), crucian carp (*Carassius carassius*)), representing different foraging styles and domestic status, were exposed to a range of particles (varying by type and colour) with or without the provision of food; the abundance of microplastics was subsequently determined in their gastrointestinal tract. These experiments revealed that visually-orientated fish ingest microplastics actively and/or accidentally with their food much more frequently than fish that are chemosensory-orientated. In addition to the microplastic concentration in the water and fish size, the colour of the plastic particles played an important role in uptake: particles were taken up significantly more often if they resembled the colour of the food. By contrast, chemosensory foraging fish were able to discriminate larger plastic particles, and only ingested microplastics on occasion, by chance. At smaller particle sizes, uptake pathways other than feeding become more relevant; statistical models showed that in large marine fish species, notable amounts of microplastics were ingested simply through drinking. Finally, these experiments showed for the first time that domestication plays an important role in the uptake of microplastics. Relative to wild fish, farmed fish discriminated less between differently coloured plastic particles, and were more likely to actively ingest microplastics when no food source was available.

The next step was to investigate the duration that microplastic particles remained in the gastrointestinal tract of fish ([Manuscript IV](#)). A special diet was developed that contained differently sized microplastic particles. The number of retained particles in the gastrointestinal tract was determined up to 72 h after administration in two fish species (rainbow trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpio*)) that have distinct gastrointestinal morphologies. The laboratory experiments showed size-dependent differences in the T_{50} value (time at which 50 % of the particles are excreted) of plastic particles in fish with a true stomach; particles with a size of $\sim 1000\ \mu\text{m}$ were excreted approximately three times faster than particles with a size of $\sim 40\ \mu\text{m}$. In fish without a stomach, the differences were substantially smaller, suggesting purely passive excretion with the chyme. It was thus concluded that the morphology of the gastrointestinal tract plays a vital role in the retention of microplastics, and that large plastic particles must be actively excreted in fish with a true stomach.

Finally, controlled laboratory experiments were conducted to investigate whether realistic microplastic concentrations have detrimental short- and long-term effects on fish ([Manuscript V](#)). In addition to an analysis of established performance and health parameters, the entire rainbow trout (*Oncorhynchus mykiss*) liver proteome

was examined and the results confirmed with the help of gene expression analysis. Two groups of fish were exposed to a realistic current environmental concentration of microplastics, and a slightly elevated microplastic concentration that reflects expected microplastic exposure levels in the near future. These two groups were then compared with a control group (no exposure to microplastics) after 120 days of continuous exposure. Microplastic exposure was shown to have a significant dose-dependent effect on growth and other performance parameters (*i.e.* specific growth rate, feed conversion rate). There were no significant differences in blood glucose, hematocrit levels and oxidative stress levels between the groups. The proteomic analysis identified over 6000 proteins, but no clear difference in their regulation or correlation with gene expression was found between treatments. However, a number of single proteins and their respective transcripts were identified as potential biomarkers for future studies. The results therefore conclusively showed that even low microplastic concentrations have a notable impact on fish with long-term exposure. Importantly, they provide the basis for future investigations of microplastic effects on health, and demonstrates the potential of novel state-of-the-art methods that are now emerging in the field.

Zusammenfassung

Die Verschmutzung der Umwelt mit Plastikabfall wurde lange Zeit ignoriert, gilt aber inzwischen als eine globale Bedrohung für aquatische Systeme und ihre Bewohner. Mikroplastik, welches Plastikfragmente, -kügelchen und -fasern kleiner als 5 mm umfasst, wird in Flüssen, Seen und Ozeanen auf der ganzen Welt nachgewiesen. Aufgrund seiner geringen Größe kann es von einer Vielzahl von Wasserorganismen, einschließlich Fischen, aufgenommen werden. Bislang ist nur wenig darüber bekannt, wie stark einheimische Süßwasserfischarten durch Mikroplastik beeinträchtigt werden. Außerdem stellt sich die Frage, wie sich der Aufbau des Verdauungstraktes und unterschiedliche Futtersuchstrategien von Fischen auf die Aufnahmemechanismen und die Verweildauer von Mikroplastik auswirken. Ziel dieser Arbeit war es, einige dieser Wissenslücken zu schließen, um so die Interaktion von Fischen mit Mikroplastik in Süßwassersystemen besser zu verstehen.

Zunächst wurde eine neue Methode zum Nachweis von Mikroplastik in Fischen entwickelt, welche eine effiziente und schnelle (<1 h) Zersetzung des gesamten Magen-Darm-Trakts von Fischen ermöglicht und zusätzlich einen optionalen Dichteseparationsschritt zur Reduzierung mineralischer Bestandteile umfasst. (Manuskript I). Diese neuartige Methode ermöglichte es, eine große Anzahl von Proben zuverlässig und schnell zu untersuchen und aufgenommene Plastikpartikel verlässlich zu identifizieren. Dadurch konnte flächendeckend die Mikroplastikbelastung einheimischer Fischarten in ganz Baden-Württemberg untersucht werden (Manuskript II). Die Auswertung der Ergebnisse ergab, dass aktuell die Gesamtbelastung mit Mikroplastik als gering eingestuft werden muss. Die durchschnittliche Prävalenz belasteter Fische lag bei ~19 % und die Intensität zwischen einem und vier Partikeln pro Individuum. Es zeigte sich zudem, dass relevante biotische und abiotische Faktoren, wie z. B. der Ort der Probenahme und der trophische Status, nur einen geringen Einfluss auf die Mikroplastikbelastung haben. Die Ergebnisse bestätigten jedoch auch eine wesentliche Einschränkung der derzeit verfügbaren Methoden zum Nachweis von Mikroplastik: Partikel <40 µm konnten im Magen-Darm-Trakt der untersuchten Fische nicht mehr zuverlässig nachgewiesen werden. Es war jedoch mit Hilfe der gewonnenen Daten möglich, die theoretische Gesamtbelastung von heimischen Fischen mit Mikroplastik über eine Größenverteilungsanalyse zu berechnen. Es zeigte sich, dass mit abnehmender Partikelgröße die Partikelkonzentration in Form eines Potenzgesetzes zunimmt. Dies deutet darauf hin, dass über 95 % aller Mikroplastikpartikel in Fischen aktuell nicht nachgewiesen werden können.

Damit würde derzeit nur ein Bruchteil des potenziellen Größenspektrums von Mikroplastik in den bis heute durchgeführten Untersuchungen berücksichtigt werden.

Es ist immer noch nicht vollständig geklärt, wie Mikroplastik von Fischen überhaupt aufgenommen wird. Um ein ganzheitliches Verständnis der Aufnahmewege zu erlangen, wurden bereits bestehende, aber auch neu entwickelte Theorien mit Hilfe einer Reihe von praktischen und theoretischen Ansätzen untersucht ([Manuskript III](#)). Vier Fischarten (Regenbogenforelle (*Oncorhynchus mykiss*), Äsche (*Thymallus thymallus*), Karpfen (*Cyprinus carpio*), Karausche (*Carassius carassius*)), die verschiedene Futtersuchstrategien und einen unterschiedlichen Grad der Domestizierung repräsentieren, wurden einer Reihe von Plastikarten und -farben bei gleichzeitiger oder ohne Futterzugabe ausgesetzt; anschließend wurde die Menge an Mikroplastik im Magen-Darm-Trakt bestimmt. Bei diesen Experimenten zeigte sich, dass visuell orientierte Fische Mikroplastik viel häufiger aktiv und/oder zufällig mit dem Futter aufnehmen als chemosensorisch orientierte Fische. Neben der Mikroplastikkonzentration im Wasser und der Fischgröße spielte auch die Farbe der Plastikpartikel eine wichtige Rolle bei der Aufnahme: Partikel wurden deutlich häufiger aufgenommen, wenn sie der Farbe des Futters ähnelten. Im Gegensatz dazu konnten chemosensorische Fische bei der Nahrungssuche größere Plastikpartikel erkennen und nahmen daher diese nur gelegentlich und zufällig auf. Bei kleineren Partikelgrößen werden andere Aufnahmewege als die Nahrungsaufnahme relevanter; statistische Modelle zeigten, dass bei großen Meeresfischarten beträchtliche Mengen an Mikroplastik durch einfaches Trinken aufgenommen werden können. Abschließend zeigten die Experimente erstmals, dass die Domestizierung von Fischen eine wichtige Rolle bei der Aufnahme von Mikroplastik spielt. Im Vergleich zu Wildfischen unterschieden Zuchtfische weniger zwischen unterschiedlich gefärbten Plastikpartikeln und nahmen Mikroplastik häufiger aktiv auf, wenn keine echte Nahrung zur Verfügung stand.

In einem nächsten Schritt wurde die Verweildauer von Mikroplastikpartikeln im Magen-Darm-Trakt von Fischen untersucht ([Manuskript IV](#)). Es wurde ein spezielles Futter entwickelt, das unterschiedlich große Mikroplastikpartikel enthielt. Die Anzahl der zurückgehaltenen Partikel im Magen-Darm-Trakt wurde bis zu 72 Stunden nach der Verabreichung bei zwei Fischarten (Regenbogenforelle (*Oncorhynchus mykiss*), Karpfen (*Cyprinus carpio*)) bestimmt, welche unterschiedliche Magen-Darm-Morphologien aufweisen. Die Laborexperimente zeigten größenabhängige Unterschiede im T_{50} -Wert (Zeit, in der 50 % der Partikel

ausgeschieden werden) von Plastikpartikeln bei Fischen mit einem echten Magen; Partikel mit einer Größe von $\sim 1000\ \mu\text{m}$ wurden etwa dreimal schneller ausgeschieden als Partikel mit einer Größe von $\sim 40\ \mu\text{m}$. Bei Fischen ohne Magen waren die Unterschiede wesentlich geringer, was auf eine rein passive Ausscheidung mit dem Chymus schließen lässt. Daraus wurde gefolgert, dass die Morphologie des Magen-Darm-Trakts eine entscheidende Rolle bei der Retention von Mikroplastik spielt und dass große Plastikpartikel bei Fischen mit einem echten Magen aktiv ausgeschieden werden müssen.

Schließlich wurde in kontrollierten Laborexperimenten untersucht, ob realistische Mikroplastikkonzentrationen schädliche kurz- und langfristige Auswirkungen auf Fische haben ([Manuskript V](#)). Neben der Analyse etablierter Leistungs- und Gesundheitsparameter wurde das gesamte Leberproteom der Regenbogenforelle (Regenbogenforelle (*Oncorhynchus mykiss*)) untersucht und die Ergebnisse mit Hilfe einer Genexpressionsanalyse bestätigt. Zwei Gruppen von Fischen wurden einer derzeit in der Umwelt messbaren Konzentration von Mikroplastik und einer leicht erhöhten Mikroplastikkonzentration, welche die in naher Zukunft zu erwartende Belastung widerspiegelt, exponiert. Diese beiden Gruppen wurden anschließend mit einer Kontrollgruppe (keine Exposition mit Mikroplastik) nach 120 Tagen kontinuierlicher Exposition verglichen. Es zeigte sich, dass die Exposition mit Mikroplastik eine signifikante dosisabhängige Wirkung auf das Wachstum und andere Leistungsparameter (d.h. spezifische Wachstumsrate, Futterverwertung) hat. Es gab keine signifikanten Unterschiede zwischen den Gruppen bei den Blutzuckerwerten, den Hämatokritwerten und dem Vorkommen von oxidativem Stress. Bei der Proteomanalyse wurden über 6000 Proteine identifiziert, aber es wurden keine eindeutigen Unterschiede in ihrer Regulation oder Korrelation mit der Genexpression zwischen den Gruppen festgestellt. Eine Reihe einzelner Proteine und ihre jeweiligen Transkripte wurden jedoch als potenzielle Biomarker für künftige Studien identifiziert. Die Ergebnisse zeigen somit schlüssig, dass selbst geringe Mikroplastikkonzentrationen bei langfristiger Exposition erhebliche Auswirkungen auf Fische haben. Zudem sind sie eine wichtige Grundlage für künftige Untersuchungen der Auswirkungen von Mikroplastik auf die Gesundheit. Abschließend demonstrieren die Ergebnisse das Potenzial neuartiger, dem neuesten Stand der Technik entsprechender Methoden, welche sich derzeit auf diesem Gebiet entwickeln.

List of abbreviations

Polymer types

EPS	Expanded polystyrene
HDPE	High-density polyethylene
LDPE	Low-density polyethylene
PA	Polyamide
PE	Polyethylene
PET	Polyethylene terephthalate
PP	Polypropylene
PS	Polystyrene
PUR	Polyurethane
PVC	Polyvinylchloride

Chemicals

BPA	Bisphenol A
CN	cellulose nitrate
KOH	Potassium hydroxide
NaCl	Sodium chloride
NaOH	Sodium hydroxide
HNO ₃	Nitric acid

Statistical analysis

ANOVA	Analysis of variance
GLM	General linear model
MCA	Multiple Correspondence Analyses
PSDA	particle size distribution analysis
RSM	Response Surface Methodology
SD	Standard deviation

Miscellaneous

ATR	Attenuated total reflection
DNA	Deoxyribonucleic acid
FTIR	Fourier-transform infrared spectroscopy
GIT	Gastrointestinal tract
IR	Infrared
MP	Microplastic
MS	Mass spectrometry
OSPAR	Convention for the Protection of the Marine Environment of the North-East Atlantic
POP	Persistent organic pollutant
PY-GC	Pyrolysis–gas chromatography
RNA	Ribonucleic acid
UV	Ultraviolet
WWTP	Wastewater treatment plant

1. General Introduction

1.1. Plastic - a versatile raw material

Plastic has become an indispensable part of our everyday life. It offers many advantages compared to other materials: it is stable, can be manufactured in a wide variety of shapes, and can be produced cheaply – if environmental costs are excluded. The inclusion of additives can give plastic special properties, such as heat resistance or malleability (Billmeyer 1984).

Plastics are synthetic or semisynthetic polymers, primarily comprising carbon and hydrogen. These polymers can be divided into different groups based on their thermomechanical behaviour: thermoplastics, thermosets and elastomers (Fig. 1.1(A)). Thermoplastics can be reshaped under heat and pressure, while thermosets cannot (Ebewe 2000). They also can be further subdivided into amorphous and semi-crystalline polymers.; amorphous polymers have randomly coiled molecular chains, whereas semi-crystalline polymers have a more organised, densely packed structure (Ebewe 2000). Elastomers possess a rubber-like elasticity, which results from long-chain structures (Billmeyer 1984). In addition to their thermomechanical behaviour, plastics can be distinguished by their density, which is between 0.9 and 1.6 g/cm³ depending on the type of plastic (Hidalgo-Ruz *et al.* 2012; Tab. 1.1). The possibility of producing a material that has clearly defined properties and can be moulded into almost any conceivable shape led to a massive increase in plastic production in the 20th century.

Table 1.1. Basic characteristics of the most commonly used plastic types (after Hidalgo-Ruz *et al.* 2012; PlasticsEurope 2020).

Plastic type	Full name	Plastic demand [%]	Density [g/cm ³]	Examples of use
PP	Polypropylene	19.4	0.90–0.91	Food packaging, pipes
LDPE	Low-density polyethylene	17.4	0.92–0.94	Food packaging, agricultural film
HDPE	High-density polyethylene	12.4	0.94–0.97	Bottles, toys
PVC	Polyvinyl chloride	10.0	1.16–1.58	Pipes, floors
PUR	Polyurethane	7.9	1.20	Insulation, foams
PET	Polyethylene terephthalate	7.9	1.37–1.45	Bottles
PS	Polystyrene	6.2	1.04–1.10	Electronics, building material
Others	-	18.8	-	-

Worldwide plastic production has increased over 200-fold between 1950 and 2019, from 1.7 million tons to approximately 370 million tons per year (PlasticsEurope 2013; PlasticsEurope 2020; Fig. 1.1(B)). In Europe, production has reached around 60 million tons per year in recent times. Nowadays, plastic is mainly used for packaging (Andrady & Neal 2009; Fig. 1.1(C)). Plastic is also an essential raw material in house building, as well as the automotive, agriculture and electronics industries. Although more than 200 different types of plastic are available, very few are used in everyday consumption (PlasticsEurope 2020; Tab. 1.1); polypropylene (PP) and polyethylene (PE) alone account for almost 50 % of plastics used.

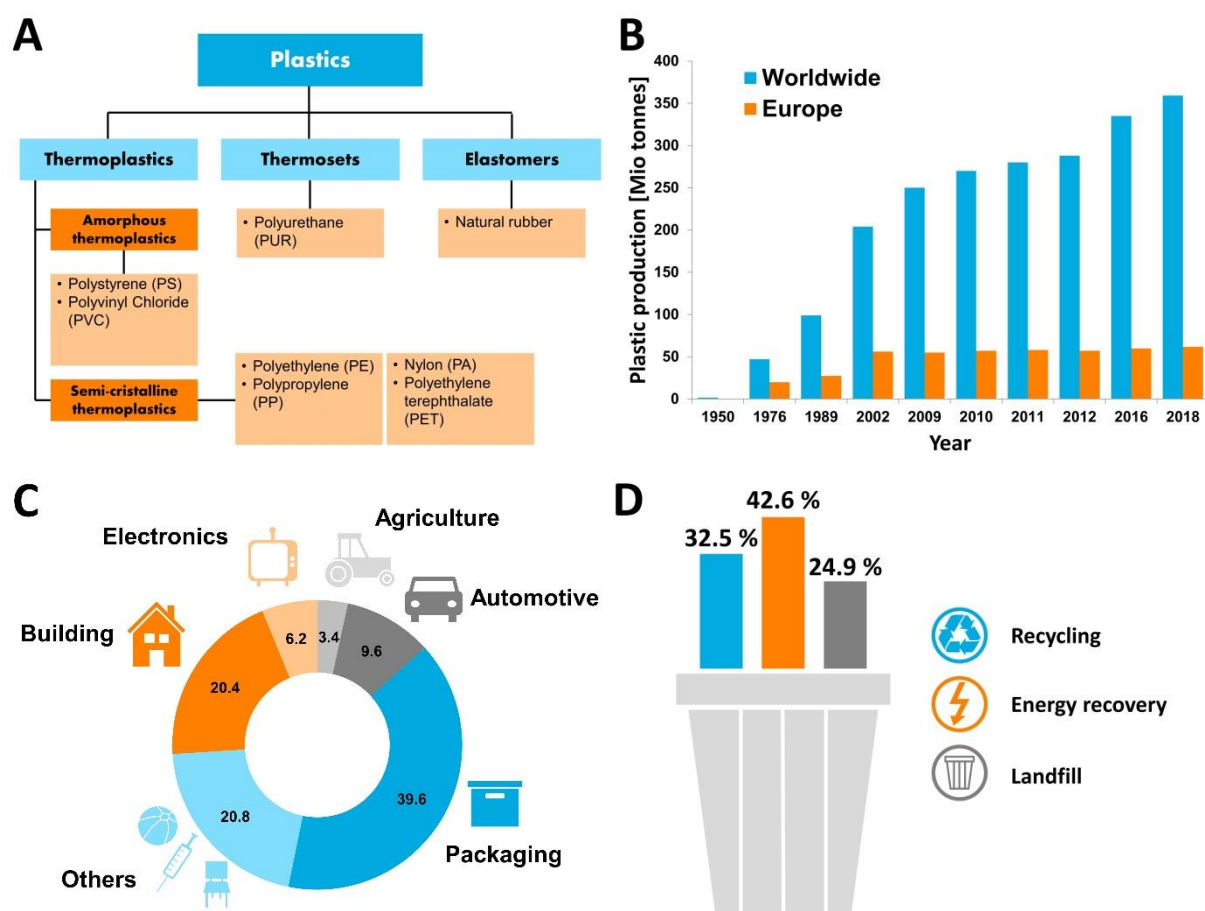


Figure 1.1. The properties, production and uses of plastic. **(A)** Overview of different types of plastic, based on their thermomechanical properties. **(B)** Worldwide and European annual plastic production (after PlasticsEurope 2020). **(C)** Plastic demand of different sectors (after PlasticsEurope 2020). **(D)** Types of disposal of plastic waste in Europe, Norway and Switzerland in 2018 (after PlasticsEurope 2020).

Logically, the increase in plastic production has also led to a dramatic increase in the production of plastic waste. It is estimated that in 2015 alone, 302 million tons of plastic waste were generated worldwide (Geyer *et al.* 2017). In principle, there are several ways of dealing with this waste: it can be recycled, used for energy recovery or sent to landfills (Fig. 1.1(D)). Recycling of plastics is still considered a

challenge, primarily because the large number of additives used means that it is challenging to reconstitute, and the lack of demand from the plastic industry in some countries (Hahladakis *et al.* 2018; Lebreton & Andrady 2019). Fortunately, new technologies have facilitated other opportunities to decrease plastic waste while simultaneously reducing the use of oil and release of carbon dioxide, especially in the area of post-consumer packaging (Mwanza & Mbohwa 2017).

Generating energy by incinerating plastic waste is often seen as a reasonable alternative to recycling, as it can replace coal-based energy, and a high energy recovery rate is ensured by currently available technology (Merrild *et al.* 2012). In Europe the aforementioned methods of dealing with plastic waste are used more or less equally, with energy recovery being slightly more prevalent ([Fig. 1.1\(D\)](#)). However, there are substantial differences between EU Member States: in Germany, Sweden and the Netherlands, there are strict restrictions on the amount of plastic waste that can be diverted to landfill (<1 %; PlasticsEurope 2020). By contrast, in Greece, Bulgaria and Croatia, plastic waste is mainly discarded in landfills and dumps (>60 %). In addition, mismanaged plastic waste (including littering) is a notable problem as it allows the plastic to enter the environment. The amount of mismanaged plastic waste worldwide is expected to increase from 99 million tons in 2015 to 155–265 million tons by 2060 (Lebreton & Andrady 2019).

1.2. Definition and emergence of microplastics

When plastic reaches the environment, its very slow degradation rate becomes a major disadvantage. Plastic can usually be detected for several hundred years after disposal (Barnes *et al.* 2009), and is broken down into smaller fragments through various processes, including mechanical friction, photo degradation and biological degradation (Andrady 2011). If a fragment size reaches less than 5 mm, it is generally termed a “microplastic” (Law & Thompson 2014). However, an official definition for microplastic does not yet exist. Due to the rapidly increasing number of studies on the subject, new size ranges and limits are suggested on a regular basis (Hartmann *et al.* 2019), influenced by factors such as the interaction with biota, the sampling method used and the detection method (Hidalgo-Ruz *et al.* 2012; Wright *et al.* 2013; Hüffer *et al.* 2017). Creating a standardised definition for microplastics was recently proposed in order distinguish them from other size categories, such as nanoplastics and macroplastics (Hartmann *et al.* 2019; [Fig. 1.2\(A\)](#)). Regardless of size, microplastics are often grouped into additional categories based on form. The most commonly used categories are: fragments,

beads/pellets/spherules, films and fibres (hereafter generally summarised as "particles"; Hidalgo-Ruz *et al.* 2012). Plastic particles are also regularly grouped according their colour (Van Cauwenberghe *et al.* 2015).

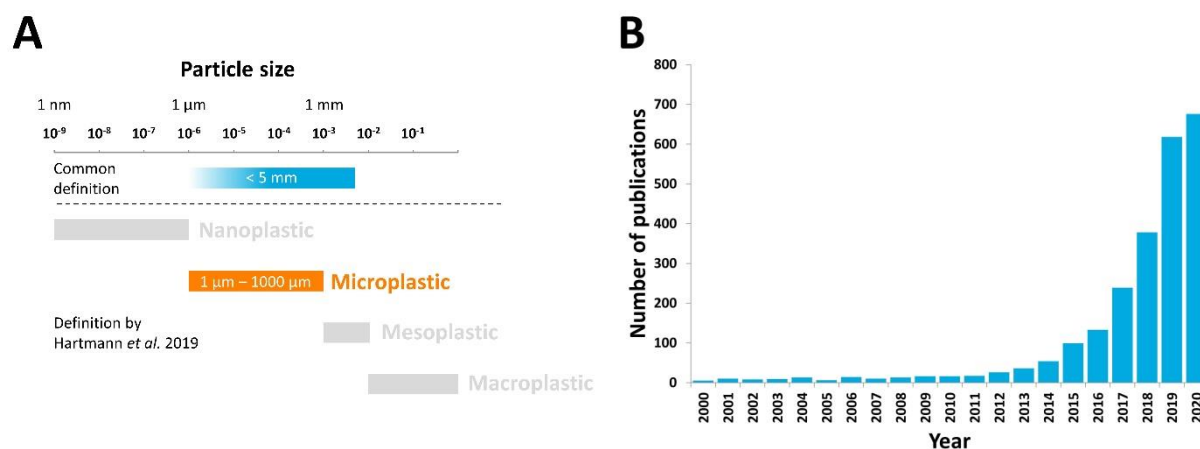


Figure 1.2. General information about microplastics. **(A)** Definition of microplastics regarding size. **(B)** Number of publications per year about microplastics (Search term: "microplastic"; Web of Science, September 2020; Clarivate Analytics 2020).

In 1972, one of the first accounts of microplastics in the environment was published in the renowned scientific journal "Science" (Carpenter & Smith 1972). Despite the increasing number of verifications from researchers across the world since then, the subject of microplastics has long remained unnoticed by the public. Over the last 10 years a now rapidly increasing number of studies have demonstrated the global scale of the problem (Fig. 1.2(B)). Plastic waste is released into the environment through littering, but also through wind drift from open landfills (Barnes *et al.* 2009). In addition, plastic particles from raw plastic processing, skin cleansing agents and clothing fibres can enter bodies of water directly from wastewater treatment plants (Cole *et al.* 2011; Browne *et al.* 2011; An *et al.* 2020). These are typically called primary microplastics because the plastic particles, fibres and beads are already a few mm in size when they enter the environment (Cole *et al.* 2011). As described earlier, microplastics can also be created by the degradation of larger plastic waste. These are usually referred to as secondary microplastics, as the waste must first break down into smaller fragments to be considered microplastic (Cole *et al.* 2011). Although sewage treatment plants can filter out a large proportion of the incoming microplastics (depending on the technology, usually >95 %), a relevant proportion still reaches the connected bodies of water (Murphy *et al.* 2016; Carr *et al.* 2016). Tyre abrasion is also an important source of microplastics, and can even reach remote regions via atmospheric transport (Evangelidou *et al.* 2020; An *et al.* 2020). Regardless of where the plastic waste originates, sooner or later it will pollute aquatic environments.

1.3. Microplastics in the aquatic environment

The increasing problem of plastic pollution in the aquatic environment first became apparent in the world's oceans. Washed up on beaches or concentrated in large sea eddies, plastic has a serious and long-term effect (Derraik 2002; Brach *et al.* 2018). It is estimated that more than 260,000 tons of plastic are found in the ocean (Eriksen *et al.* 2014). However, this quantity is probably greatly underestimated as a large number of microplastics sink and remain on the sea floor (Woodall *et al.* 2014). Research has shown that rivers and lakes are similarly affected (Wagner *et al.* 2014; Horton *et al.* 2017). Although the majority of microplastic sources are land based, it is now known that rivers are important transport routes (Lebreton *et al.* 2017; Fig. 1.3). It has also become clear that freshwaters, similar to the oceans, are important sinks, and that even remote bodies of water are affected (Free *et al.* 2014; Li *et al.* 2018).

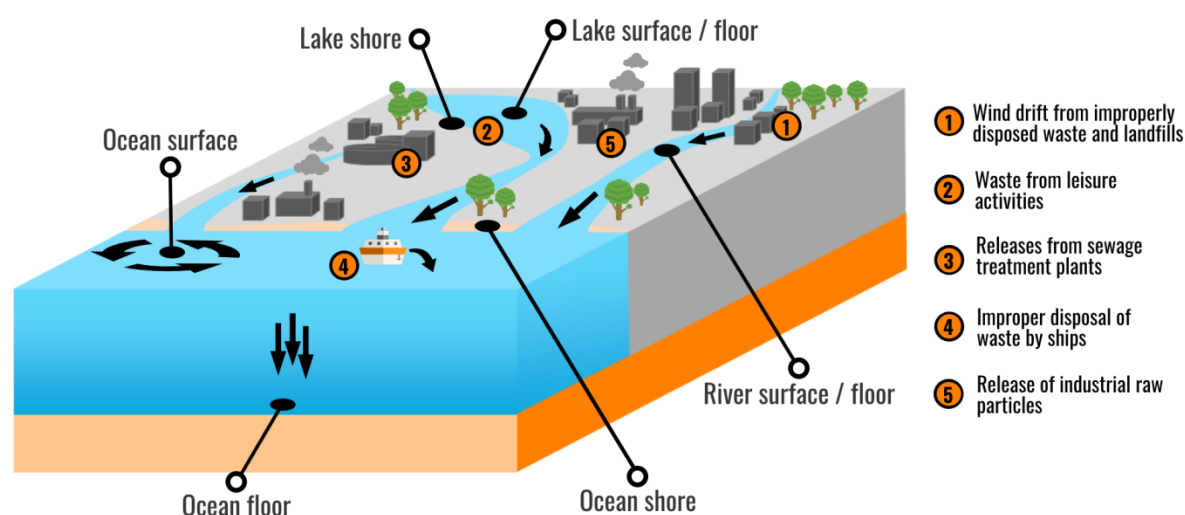


Figure 1.3. Sources, detection and distribution of microplastics in lakes, rivers and the ocean.

Unfortunately, when trying to compare the results from individual studies that examine microplastic pollution in aquatic systems, a fundamental problem becomes apparent: there is no standardised sampling protocol available, meaning that investigations vary greatly in their setup and accuracy. First of all, differences exist in the size range investigated, which mostly depends on the selected mesh size of the nets (surface water) or sieves (sediment) during sampling (Koelmans *et al.* 2020; Hidalgo-Ruz *et al.* 2012). Furthermore, there are several steps necessary to separate plastic particles from collected sediment, and/or the organic components of the water sample must be decomposed (Van Cauwenberghe *et al.* 2015; Li *et al.* 2018). The methods chosen influence whether the entire microplastic

fraction can be isolated, or whether certain polymer types and particle sizes are lost. The next challenge is the actual identification of microplastics. In recent years, numerous methods have been developed for the detection of microplastics. These range from simple visual identification based on shape and colour, to complex spectroscopic analyses (Schwaferts *et al.* 2019). Depending on the method, different information about the plastic particles can be obtained. In the case of purely optical identification, conclusions can be drawn about the shape, size and colour; however, very small particles can easily be overlooked and definitive differentiation from natural materials is not possible (Vandermeersch *et al.* 2015; Hidalgo-Ruz *et al.* 2012). Spectroscopic analyses, such as FTIR (Fourier transform infrared spectroscopy) and Raman, allow the determination of the polymer type but are very cost-intensive and time-consuming (Löder & Gerdts 2015). Another complicating factor is the risk of contamination during the processing of a sample (Nuelle *et al.* 2014; Woodall *et al.* 2015); as plastic is omnipresent in the modern laboratory environment, and plastic fibres from clothing can be dispersed via the air, contamination cannot be completely prevented despite precautions (Woodall *et al.* 2015). It is therefore essential that so-called "procedural blanks" are also examined during processing, and that any contamination that has occurred is accounted for in the analysis (Wesch *et al.* 2017).

All the aforementioned complications make it difficult to accurately evaluate the global microplastic pollution. What is certain, however, is that particle concentrations are highly variable and can depend on a large number of factors (Shahul Hamid *et al.* 2018). Since the majority of plastic waste originates on the land (Schmidt *et al.* 2017), one factor that is regularly considered to be crucial in determining plastic pollutions levels in both marine and freshwater systems is population density. In marine environments, the highest microplastic concentrations are measured in densely urbanised areas (Tsang *et al.* 2017; Kang 2015). Similar relationships have been observed in rivers, but influences on microplastic concentrations there appear to be more complex in most cases (Mani *et al.* 2015; van Emmerik *et al.* 2019). New approaches and common guidelines are urgently needed to reliably determine the microplastic burden in the aquatic environment and to better understand the pathways of entry and dispersal (Koelmans *et al.* 2020). This knowledge is critical for assessing the risks to the aquatic environment and its inhabitants.

1.4. Impact of microplastics on aquatic organisms

One of the most concerning impacts of the plastic pollution is its interaction with aquatic organisms. It became clear quite early on that animals can become entangled in larger plastic waste, but also that it can be colonised and, in some cases, help to spread invasive species (Gregory 2009; Gall & Thompson 2015). However, the smaller the waste becomes, the more organisms can potentially ingest it. Evidence of microplastics in the digestive tract of animals is extensive and covers the entire range of the animal kingdom (Laist 1997). Plastic particles have been found in varying numbers and shapes in zooplankton, macrozoobenthos, fishes, birds, seals and even whales (Ivar do Sul & Costa 2014). They not only enter the animals directly, but can be passed on through the food chain (Setälä *et al.* 2014; Santana *et al.* 2017; Welden *et al.* 2018; Fig. 1.4).

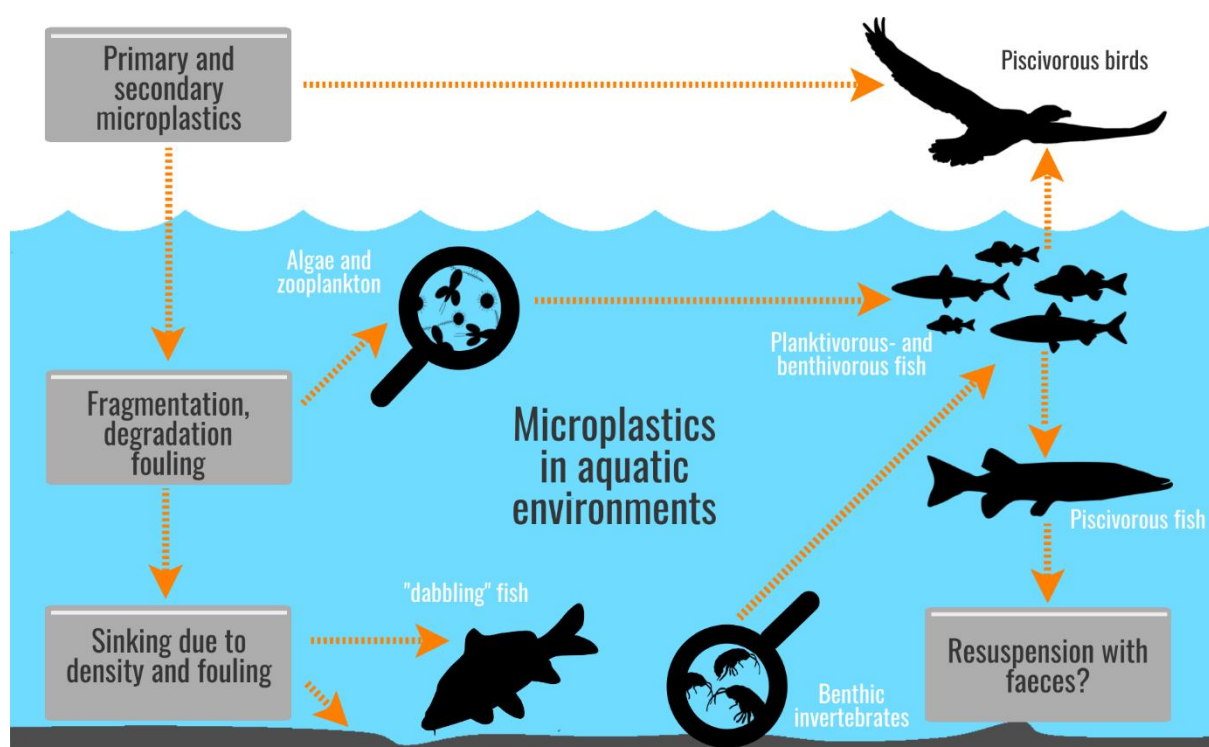


Figure 1.4. Illustration of several possible transport routes for microplastics in the aquatic food chain.

To date, a consensus on the uptake pathways, residence time and potential negative health effects is yet to be established. The diversity and complexity of the pollutant, and the sheer number of different taxa affected, makes a reliable assessment of the microplastic burden and its effects difficult. Similar to environmental samples, pre-treatment of the affected tissue is necessary prior to the identification of microplastic particles (Lusher *et al.* 2017). Likewise, appropriate methods must be chosen to verify the human origin of the particles and prevent contamination (Hermesen *et al.* 2018). Teleost fish are one of the best-

studied aquatic organisms in terms of microplastic pollution: as early as the 1970s, it was shown that they regularly ingest plastic particles (Carpenter & Smith 1972). Today, there are records of microplastics in the intestinal tracts of fish all over the world, both in freshwater and in the world's oceans (Barboza *et al.* 2018; Collard *et al.* 2019).

1.5. Microplastics in fish

There are several hypotheses regarding the uptake pathways for microplastics in fish. If the particles are similar in size and colour to the preferred food, direct ingestion due to confusion with food may occur (Mizraji *et al.* 2017; Ory *et al.* 2017). Alternatively, small plastic particles may be ingested unintentionally during foraging (Hoss & Settle 1990; Jovanovic 2017). Lastly, particles can be passed on through the food chain (Setälä *et al.* 2014; Santana *et al.* 2017; Welden *et al.* 2018). One factor that has been largely ignored in research thus far is the effect of domestication on food selection behaviour. In freshwater environments, billions of fish are stocked every year for conservation purposes (Brown & Day 2002). These fish are often artificially hatched, then reared for some time in hatching facilities before being released; they may also be descended from breeding lines (Araki & Schmid 2010). There is concern that the genetic origin and/or rearing fish in non-natural conditions may alter their ability to recognise and avoid inedible items (Araki *et al.* 2008; Thodesen *et al.* 1999; Rikardsen & Sandring 2006), making them more susceptible to the ingestion of plastic particles.

Although it has been shown that microplastics are regularly ingested by fish, so far there is no evidence of accumulation in an individual over time or along the food chain, as is the case with many other toxic pollutants (Jovanovic 2017). The reason for this is that microplastics are normally excreted with the chyme after a certain period of time (Cong *et al.* 2019; Grigorakis *et al.* 2017; Mazurais *et al.* 2015). Thus far, the available research on the uptake pathways and the residence time of microplastics is mostly based on case studies. A holistic understanding is lacking, which has been challenging to reach given the diversity of the pollutant and the target species.

To date, the microplastic concentrations measured in fish are highly variable, similar to environmental samples. Particle intensities are typically fewer than 10 particles per individual, but in some marine and freshwater hot spots, up to 20 particles per individual have been detected (Barboza *et al.* 2018; Collard *et al.* 2019). Prevalence also varies widely, ranging from a few percent to all examined

individuals being burdened. Here, too, it must be noted that the detection methods used have a decisive influence on the results and comparisons are difficult to make for this reason (Collard *et al.* 2019).

To identify smaller plastic particles, digestion of the stomach or gut contents, or the entire gastrointestinal tract, is necessary. Numerous methods and protocols have been developed for this purpose (Vandermeersch *et al.* 2015; Cole *et al.* 2014; Avio *et al.* 2015). Acidic and basic chemicals are most commonly used, as they are able to decompose the organic material (Vandermeersch *et al.* 2015). However, care must be taken to ensure that the digestion process does not damage the plastic particles, as some polymer types are sensitive to certain chemicals (Lusher *et al.* 2017). Depending on the fish species, another required step is to separate mineral components from potential plastic particles. Benthic fish species in particular regularly ingest mineral debris and other non-digestible items while feeding (Brewer *et al.* 2001; German 2009; Mauchline & Gordon 1984). Furthermore, the bony structures and shells of mussels and snails can make identification of microplastics difficult (Hidalgo-Ruz *et al.* 2012). To confirm whether the remaining particles in the sample are microplastics, the same methods are used as for environmental samples (Lusher *et al.* 2017).

1.6. Effects of microplastic pollution on fish

That fishes are regularly exposed to microplastics both in freshwater and marine systems is undisputed. What effects this exposure has on the health of the fish is controversial and still subject to ongoing research (Parker *et al.* 2021). In general, microplastic ingestion is associated with a number of negative effects on fish (Fig. 1.5). It was suspected early on that physical damage to the gills and digestive tract could occur due to the material properties of plastics (*e.g.* sharp edges after fragmentation; Wright *et al.* 2013; Holm *et al.* 2013). Furthermore, increased levels of exposure might result in blockages of the gastrointestinal tract (Lusher *et al.* 2013; Hoss & Settle 1990). Associated with this was the concern that microplastic consumption could lead to reduced appetite as the gastrointestinal tract becomes filled with indigestible items (Hoss & Settle 1990). However, to date there is no substantiated evidence that microplastics cause any of these direct physical impairments. Fish are regularly exposed to solid materials, such as fine sediments during heavy rain events, without experiencing physical damage (Lake & Hinch 1999; Redding *et al.* 1987). They also regularly ingest non-digestible items when foraging (Doyle *et al.* 2011; Johansson 1991; Mauchline & Gordon 1984). Some laboratory experiments have demonstrated cellular-level physical damage from

microplastics (Lei *et al.* 2018; Ahrendt *et al.* 2020), but the results have been questioned by experts, and attributed to post-mortem autolytic processes and improper sample preparation (Batel *et al.* 2020; Baumann *et al.* 2016; De Sales-Ribeiro *et al.* 2020).

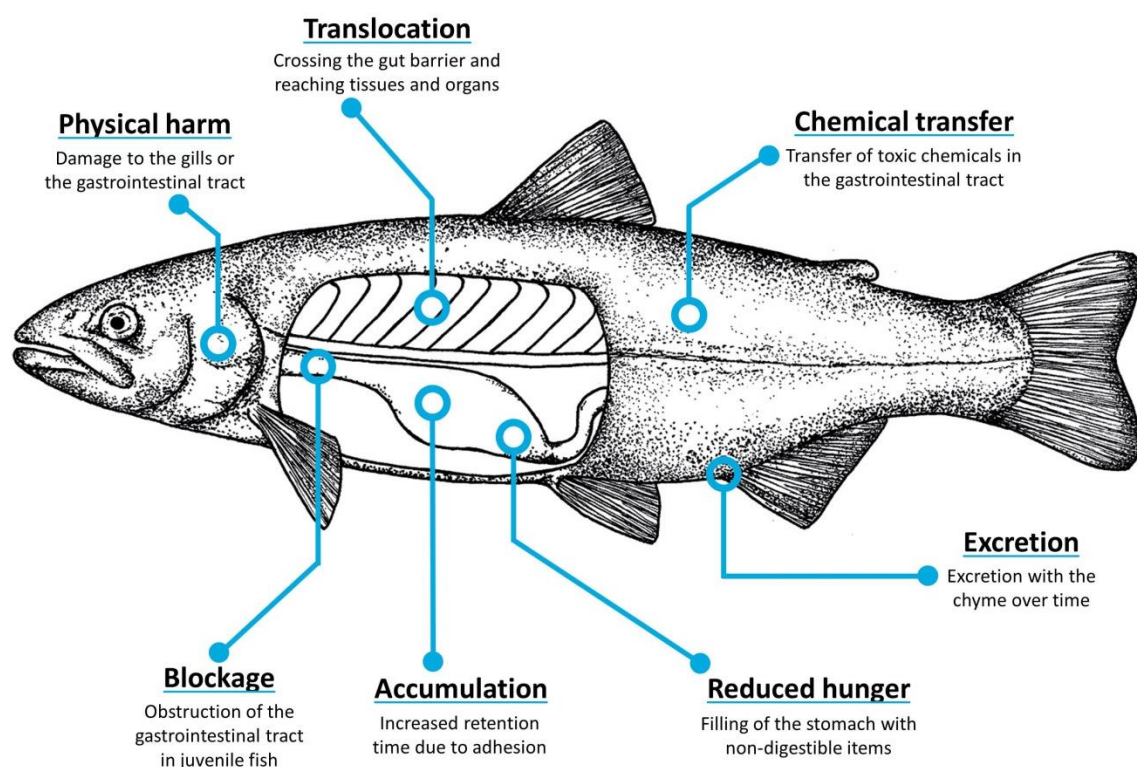


Figure 1.5. Commonly suggested direct and indirect health effects of microplastics on marine and freshwater fish.

Blockage of the gastrointestinal tract is possible, but is likely to be restricted to early life stages (Mazurais *et al.* 2015); for most fish species, typically ingested particles are small relative to the body size of the fish and can therefore be excreted without complications (Cong *et al.* 2019; Grigorakis *et al.* 2017; Mazurais *et al.* 2015). Moreover, one species of fish has been shown to recognise larger plastic particles (~1 mm) as inedible and spit them out immediately, preventing these particles from entering the digestive tract (Ory *et al.* 2018). These same reasons also render the accumulation of particles over time unlikely. That said, it is not yet clear whether the transit time of microplastics depends solely on that of food, or whether it differs based on the size or properties of the plastic particles ingested (Grigorakis *et al.* 2017; dos Santos & Jobling 1991).

Microplastics can also have indirect negative effects on fish through the transfer of certain pollutants. This is in part due to the hydrophobic properties of plastic (Teuten *et al.* 2009), which allow persistent organic pollutants (POPs) from the surrounding water to adhere to microplastics and accumulate (Rochman *et al.*

2013; Lee *et al.* 2019). In addition, a number of additives are regularly used in plastic production, which can be harmful to aquatic organisms above a certain concentration (Teuten *et al.* 2009; Hahladakis *et al.* 2018). These include plasticisers, flame retardants and stabilisers (Hahladakis *et al.* 2018). If these particles are ingested, the pollutants can be released into the animals' tissues due to the acidic conditions in the gastrointestinal tract (Sleight *et al.* 2017). Numerous studies have shown that this certainly occurs in laboratory conditions (Rochman *et al.* 2014; Oliveira *et al.* 2013; Batel *et al.* 2016). However, one must be careful when translating laboratory results to the real-world environment. Recreating realistic exposure levels in the laboratory is challenging and often inaccurate (Connors *et al.* 2017); the microplastic concentrations used are often two to nine orders of magnitude higher than those found in environmental samples (Lenz *et al.* 2016; Connors *et al.* 2017; de Sá *et al.* 2018). Mathematical models were able to show that exposure to POPs and additives via the ingestion of microplastics in the environment contributes only negligibly to the toxification of fish (Koelmans *et al.* 2014). The reason is that the average microplastic concentration in fish is too low relative to the high concentrations of these pollutants in the surrounding water (Herzke *et al.* 2016; Beckingham & Ghosh 2017; Koelmans *et al.* 2016).

As detection methods have improved in recent years, another potential problem of microplastic pollution for aquatic organisms has become apparent. Small plastic particles have been detected in the organs, muscle tissue and even the brains of fish (Collard *et al.* 2018; Lu *et al.* 2016; Zeytin *et al.* 2020). It has therefore been suggested that a translocation process may take place, wherein microplastics pass through the intestinal barrier (Jovanovic 2017). At what particle size and how frequently this occurs is not yet conclusively clarified. For example, two studies detected single particles of up to nearly 600 μm in the livers of European anchovies (*Engraulis encrasicolus*) and chub (*Squalius cephalus*) (Collard *et al.* 2017; Collard *et al.* 2018). Laboratory studies, on the other hand, have demonstrated that particles smaller than 5 μm are able to pass the intestinal barrier in fish (Lu *et al.* 2016; Zeytin *et al.* 2020). Generally, there are two suggested pathways for the translocation of microplastics: transcellular and paracellular. In the transcellular route, microplastic particles are transported through the intestinal epithelium by endocytosis (Wright & Kelly 2017). In the paracellular route, the particles are transported through the tight junctions between cells (Handy *et al.* 2008). For now, these findings must be viewed with caution, since contamination during sample processing cannot be ruled out; it is also not clear which tissues are actually affected (De Sales-Ribeiro *et al.* 2020; Jovanović *et al.* 2018).

1.7. Relevance of microplastics for human health

With the extent of plastic pollution becoming more and more evident, concerns arose around seafood as a potential pathway for microplastics to reach humans. Several studies have detected microplastics in aquatic species used for human consumption (Barboza *et al.* 2018; Santillo *et al.* 2017; Van Cauwenberghe & Janssen 2014). In addition to physical damage, there were fears of increased exposure to adsorbed pollutants and plastic additives (Thompson *et al.* 2009; Wright & Kelly 2017; Bouwmeester *et al.* 2015). Transmission from fish was considered unlikely, as plastic particles are mainly detected in the gastrointestinal tract, which is usually not consumed (Lusher *et al.* 2017). However, the recently discovered translocation processes (described in [section 1.6](#)) could lead to a greater accumulation of plastic particles in fish than previously assumed, while simultaneously increasing the probability of transfer (*e.g.* via muscle tissue) to human consumers.

At present, it is not possible to conclusively assess how and whether exposure to seafood containing microplastics affects human health. Since it is not yet possible to adequately quantify the microplastic burden of aquatic species, it likewise remains unknown whether relevant quantities of microplastics reach humans. The fact that we are surrounded by plastic in our everyday lives and therefore directly exposed to sources of microplastic must also be taken into account. Plastic particles and fibres have been detected in drinking water, beer, honey and salt (van Raamsdonk *et al.* 2020). Artificial fibres are also found in the air both indoors and outdoors, and are regularly inhaled (Wright *et al.* 2020; Dris *et al.* 2017). As in other parts of the field, there is a lack of standardisation that may distort results, impeding a sound assessment of risk (Wright *et al.* 2021; Lachenmeier *et al.* 2015).

1.8. Motivation and aim of the thesis

Despite intensive research in recent years, many questions remain unanswered about the interaction and possible detrimental effects of microplastics on fish. The diversity of the pollutant “microplastic” and resultant large number of differing approaches to the topic has made it difficult to find conclusive answers. This was especially the case in 2014, when this present thesis was planned and conducted. At that time, only sparse information was available about the pollution of freshwater systems, especially in Europe. However, a then-recent study published in 2013 that examined the beach sediments of Lake Garda (Imhof *et al.* 2013) raised concerns that local fish fauna could be affected by microplastics, similarly to those in the marine environment. To learn more about the microplastic exposure of fish in south-western Germany, and to better understand the interaction of plastic particles with fish, the following main objectives were set:

- (A) Systematic evaluation of the microplastic burden of wild fishes in the federal state Baden-Württemberg, south-western Germany
- (B) Investigation of the uptake pathways and transit time of microplastics in freshwater fish under controlled laboratory conditions
- (C) Examination of the effects of microplastics on fish health during short- and long-term exposure with environmentally relevant particle concentrations

In order to systematically investigate the burden of the local fish fauna, a suitable method for isolating microplastics from the gastrointestinal tract of fish had to be developed as part of this thesis. At that time, only time-consuming digestion protocols were available (Claessens *et al.* 2013); these would have only allowed the analysis of a limited number of samples. By keeping the number of processing steps low as low as possible, this newly developed method would both ensure increased efficiency and reduce the risk of contamination. At the same time, it was key to keep any damage to the microplastic itself as minimal as possible. Finally, an optional additional separation step was developed, which allowed the separation of mineral substances (*e.g.* sediment or mussel shells) from the sample.

Once this novel digestion protocol had been established, it was used to facilitate a state-wide survey of fish in Baden-Württemberg. The general aim was to study two fish species with differing life histories at each sampling site. It was hypothesised that benthic and pelagic fish species might differ in their exposure to microplastics, as they use different habitats and are thus subjected to different microplastic concentrations. The sampling sites were chosen in such a way that the entire state and its relevant catchment areas were covered: both large and small rivers, as well

as natural lakes, were investigated. Due to a large-scale survey conducted in 2014 ("Projet Lac"; Alexander *et al.* 2016), it was also possible to study a number of fish species with different life histories in Lake Constance. The results were then used to examine the spatial differences and influence of a number of biotic and abiotic factors on the microplastic burden.

The next step was to investigate and test possible uptake pathways of microplastics through controlled laboratory experiments. The main question was whether fish actively ingest microplastic particles and/or ingest them accidentally during feeding. To this end, fish were exposed to a range of plastic particles with different colours (food-like, inconspicuous or brightly coloured) and of different densities (floating and sinking). The exposure took place once without food and once with simultaneous feeding. In addition, it was tested whether foraging style (visual or chemosensory) had an influence on microplastic uptake. Finally, "domestic status" was investigated as a potential factor; there was speculation that farmed fish might be not as good at avoiding non-edible items as wild fish (Thodesen *et al.* 1999). Given the regular intake of water by marine fish species in order to maintain homeostasis (Fuentes & Eddy 1997), a model was also used to simulate whether microplastic ingestion through drinking is conceivable.

Further to questions about the uptake of microplastics is the question of transit time of through the gastrointestinal tract of fish. Some studies had already shown that microplastics are excreted over time, but there was no information about whether the morphology of gastrointestinal tract (*e.g.* presence or absence of a true stomach) has an influence on the excretion of the particles. In addition, it was unclear whether the size of the ingested plastic particles had an effect on their retention time. To ensure that the fish ingested microplastics of different sizes, a special diet was developed which contained plastic particles with a defined size range. This diet was provided to two fish species in a controlled laboratory experiment, and the microplastic concentration was examined at regular intervals. The data obtained were then used to determine size-dependent transit times for fish with and without true stomach.

A final experiment was carried out to investigate whether microplastics have negative effects on fish health. Critically, the microplastic exposure levels had to reflect those found in environmental samples today and in the near future. Because the actual microplastic burden is quite low, there was a concern that established parameters for evaluating fish health status (*e.g.* performance or stress markers) might lack the sensitivity to identify potential and relevant physiological reactions. For this reason, as part of a screening approach, the complete proteome of the fish

liver was also examined in selected fish. If certain proteins were found to be significantly more or less abundant compared to a control, the differences were confirmed by gene expression analysis. These two groups were continuously exposed to microplastics via their diet for approximately four months. A control group received the same diet but without plastic particles. Therefore, in addition to answering questions about the impact of microplastics on fish, an evaluation of these results should provide new perspectives and recommendations for studying the impact of microplastics on fish.

In summary, the aim of this dissertation was to decisively expand the knowledge about the interaction of teleost fish with microplastics in freshwater systems, while also providing new insights into the study of this complex topic. As microplastics have become a public issue and are seen as an additional biodiversity stressor in a long list of issues threatening fishes worldwide, it is all the more vital that well-founded information is provided about their effect on the environment and wildlife.

2. Manuscript I: Rapid and Efficient Method for the Detection of Microplastic in the Gastrointestinal Tract of Fishes



Article

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Rapid and Efficient Method for the Detection of Microplastic in the Gastrointestinal Tract of Fishes

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2.1. Abstract

The rising evidence of microplastic pollution impacts on aquatic organisms in both marine and freshwater ecosystems highlights a pressing need for adequate and comparable detection methods. Available tissue digestion protocols are time-consuming (>10 h) and/or require several procedural steps, during which materials can be lost and contaminants introduced. This novel approach comprises an accelerated digestion step using sodium hydroxide and nitric acid in combination to digest all organic material within 1 h plus an additional separation step using sodium iodide which can be used to reduce mineral residues in samples where necessary. This method yielded a microplastic recovery rate of $\geq 95\%$, and all tested polymer types were recovered with only minor changes in weight, size, and color with the exception of polyamide. The method was also shown to be effective on field samples from two benthic freshwater fish species, revealing a microplastic burden comparable to that indicated in the literature. As a consequence, the present method saves time, minimizes the loss of material and the risk of contamination, and facilitates the identification of plastic particles and fibers, thus providing an efficient method to detect and quantify microplastics in the gastrointestinal tract of fishes.

2.2. Introduction

The plastic burden on the environment is a rising threat, affecting aquatic ecosystems worldwide (Lambert *et al.* 2014), and its impact on marine systems has been studied intensively in recent years (Ivar do Sul & Costa 2014). It has been estimated that between 4.8 and 12.7 million metric tons of land-based plastic debris entered the oceans in 2010 (Jambeck *et al.* 2015). Larger pieces, mostly originating from landfilled waste and litter (Barnes *et al.* 2009), are often degraded into smaller fragments by mechanical disintegration, UV light and biological processes (Lambert *et al.* 2014). Those plastic fragments are commonly termed as microplastics and comprise all polymer particles and fibers smaller than 5 mm (Law & Thompson 2014). Microplastics can also stem from other sources, including cleansing (Cole *et al.* 2011) and laundry products (Browne *et al.* 2011). While there is relatively little data available to quantitatively evaluate the microplastic burden in freshwater systems (lakes, rivers), microplastic loads are estimated to be similar to that reported in marine systems (Wagner *et al.* 2014; Free *et al.* 2014). Furthermore, rivers are thought to be important pathways for land-based microplastic debris into the oceans, which is reflected by high concentrations in estuaries worldwide (Lima *et al.* 2014; Naidoo *et al.* 2015). While it is important to further examine the rising threat of microplastics, especially the consequences for aquatic organisms, the techniques used to identify and quantify microplastic burdens lack consistency, making it difficult to compare results between studies (Van Cauwenberghe *et al.* 2015).

The potential risks of microplastics for aquatic organisms mainly stem from direct ingestion of particles and fibers (Gall & Thompson 2015) and from associated toxins, such as those released from additives like plasticizers (Koelmans *et al.* 2014) or persistent organic pollutants (POPs) that accumulate on the surfaces of microplastics (Teuten *et al.* 2009). The problem is a complex one, likely to affect virtually all aquatic fauna, including zooplankton, macro invertebrates, mussels, birds, and fishes (Wright *et al.* 2013). Marine plastic burdens have been documented for at least 639 different species to date (Gall & Thompson 2015). These include numerous examples from the stomach contents of fish (Hoss & Settle 1990; Boerger *et al.* 2010; Lusher *et al.* 2013), and a growing body of evidence indicates such ingestion can have a negative influence on fish health (Rochman *et al.* 2013; Rochman *et al.* 2014; Mazurais *et al.* 2015; Lönnstedt & Eklöv 2016). Thus far, however, most records pertain to marine fishes, and evaluation of freshwater species has been limited (Sanchez *et al.* 2014; Phillips & Bonner 2015; Peters & Bratton 2016).

A major challenge in the quantification of microplastics in aquatic organisms centers on the methodologies for identifying the plastic debris. Visual inspection of stomach contents alone, even with the aid of a microscope, brings a high risk of small and inconspicuous particles and fibers being overlooked (Hidalgo-Ruz *et al.* 2012; Dekiff *et al.* 2014; Vandermeersch *et al.* 2015). To increase the chance of identifying and quantifying all important types of plastic, it is necessary to digest away organic matter; however, previously developed digestion techniques and protocols have been shown to be less effective and/or very time-consuming (Vandermeersch *et al.* 2015; Cole *et al.* 2014; Collard *et al.* 2015; Avio *et al.* 2015). There are also potential drawbacks associated with the series of steps required to purify samples, including the loss of certain types of polymers and the possibility of contamination, for example, by airborne fibers and fragments, which are omnipresent even in laboratory environments (Nuelle *et al.* 2014; Woodall *et al.* 2015). Even with special precautions such as working under a fume hood, precleaning tools, and filtering liquids, complete prevention of contamination is not possible, and the need has to be considered in the assessment of samples (Woodall *et al.* 2015). Furthermore, it is possible that other inorganic debris might impede the identification and characterization of microplastics and trigger false positive results, especially if the debris is not verified as plastic using established methods such as spectroscopy (Remy *et al.* 2015; Wesch *et al.* 2016).

The aim of this work was to develop a reliable method for the digestion of the gastrointestinal tract (GIT) of fishes, which (i) uses chemicals that digest organic matter efficiently while causing minimal damage to important polymer types, (ii) minimizes the number of digestion steps to reduce the loss of material and contamination, and (iii) minimizes potential interference from other nonplastic inorganic materials. The method was then tested for efficiency and degradation effects on polymer types commonly encountered in microplastics and finally evaluated under real conditions using fishes from a German river.

2.3. Material and Methods

2.3.1. General digestion method

All steps described were performed under a fume hood with synthetic-free clothing, and gloves were worn at all times. All labware was rinsed three times with filtered ultrapure water (0.2 μm pore size, Arium 611, Sartorius Stedim Biotech, Goettingen, Germany). Samples were placed in 250 mL glass beakers which were

covered by watch glasses to prevent contamination. Sodium hydroxide solution (NaOH 1 mol L^{-1} , Chemsolute, Th.Geyer, Renningen, Germany) was added according to the weight of sample in each beaker, as specified in Table 2.1. The solutions were heated to 50 °C on a hot plate for 15 min while being mixed with a stirring bar. Next, appropriate volumes of nitric acid (HNO_3 65 %, Chemsolute, Th.Geyer, Renningen, Germany) and ultrapure water were added as specified in Table 2.1, and the samples were heated for another 15 min at 50 °C (final concentration of HNO_3 : 10 mol L^{-1} / ~49 %). The temperature was then increased to 80 °C during 15 min to ensure the removal of more resistant suspended solids. For filtration, the samples were diluted at a 1:2 ratio (v:v) with ultrapure water heated to 80 °C and filtered on a cellulose nitrate (CN) filter ($\varnothing$ 47 mm, 8 μm pore size, Sartorius Stedim Biotech, Goettingen, Germany). The beakers and filtration devices were also rinsed with warm ultrapure water to ensure no loss of material.

Table 2.1. Amount of chemicals used in the described digestion method for different sample weights.

Weight of sample [g]	NaOH volume ^a [mL]	HNO_3 volume ^b [mL]	Water volume [mL]	Final volume ^c [mL]
< 1 g	5	17.5	2.5	25
1 - 3 g	10	36	4	50
3 - 5 g	25	72	3	100
5 - 10 g	50	144	6	200
10 - 15 g	75	221	4	300

^a Sodium hydroxide, 1 mol L^{-1} . ^b Nitric acid, 65 %. ^c Final concentration of HNO_3 is 10 mol L^{-1} .

When saponins derived from the first two digestion steps remained on the filters with no trace of mineral residues (see below), the CN filters were placed in 150 mL glass beakers with 20 mL of 1 mol L^{-1} NaOH , and the solutions were heated at 50 °C until the filters were dissolved completely. The samples then were diluted with 100 mL of 80 °C ultrapure water and filtered on quartz filters ($\varnothing$ 25 mm, grade T293, Sartorius Stedim Biotech, Goettingen, Germany), which were dried at room temperature and subsequently analyzed.

2.3.2. Reduction of mineral residues on the filter by density separation

To reduce the quantity of mineral residue on the filters, a density separation was performed after the first two digestions steps. Each CN filter was placed in a 50 mL separating funnel, and 25 mL of 80 °C NaOH (1 mol L⁻¹) was added. The separation funnel was shaken until the filter was completely dissolved. Then 20 g of sodium iodide (NaI pure, Bernd Kraft, Duisburg, Germany) was added, and the separation funnel was shaken again to achieve a solution with a final density of ~1.6 g cm⁻³. After 5 min of resting, 15 mL of the solution was drained and discarded. The supernatant was diluted 1:2 (v:v) with 80 °C ultrapure water, and samples were filtered on 25 mm quartz filters. The separation funnel and the filtration device were rinsed with warm ultrapure water to ensure no loss of material. The filters were dried at room temperature and subsequently analyzed.

2.3.3. Testing the efficiency of the digestion method

The efficiency of the digestion method was tested by spiking the GIT of fish with known quantities of polystyrene (PS) particles. The GIT of six whitefish *Coregonus lavaretus* L. caught in Lake Constance during a routine monthly survey in August 2015 were sampled. Mean total length and fresh weight of the whitefish was 33.5 ± 4.6 cm ($\bar{x} \pm$ SD) and 223.5 ± 72.3 g. The GIT was dissected and stored at -20 °C until further use. On the day of the experiment, the GITs were rinsed with filtered ultrapure water, chopped and placed into 250 mL glass beakers. For the spiking, three different size classes of commercial fluorescent PS particles (Glow-Side, Kretz, Germany) were used. The size classes differed in color as follows: yellow (900-550 µm, 10 particles), red (549-300 µm, 10 particles) and blue (299-100 µm, 20 particles). The particles were mixed with whitefish GIT constituents and treated as mentioned above. For the digestion procedure 25 mL of 1 mol L⁻¹ NaOH, 72 mL of 65 % HNO₃ and 3 mL of filtered ultrapure water were used (Tab. 2.1). After the first digestion step, the particles were counted using a dissecting microscope (Zeiss, Stemi SV6, 8-50x magnification) under UV-light. The count was repeated following the density separation described above and the plastic recovery rates were calculated.

2.3.4. Examination of degradation effects on common polymer types

The effects of the digestion process were tested on a range of polymers derived from common household and laboratory objects: high density polyethylene (HDPE, Falcon tube cap), low density polyethylene (LDPE, plastic bowl), polypropylene (PP, shampoo bottle), polystyrene (PS, cd case), expanded polystyrene (EPS, packaging material), polyethylene terephthalate (PET, drink bottle), polyamide (PA, monofilament cord), plasticized polyvinyl chloride (PVC-P, cable coating) and unplasticized polyvinyl chloride (PVC-U, water pipe). The plastic was chopped to approximately square fragments 1-2 mm wide (with exception of EPS granules, which had a diameter of 1-5 mm). For each plastic type, 6 replicate tests were carried out using 10 particles for each replicate. Each sample was weighed, the particles were photographed digitally under 20-200x magnification and the surface area of each particle was measured to the nearest 0.001 mm using the onboard software of the digital microscope (Keyence, VHX-700F). The samples were digested in 5 mL of 1 mol L⁻¹ NaOH, 17.5 mL of 65 % HNO₃ and 2.5 mL of ultrapure water (Tab. 2.1) as described above. After drying the filters overnight, the particles were weighed and the surface area remeasured. Changes in color and shape were noted and weight changes calculated.

To examine possible changes in spectroscopical properties, FTIR (Fourier transform infrared spectroscopy) spectra of 5 particles were recorded for each plastic type before and after digestion using a FTIR spectrometer (Bruker, Vector 22, coupled with an ATR IR-unit) at a resolution of 8 cm⁻¹ in a wavenumber range of 400-4000 cm⁻¹. Each spectrum was the result of 32 digitally combined scans (software: Bruker, OPUS 6). Before comparing results for each plastic type, the spectra generated from 5 particles were averaged, baseline-corrected, smoothed and normalized using the KnowItAll software V15 (Bio-Rad).

2.3.5. Estimation of the contamination during the digestion procedure

To evaluate the amount and type of contamination that might occur during the digestion procedure, 10 procedural blanks were conducted according to the digestion and density separation methodologies described above but without any GIT samples. In summary, 25 mL of 1 mol L⁻¹ NaOH, 72 mL of 65 % HNO₃ and 3 mL of filtered ultrapure water were used (Tab. 2.1). The resulting quartz filters

were examined for contamination under a digital microscope and the number, color and length of any particles and fibers observed were determined.

2.3.6. Evaluation with field samples

The digestion method was tested under real conditions using the GIT from two different benthic fish species. Fifteen individuals of round goby (*Neogobius melanostomus*) and 10 of common barbell (*Barbus barbus*) were caught during a routine survey in June 2015 at the old passage of the Rhine near Basel (border between Germany and France). On the day of capture, fresh weight and total length of each individual was measured. The whole GIT was dissected under a fume hood, vestiges of other internal organs and fatty tissue were removed and the GIT was weighed. The samples were stored in centrifuge tubes at -20 °C until required for further use. The extraction of microplastics was conducted using the digestion method described above and the additional density separation step to remove remaining minerals (for the quantities of chemicals, see [section 2.3.1](#)). The filtered material was examined under a dissecting microscope and the efficacy of the methodology under real conditions evaluated. Suspicious particles and fibers were verified as plastic using a hot needle point, which leaves a mark on plastic debris (Vandermeersch *et al.* 2015; Devriese *et al.* 2015). The number of microplastic particles per fish (intensity) was calculated and the maximum length of each particle or fiber was measured. No further characterization of polymer type was conducted.

2.3.7. Statistical Analysis

Data was tested for homoscedasticity (Levene-Test; Levene 1960) and parametric or nonparametric tests were chosen accordingly. Differences in recovery rate between particle sizes in the spiking experiment were analyzed using an ANOVA or a Steel-Dwass All Pairs test. To analyze the weight differences after digestion, data was tested for normal distribution and a Grubbs' test was used to identify outliers (Grubbs 1950), which were excluded from the calculation of the mean weight change. A t-test or Wilcoxon test was performed to analyze differences in surface area before and after digestion. To examine differences in weight, the paired t-test or the Wilcoxon signed-rank test was used. All statistical analyses were run using JMP Pro 12 (Vers. 12.2.0, SAS Institute Inc.).

2.4. Results

2.4.1. Efficiency of the digestion method

All GITs were completely digested by the method described, leaving negligible traces of tissue (Fig. 2.1(A, B)). The fluorescent particles were clearly visible under UV-light and their color facilitated counting considerably (Fig. 2.1(C)). In general, recovery rates for each size class of PS particles were high, ranging between 95 and 100 % (Fig. 2.1(D)). There was a slight and nonsignificant decrease in recovery for the smallest particles (first and second digestion step: Steel-Dwass All Pairs test, $P > 0.05$; density separation step: ANOVA, $P > 0.05$).

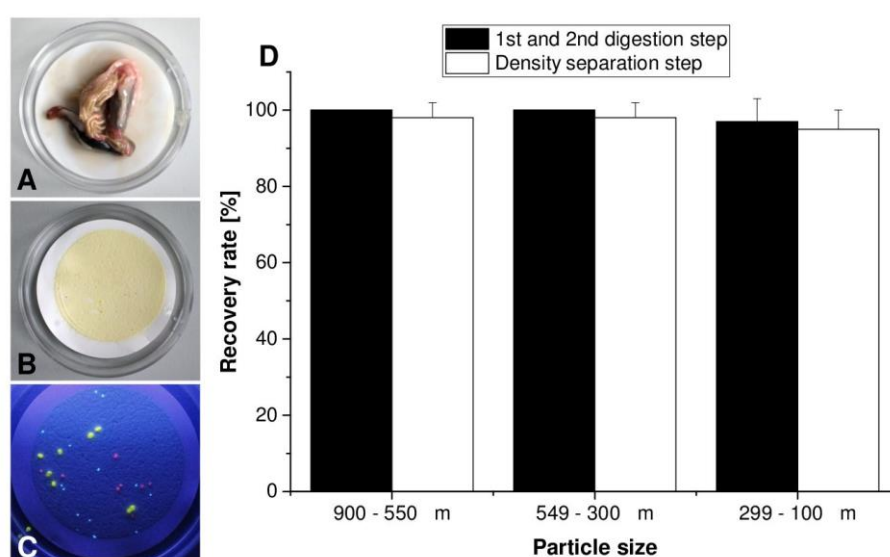


Figure 2.1. Efficiency of the digestion method. **(A)** Gastrointestinal tract (GIT) of a whitefish (*Coregonus lavaretus*) before digestion. **(B)** Filter after the completion of digestion steps. **(C)** Recovered polystyrene (PS) particles after digestion fluorescing under UV light. **(D)** Mean (+SD) recovery rates of PS particles after the first and second digestion steps (black bars) and after density separation (white bars) for each size class ($n = 6$).

2.4.2. Degradation effects on common polymer types

All polymer types, except polyamide, appeared largely resistant to degradation effects during the digestion process (Tab. 2.2). There were however slight color changes to PET and PVC particles and PET showed a statistically significant weight loss of 15.8 ± 3.3 % ($P < 0.05$, paired t-test). Weight changes of other examined polymer types ranged between -2.0 ± 4.0 % and $+3.1 \pm 2.2$ % and were not statistically significant (Tab. 2.2). With some polymer types, minor agglutination or corrosion was apparent (Tab. 2.2). The polymer PA was completely dissolved by the digestion procedure and could not be recovered. Furthermore, there were statistically significant changes in the surface area of EPS, LDPE, PVC-

P and PVCU before and after the digestion procedure (Fig. 2.2). A significant decrease was found in the surface area of EPS granules (Wilcoxon test, $P < 0.0001$), while the surface areas of LDPE, PVC-P and PVC-U particles increased (HDPE: Wilcoxon test, $P < 0.0001$; PVC-P, PVC-U: t-test, $P < 0.0001$). No statistically significant changes were apparent in the other polymer types (Fig. 2.2). The FTIR spectra before and after the digestion method showed no recognizable differences and in all cases the particles could be clearly identified (Fig. 2.3).

Table 2.2. Degradation effects of applied chemicals and temperature on common polymer types. n = number of replicates (each with 10 particles). Mean particle weight change is given as a percentage ($\bar{x} \pm SD$). Changes in weight were statistically analysed, using a paired t-test or Wilcoxon signed-rank test.

Polymer type ^a	n	Colour change (before / after)	Weight change [%]	P weight change	Other changes
EPS	6	no	-0.8 ± 11.6	ns	-
HDPE	6	no	-2.0 ± 4.0	ns	-
LDPE	6	no	$+3.1 \pm 2.2$	ns	agglutination of particles
PA	6	n.a.	-100	n.a.	completely dissolved
PET	6	yes (green / white)	-15.8 ± 3.3	< 0.05	-
PP	6	no	-1.8 ± 2.5	ns	-
PS	6	no	-1.0 ± 1.9	ns	slight corrosion at the edges
PVC-P	6	yes (blue / white)	-1.0 ± 2.3	ns	-
PVC-U	6	yes (brown / white)	$+1.9 \pm 2.5$	ns	-

^a HDPE = high density polyethylene (Falcon tube cap), LDPE = low density polyethylene (plastic bowl), PP = polypropylene (shampoo bottle), PS = polystyrene (cd case), EPS = expanded polystyrene (packaging material), PET = polyethylene terephthalate (drinking bottle), PA = polyamide (monofilament cord), PVC-P = plasticized polyvinyl chloride (cable coating), PVC-U = unplasticized polyvinylchloride (water pipe).

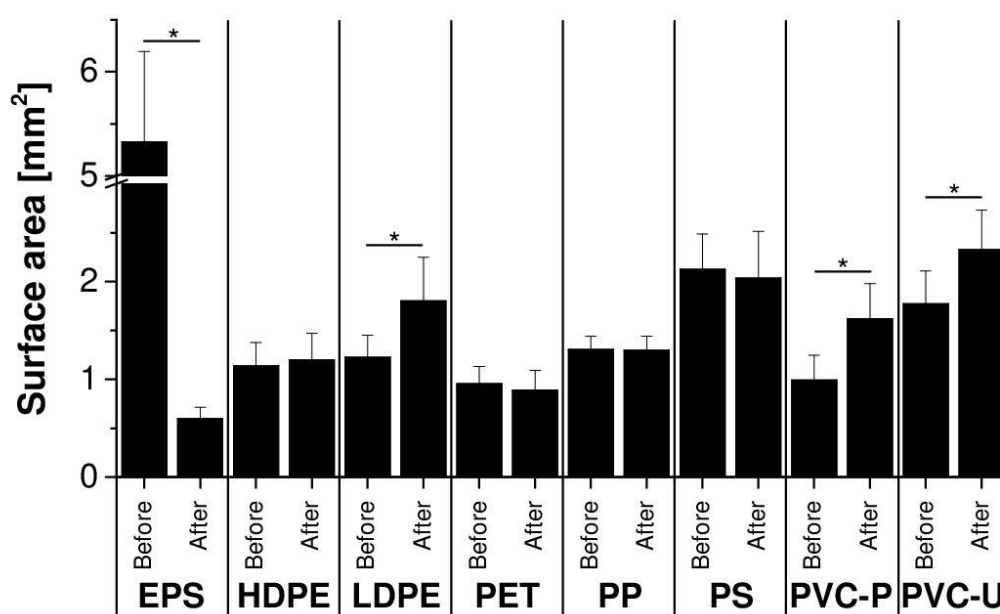


Figure 2.2. Surface area of common polymer types before and after the digestion procedure. * = statistical significant differences (EPS, LDPE: Wilcoxon test, $P < 0.0001$, PVC-P, PVC-U: t-test, $P < 0.0001$). Note the axis break on the y-axis.

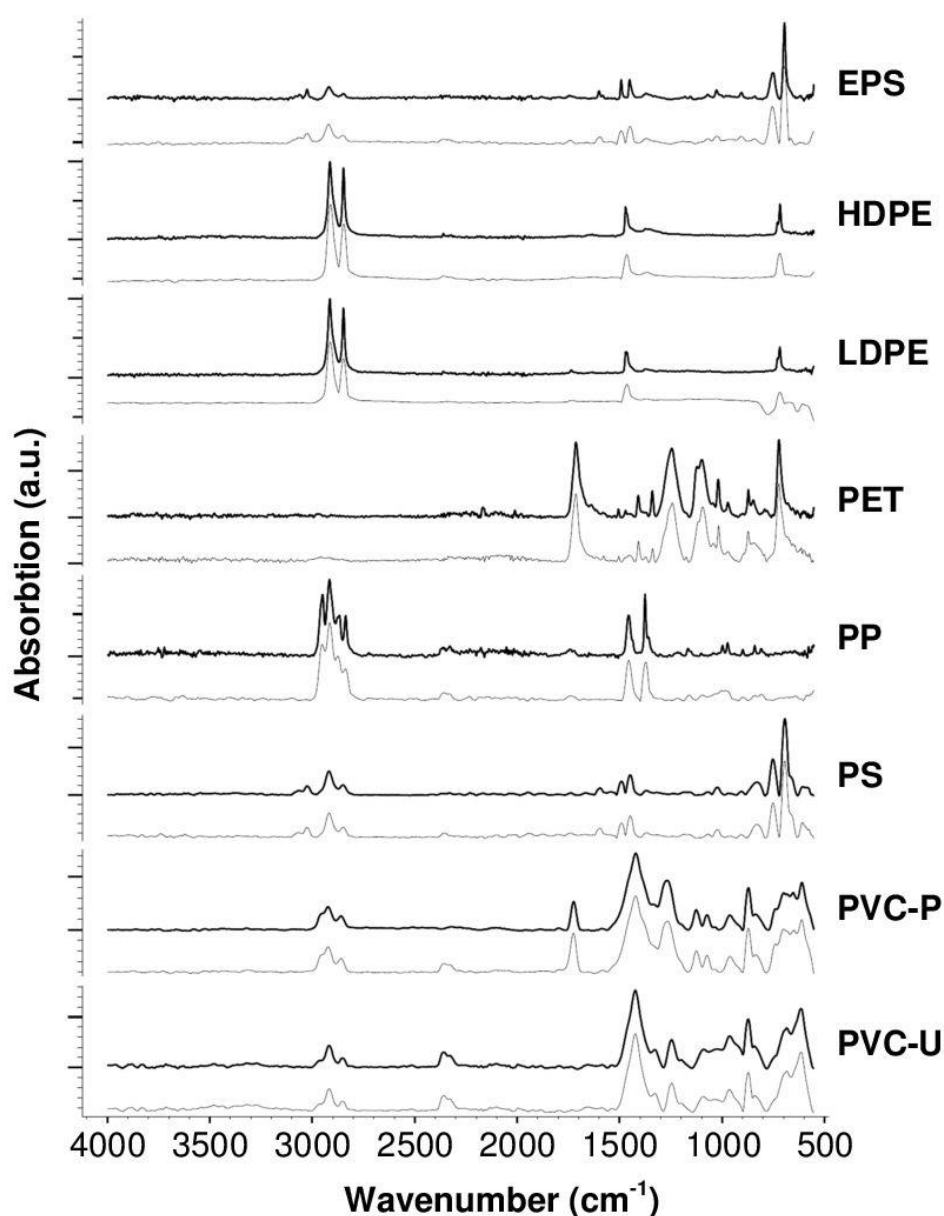


Figure 2.3. Fourier transform infrared spectroscopy (FTIR) spectra of common polymer types before (gray lines) and after (black lines) digestion. The spectra were averaged from five particles of each type, baseline-corrected, smoothed, and normalized.

2.4.3. Contamination during the digestion procedure

The contamination detected in the procedural blanks consisted exclusively of fibers. The average number of fibers per replicate was 12.8 ± 6.3 , and they came in a variety of different sizes and colors (Fig. 2.4). Sizes ranged between 101 and 4774 μm , but 96 % of all fibers examined were less than 3000 μm long. In total, six different colors were identified (Fig. 2.4), with white being the most abundant color (43 %). Other colors appeared more rarely (blue: 17 %, black: 17 %, gray: 11 %, yellow: 8 %, red: 4 %). The chemical nature of recovered fibers was not further evaluated.

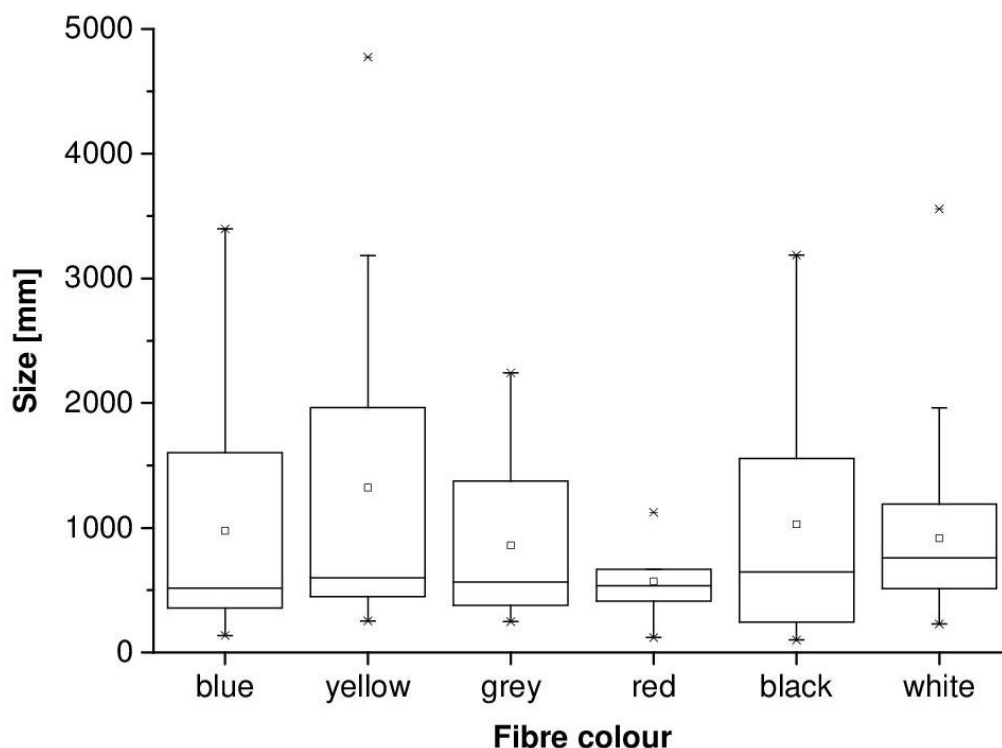


Figure 2.4. Analysis of the procedural blanks to examine the fiber contamination during the digestion procedure (n = 10). Box plots indicate distribution of fiber sizes from all replicates for each fiber color.

2.4.4. Evaluation with field samples

Mean total length and fresh weight was 12.1 ± 1.6 cm and 26.5 ± 10.0 g for round goby and 14.7 ± 2.8 cm and 27.9 ± 13.9 g for common barbel. All GITs (mean weight: 2.1 ± 1.2 g (round goby) and 0.8 ± 0.6 g (barbel)) were completely digested by the method applied.

Digested samples contained no tissue residues, and mineral residues were distinctly reduced after performing the density separation step. Microplastics were detected in 27 % of round goby samples and 20 % of common barbel (Fig. 2.5). The plastic debris was identified by the hot point test and comprised seven particles and one fiber (only fibers that were clearly distinguishable from potential contaminants, *i.e.* because of their shape (Murphy *et al.* 2016), were considered). The mean number of microplastics per affected fish (intensity) was 1.0 ± 0.0 for common barbel and 1.25 ± 0.5 for round goby. The length of the particles ranged from 264 to 2907 μm (mean 1052 ± 975 μm); the single fiber was 3063 μm long. Differences in color and shape between the particles were apparent (Fig. 2.5).

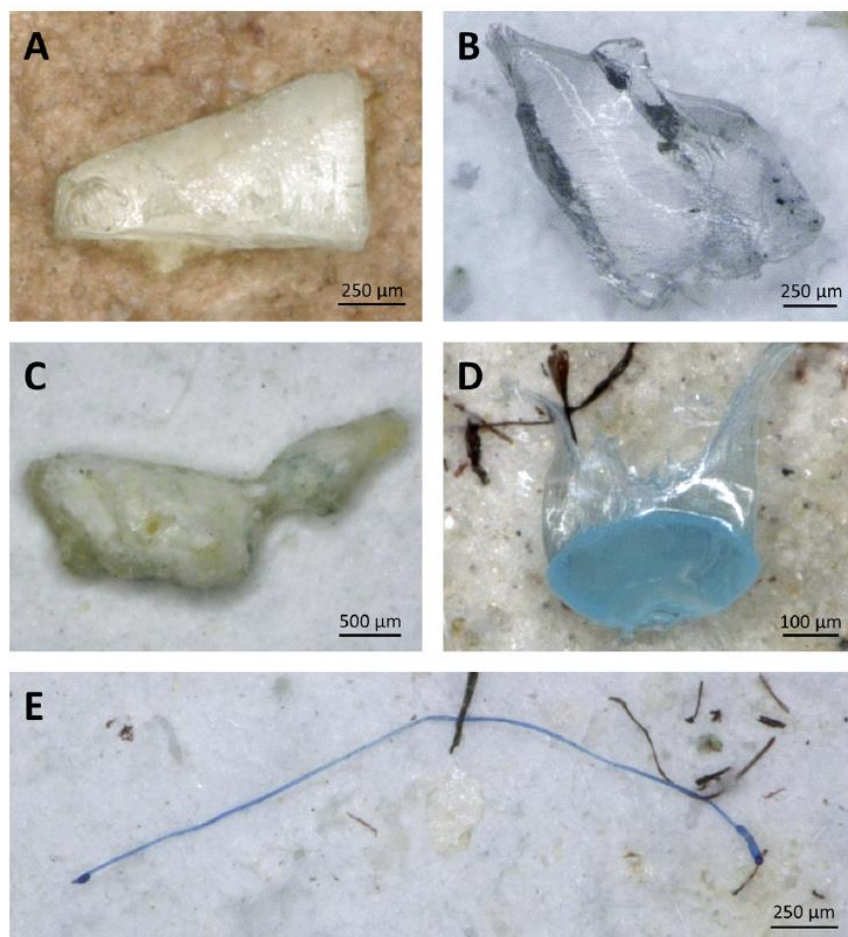


Figure 2.5. Selection of microplastics recovered from round goby (*Neogobius melanostomus*) and common barbel (*Barbus barbus*) using the described digestion method. The fishes were caught during a routine survey in June 2015 at the old passage of the Rhine near Basel (border between Germany and France). The microplastic debris can be categorized into fragments (**A–C**), beads (**D**), and fibers (**E**).

2.5. Discussion

2.5.1. Efficiency of the digestion method

The detection of plastic particles and fibers in aquatic organisms is of growing interest and adequate standardized detection methods are urgently needed (Vandermeersch *et al.* 2015). The novel digestion method described takes a combined approach to reduce the overall processing time and the number of procedural steps while maintaining a high efficacy of digestion. The protocol was developed and optimized to completely dissolve fish digestive tract tissues and enable the identification of important polymer types relevant in environmental pollution (Ivar do Sul & Costa 2014; Cole *et al.* 2011; Van Cauwenberghe *et al.* 2015). The combined use of a base (NaOH) and an acid (HNO₃) meant that intermediate steps required in other protocols, including filtration, removal of the sample from the filter, and changing of glassware (Tab. 2.3), could be bypassed.

HNO₃ has been used previously in several digestion protocols because, given time, it leads to the complete dissolution of the organic matter (Collard *et al.* 2015; Claessens *et al.* 2013; De Witte *et al.* 2014). However, the time required for complete dissolution using HNO₃ alone ranges from overnight to several weeks unless the process is catalyzed by increasing the working temperature or concentration (Tab. 2.3). The addition of a preliminary NaOH step reduced the digestion time to less than 1 h and allowed for the use of less concentrated chemicals than required by other approaches (Tab. 2.3). By filtering the sample on CN filters, which can be completely dissolved by NaOH, additional purification steps can be conducted without any loss of material. To further facilitate the identification of microplastics, an optional density separation step was added to remove mineral residues. Depending on foraging preference and habitat, the GIT of certain fish species may contain natural inorganic residues such as sand (Peters & Bratton 2016; Skora *et al.* 2012). Avio *et al.* (2015) performed an additional density separation step to remove these, using a hypersaline sodium chloride (NaCl) solution with a density of $\sim 1.2 \text{ g cm}^{-3}$ prior to the digestion of organic tissue. However, this only separated particles and fibers with lower densities (Van Cauwenberghe *et al.* 2015; Claessens *et al.* 2013) and left important polymers, such as, PVC and PET. As an alternative, NaI might be used to increase the density of the separation solution (Nuelle *et al.* 2014; Claessens *et al.* 2013; Karami *et al.* 2017) and aid the recovery of all relevant polymer types while reducing the quantity of other heavier residues.

To test the efficacy of this novel method, the GITs of whitefish were spiked with different-sized PS particles. The mean recovery rate was $\geq 95 \%$, depending on particle size. This is comparable with other approaches using similar polymer types and size classes (Avio *et al.* 2015; Karami *et al.* 2017). As the present experiments indicated, recovery rate might decrease with lower particle size for certain polymer types. The use of fluorescent color coded particles and UV light can greatly aid the evaluation of recovery rates and enables the usage of small particles in such spiking experiments.

2.5.2. Degradation effects on common polymer types

The digestion method described caused only minor degradation effects on the common polymer types tested, with the exception of PA. This is a general problem with protocols using HNO₃, as PA is easily dissolved by this corrosive chemical (Claessens *et al.* 2013; Bürkle 2016).

Table 2.3. Overview of available protocols to digest organic tissue for the identification of microplastics in water organisms.

Reference	Duration of the digestion method	Chemicals used	Reaction Temperature [°C]	Polymer types used	Additional density separation step	Changes of surface area examined	Contaminations evaluated	Validation with field samples (type)
present study	< 1 h + 0.5 h (density separation step)	NaOH (~4 %), HNO ₃ (~49 %)	50 °C, 80 °C	HDPE, LDPE, PET, EPS, PS, PP, PA, PVC-U/-P	yes (NaI) ~ 1.6 g cm ⁻³	yes	yes	yes (whole gastrointestinal tract of fishes)
Claessens <i>et al.</i> 2013	Overnight + 2 h	HNO ₃ (~95 %) ^a	Room temperature, 100 °C	PS, PA	no	no	no	no
Foekema <i>et al.</i> 2013	2 - 3 weeks	KOH (10 %)	Room temperature	n.a.	no	no	no	yes (content of the gastrointestinal tract of fishes)
De Witte <i>et al.</i> 2014	Overnight + 0.5 h	mixture of HNO ₃ (65 %) and HClO ₄ (68 %) in a 4:1 v/v ratio	Room temperature, 100 °C	n.a.	no	no	yes	yes (mussels)
Avio <i>et al.</i> 2015	n.a.	H ₂ O ₂ (15 %)	n.a.	PE, PS	yes (NaI) ~ 1.2 g cm ⁻³	no	no	yes (complete gastrointestinal tract of fishes)
Collard <i>et al.</i> 2015	Overnight	NaClO (9 %), mixture of NaClO (9 %) and HNO ₃ (65 %) in a 1:10 v/v ratio	n.a.	PET, PVC, PE, PP, PS, PC, PA	no	no	no	yes (stomach content of fishes)
Karami <i>et al.</i> 2017	48-72 h	KOH (10%)	40 °C	LDPE, HDPE, PP, PS, PET, PVC, nylon-6/-66	yes (NaI) ~ 1.5 g cm ⁻³	yes	yes	yes (whole fishes)

^a The concentration mentioned by Claessens *et al.* (2013) was 22.5 mol L⁻¹ (= 95 %), which is most likely an error in the manuscript. ^b GIT = gastrointestinal tract.

While PA represents a very small part of the total plastic production and consumption (Fischer 2004, Plastics Europe 2014), its availability in marine and freshwater systems is quite ambiguous (Wright *et al.* 2013; Dris *et al.* 2015). Depending on the local situation, PA might be a potentially important aquatic pollutant due to its use in fishing gear (Possatto *et al.* 2011; Dantas *et al.* 2012; Ramos *et al.* 2012). Therefore, before applying the introduced method, it is crucial to establish whether PA is playing a considerable role in the investigated waters. In cases where PA is of special relevance, other protocols will be needed (*e.g.*, using enzymatic digestion techniques; Cole *et al.* 2014). Degradation of the other polymer types studied was limited mainly to slight color and weight changes, and minor corrosion and agglutination effects. There were also statistically significant changes in the surface area of some particles. The foamlike properties of EPS meant that it showed considerable shrinkage and surface area loss (PlasticsEurope 2016). Such effects must be considered when analyzing field samples and measuring recovered particles. With LDPE, PVC-P, and PVCU, a statistically significant increased surface area indicates minor melting due to the corrosive nature of the chemicals deployed. However, these effects might be reduced when the plastics are embedded in tissue (Claessens *et al.* 2013). Unfortunately, surface area is rarely measured when testing digestion protocols (Tab. 2.3) despite its relevance for organic pollutant uptake and release (Teuten *et al.* 2009). It might become necessary to develop respective regression functions for relevant polymer types to calculate the naturally occurring size of recovered particles before samples are subjected to treatment.

After identifying possible microplastic debris in animal tissue, further analysis of the recovered particles and fibers is normally necessary (Rocha-Santos & Duarte 2015). Spectroscopic approaches are often used to confirm anthropogenic sources and identify individual polymer types (Harrison *et al.* 2012; Song *et al.* 2015; Löder & Gerdt 2015). The present method had no detectable effect on the FTIR spectra of the common polymers tested, each of which could be still clearly identified by its specific peaks without restrictions. Therefore, this digestion method can be used to further characterize isolated microplastics.

2.5.3. Contaminations during the digestion procedure

Though often overlooked, contamination is commonly observed during laboratory processing of samples (Woodall *et al.* 2015). The risk of contamination rises with the number of digestion steps, and the problem is especially important in animal tissue samples, as here the overall microplastic burden is comparably low and the

risk of overestimation is therefore high. It is important to evaluate the quantity and type of likely contamination (Nuelle *et al.* 2014; Fries *et al.* 2013). The analyses of the procedural blanks after performing the digestion protocol revealed that white and colored fibers smaller than 3000 μm were abundant. These most likely stem from clothing and are likely to be transferred to samples from the ambient air (Vandermeersch *et al.* 2015; De Witte *et al.* 2014; Foekema *et al.* 2013). It is crucial that appropriate measures are taken to minimize the entry of airborne fibers by working under a fume hood, wearing synthetic-free clothing, and cleaning all lab equipment used (Hidalgo-Ruz *et al.* 2012; Woodall *et al.* 2015). It is also suggested that filtering of the chemicals used might further reduce any contamination (Vandermeersch *et al.* 2015; De Witte *et al.* 2014), but there was no indication of any particles and fibers originating from that source in the present study (data not shown). As it stands, small colored fibers are often excluded from analyses because of the problems mentioned above. As contamination is very difficult to eliminate and levels of contamination vary between laboratories, alternative strategies have to be developed. One possibility would be to perform relative comparisons between samples, rather than using a purely quantitative approach. However, further studies are necessary to examine ways of minimizing or controlling contamination effects and to allow small fibers to be included in the analyses.

2.5.4. Evaluation with field samples

To test the present method under real-life conditions, complete GITs of round goby and common barbel were digested and examined for plastic particles and fibers. The described method removed all organic tissue, and the subsequent density separation considerably reduced the quantity of mineral residues. The isolated microplastics were easily identified, and the results were comparable with other findings in freshwater fishes (Sanchez *et al.* 2014; Phillips & Bonner 2015; Peters & Bratton 2016).

As the field samples were used for evaluation purposes only, no ecological information about examined fish was taken into account, and a further characterization of found microplastics was not conducted. The shape and color of recovered particles and fibers did not allow any conclusions about their origin, but they are likely to be mainly fragments of larger plastic debris (Fig. 2.5). The new method might facilitate the detection of microplastics in animal tissue and improve the knowledge of actual burden in fishes. Knowledge about the uptake routes and effects of direct ingestion of microplastics by fishes remains limited. However, it is

suggested that small particles and fibers are either confused with prey or ingested accidentally with other food (Hoss & Settle 1990) and likely consequences are thought to include (i) blocking of the stomach or gut, (ii) reduced appetite, and (iii) accumulations in the stomach or gut (Lusher *et al.* 2013). Any of these effects might lead to digestion problems, starvation effects, or direct damage to internal organs. Indirect consequences of plastic ingestion by fishes include the transfer and bioaccumulation of additives and POPs into fish tissue (Rochman *et al.* 2013; Rochman *et al.* 2014).

2.6. Acknowledgments

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3. Manuscript II: A systematic study of the microplastic burden in freshwater fishes of south-western Germany **- Are we searching at the right scale?**

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A systematic study of the microplastic burden in freshwater fishes of south-western Germany - Are we searching at the right scale?



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3.1. Abstract

In a comprehensive study of microplastic contamination in southern Germany, 1167 individual fish of 22 different species were sampled from 11 rivers and 6 lakes across the state. The microplastic burden of investigated fish was analyzed on the basis of habitat type, location, and a number of abiotic and biotic factors. A particle size distribution analysis of the detected microplastics was carried out. The results showed a relatively low plastic prevalence of 18.8 %, with significant differences between rivers (20.6 %) and lakes (16.5 %). The number of ingested plastic particles ranged between 1 and 4 particles per fish. The majority of abiotic and biotic factors seem to play little or no role in the ingestion of microplastics, suggesting that in most cases uptake is passive or accidental. It is notable that piscivorous fish appeared significantly less burdened, suggesting a low transfer rate and no accumulation in the food web. However, size distribution analysis identified a power law growth fit in particle numbers at the smallest end of the distribution. This carries a worrying implication, that >95 % of particles are likely to be smaller than 40 µm and thereby beyond the detection range of this and most other microplastic surveys conducted so far. When the frequency development of

small particles is taken into account, the likely microplastic prevalence in the present study increases to 100 %, with an average intensity of around 23 predominantly small particles per fish. A striking 70 % of those particles would be smaller than 5 μm and therefore eligible for translocation into tissues, with critical implications for fish health and consumer exposure. This raises a question as to whether current estimates of microplastic burden in fishes generally might be overlooking a majority of potential contamination within the critical smaller particle size classes.

3.2. Introduction

The long ignored issue of plastic pollution of aquatic systems is now recognised as a severe threat to the global environment (Rochman *et al.* 2013). Plastic particles (comprising fragments, fibres, beads, films) smaller than 5 mm are commonly referred to as microplastics (Hartmann *et al.*, 2019; Law and Thompson, 2014), and are found in oceans, rivers and lakes worldwide (Lambert *et al.* 2014; Zhu *et al.* 2018). However, the majority of microplastic research has focused on marine ecosystems (Wagner *et al.* 2014), where particles are known to be ingested by zooplankton, macroinvertebrates, fishes and birds (Gall and Thompson, 2015; Zhu *et al.*, 2019). Knowledge of the issue in freshwater systems is still sparse (Horton *et al.* 2017), but the small size of microplastics suggests the problem is likely to impact all aquatic fauna (Wright *et al.* 2013). In recent years, it has become evident that freshwater systems carry a microplastic burden comparable to that in marine environments (Dris *et al.* 2015) and that rivers serve as important distribution pathways (Schmidt *et al.* 2017).

Estimates for microplastic burden in fish have to be treated with some care, as there is currently no standardised method for detection (Hermsen *et al.* 2018). Different methods for the isolation of plastic particles do exist. These range from simple dissection to the complete digestion of organic tissue using dissolving chemicals or enzymes (Lusher *et al.* 2017). However, the identification of microplastics can be challenging, as small and inconspicuous particles might be overlooked (Hidalgo-Ruz *et al.* 2012; Vandermeersch *et al.* 2015). Therefore, several additional analytical methods, such as spectroscopic approaches, are regularly used (Löder & Gerdts 2015). Another problem is the risk of contaminations (*e.g.* through airborne plastic particles), which might significantly bias outcomes (Nuelle *et al.* 2014), but which given the ubiquity of plastics is very difficult to prevent.

The abundance and size of ingested microplastic particles both have a bearing on the severity of adverse health effects on fish. Larger particles may accumulate or cause direct mechanical damage, injuring or obstructing the gastrointestinal tract (Jovanovic 2017), while smaller particles might be incorporated and translocated into tissues (Avio *et al.* 2015; Collard *et al.* 2018), and ultimately find their way to the human consumer (Wright & Kelly 2017).

Particle size is also highly pertinent to the potential transfer of toxic chemicals associated with microplastics, because smaller particles exhibit an exponentially increasing surface area to volume ratio (Lee *et al.* 2014). Additives such as plasticisers or surface accumulated persistent organic pollutants (POPs), are thought to cause adverse effects (Koelmans *et al.* 2014; Teuten *et al.* 2009). Whether the quantities associated with current concentrations of environmental microplastics are high enough to elicit a significant effect is not known (Koelmans *et al.* 2016).

While it is generally assumed that the abundance of microplastics in the environment increases exponentially with declining particle size (Imhof *et al.* 2016; Steer *et al.* 2017), the limitations of the sampling methods and identification techniques applied in a majority of studies mean that the majority of work to date has focussed on larger particles (Lusher *et al.* 2017).

The current study involved a systematic evaluation of microplastic occurrence in freshwater fish in south-western Germany, with a focus on particle number and size. Sampling took place in a range of relevant surface waters and included fishes from diverse habitats. An in-depth survey was conducted in Lake Constance looking at a wide range of species from all food web positions and lake habitats. The data gathered was used to identify and appraise the importance of biotic and abiotic factors in microplastic occurrence and uptake pathways. Furthermore, the results from fish digestive tracts were examined with previous data describing microplastic contamination of surface water, comparing the abundance, size distribution and types of plastic identified. Finally, a particle size distribution analysis (PSDA) was used to extrapolate the likely distribution of microplastic distribution beyond the current size limits of physical detection.

3.3. Material and methods

3.3.1. Sampling sites and procedure

Fish of several species were sampled between 2014 and 2018 at different locations in the federal state of Baden-Württemberg (Germany), spread across an area of approximately 35,700 km² (Fig. 3.1, Tab. S3.1). Baden-Württemberg is located in southwestern Germany, bordered to the south by Lake Constance, a large, deep and formerly warm monomictic lake (Petri 2006).

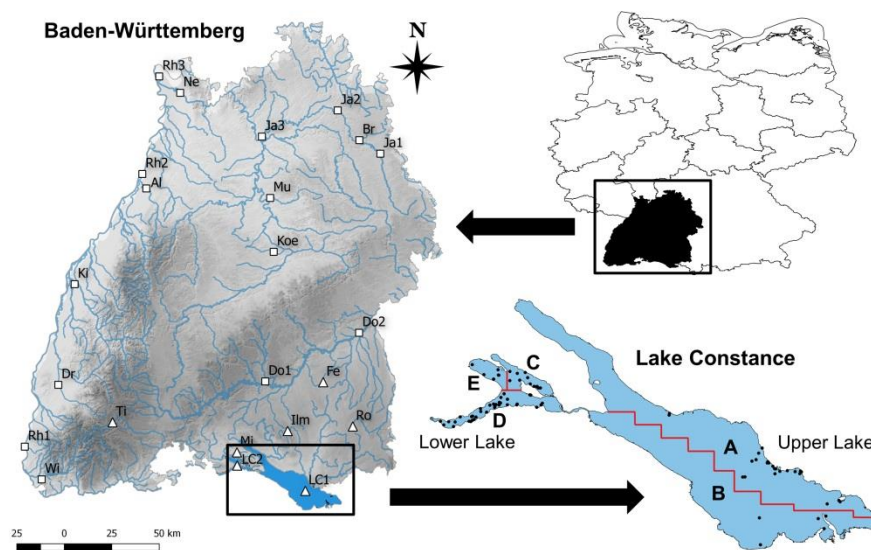


Figure 3.1. Overview of the sampling sites in Baden-Württemberg (left; river sampling sites: squares, lake sampling sites: triangles) and Lake Constance (lower right; sampling locations: circles) in Germany. Upper Lake Constance was divided into “eastern shore” (**A**) and “western shore” (**B**) and the Lower Lake into “Gnadensee” (**C**), “Rheinsee” (**D**) and “Zellersee” (**E**). For more details, see Table S1.

Fish were collected from 16 river and 7 lake sampling sites (*cf.* [supplementary material and Table S1](#)). At most sites, two fish species with either benthic or pelagic habitat preferences were studied. The exception was Lake Constance, from which 15 fish species were examined (Tab. S3.1). Each fish was weighed to the nearest 0.1 g and measured for total length to the nearest 0.1 cm. The whole gastrointestinal tract (GIT) was dissected, vestiges of other internal organs and fatty tissue were removed and the GIT was weighed to the nearest 0.1 g. The samples were stored in centrifuge tubes at –20 °C until further use. Each species was allocated to a series of biotic categories describing preferred habitat (benthic, benthopelagic, pelagic), trophic group (inverte-piscivorous, invertivorous, omnivorous, piscivorous, planktivorous) and stomach presence or absence (Kottelat & Freyhof 2007).

3.3.2. Extraction and identification of microplastics

The extraction of microplastics was performed according to (Roch & Brinker 2017); *cf.* [supplementary material](#)). All extraction steps were performed under a fume hood, and synthetic-free clothing and gloves were worn at all times. All labware was rinsed three times with filtered ultrapure water. Procedural blanks were used to exclude potential contamination.

To identify plastic particles, the filtered material was examined under a dissecting microscope. Suspicious particles and fibres were verified as plastic using a hot needle point test (Devriese *et al.* 2015; Vandermeersch *et al.* 2015). The percentages of burdened fish (prevalence), numbers of microplastic particles per fish (abundance) and numbers of microplastic particles per burdened fish (intensity) were calculated. The particles were photographed digitally (Keyence, VHX- 700F) under 20-200x magnification and the maximum length and surface area of each particle was measured to the nearest 0.001 mm using the on-board software.

Only plastic particles smaller than 5 mm were used for further characterization and statistical analysis. The digital photographs were used to categorize identified particles by shape (fibre, fragment, film, and bead) and colour (blue, clear, yellow, grey, green, red, black, white), with colours that occurred <5 % summarized as “others”. These designations are hereafter referred to as particle shape and plastic colour distribution. The minimum particle size threshold for detection was 20 µm, but particles smaller than 40 µm were not consistently detected and might thus be underrepresented (Lusher *et al.* 2017).

3.3.3. Comparison with surface water concentrations

The data derived from our fish samples were compared with the concentrations of microplastics recorded in surface water on nearby sampling sites being part of a recent survey by (Heß *et al.* 2018). The maximum distance between fish and surface water sampling sites was 15 km along the water course. The type and particle size distribution of plastics detected in each study was compared. Particle classes from fish and surface water were identified and graded by size as macroplastics (>5 mm), grade I microplastics (5-1 mm), grade II microplastics (1000-300 µm) and grade III microplastics (300-20 µm).

3.3.4. Particle size distribution analysis

To calculate the probable size distribution of particles below the detection limit, a PSDA was carried out according to (Patterson *et al.* 1999). Methodological limitations meant that fibres and particles smaller than 40 µm could not be reliably detected and so these were not included in the analysis. Equivalent Circular Area Diameters (ECAD) were calculated for all larger microplastic particles according to (Li *et al.* 2005), normalizing the data and grouping particles into 37 size classes ranging from 1 to 5100 µm. The size class range was increased by a factor of 1.26 to satisfy the ratio conditions stipulated by (Kavanaugh *et al.* 1980). Further calculations were performed to determine particle size class boundaries (l_i, l_{i+1}), size of each particle size class (Δl_i), volume equivalent diameter (l_i^*), ratio ($\Delta l_i/l_i^*$), number of particles per size class (ΔN_i) and particle frequency per size class ($\Delta N_i/\Delta l_i$). Volume equivalent diameter was plotted against particle frequency and linearized by log10-log10 transformation. A linear regression was performed and the resulting regression function was used to extrapolate particle frequency values ($\Delta N_i/\Delta l_i$) for size classes under 40 µm. Finally, the number of microplastic particles per fish was estimated by dividing the number of particles per size class by the number of fishes burdened with microplastic particles <40 µm.

3.3.5. Statistical analysis

To examine differences in the prevalence of microplastic ingestion and the distributions of colour and shape among identified plastic particles, a Fisher's exact test or Chi-Squared test was applied according to (Sokal & Rohlf 2003). When comparing the abundance, intensity, maximum length, data was first tested for homoscedasticity (Levene-Test; Levene 1960) before choosing a parametric (t-Test or ANOVA) or non-parametric (Wilcoxon test or Steel-Dwass-Method) test (Sokal & Rohlf 2003). Multiple Correspondence Analyses (MCA) were performed to reveal patterns between abiotic factors such as “water type”, or “sampling site”; biotic factors such as “preferred habitat”, “trophic state” and “stomach presence or absence” and the shape and colour of plastic particles discovered (Greenacre 2017). The first two dimensions were plotted as a map. Cumulative percentages of adjusted inertia were calculated as a measure of how well the first two dimensions were able to explain variances in the data, after (Greenacre 1984). Continuous data were presented as mean ± standard deviation (SD).

Finally, general linear models (GLM) were applied to investigate the influence of the biotic factors “fresh weight”, “preferred habitat”, “trophic state” and “stomach

presence or absence” on observed frequencies of microplastic ingestion of fishes in Baden-Württemberg. The factors “Water type” (*i.e.* river or lake) and individual fresh weight of examined fishes were also added to the model, and the GLM was applied to both datasets individually to take account of any bias between them. All statistical analyses were performed using JMP Pro 14 (Vers. 14.0, SAS Institute Inc.).

3.4. Results

3.4.1. The microplastic burden in fishes from Baden-Württemberg

In total, 1167 fishes representing 22 different species were sampled ([Table S3.1](#)), and plastic particles were found in 18.8 % of individuals. Overall plastic abundance was 0.2 ± 0.5 particles per fish and intensity ranged from 1 to 4 particles (mean: 1.2 ± 0.5). Of the ingested plastic particles, 97.7 % were smaller than 5 mm (minimum: 22 μm , mean: $899 \pm 1050 \mu\text{m}$) and were thus categorized as microplastics. Particles longer than 5 mm consisted exclusively of fibres. A small fraction of microplastics (1.9 %) was destroyed during the detection procedure and was excluded from further characterisation and analysis. The results for the individual sampling sites are summarized in [Table S3.2](#) and an overview of the plastic burden in Baden-Württemberg is given in [Figure 3.2](#).

The prevalence of plastic in fish from river sites ranged from 7.5 % (Wiese, Neckar) to 42.9 % (Jagst; [Tab. S3.2](#)). There were statistically significant differences in plastic abundance between the River Jagst (Ja2) and the rivers Dreisam, Neckar, Murr and Wiese (Steel-Dwass-Method, $P < 0.05$; [Tab. S3.2](#)). In rivers with more than one sampling site (the Rhine, Danube, Jagst) there was no apparent increase in prevalence along the course of the river (Fisher's exact test, $P > 0.05$), nor any significant difference in plastic intensity between sites (Steel-Dwass-Method, $P > 0.05$). In lake sites, plastic prevalence ranged from 12.5 % (Federsee) to 20.0 % (Ilmensee; [Tab. S3.2](#)), but again with no statistical significant difference in plastic abundance and intensity between sampling sites (abundance: ANOVA, $P > 0.05$; intensity: Steel-Dwass- Method, $P > 0.05$).

There was a statistically significant difference in microplastic prevalence between fishes from river and lake sampling sites at 20.6 % and 16.5 % respectively (Fisher's exact test, $P = 0.0442$). No significant differences were found between rivers and lakes in either the abundance or intensity of plastic per fish ([Tab. S3.2](#)).

In Lake Constance, 331 individual fish were examined from 84 sampling sites, representing 15 species. With the exception of dace (*Leuciscus leuciscus*), all sampled species contained plastic debris. In both parts of the lake, whitefish (*Coregonus wartmanni*) exhibited the highest prevalence of plastic ingestion (Fig. 3.3). Some individuals of burbot (*Lota lota*), ruffe (*Gymnocephalus cernua*), bleak (*Alburnus alburnus*) and bream (*Abramis brama*) had ingested plastic particles exhibiting similar distinctive shape and colouration (Fig. 3.3). These were classified as “paint particles”, but since they were found only in fishes from the Upper Lake, they were treated as contamination as a precaution.

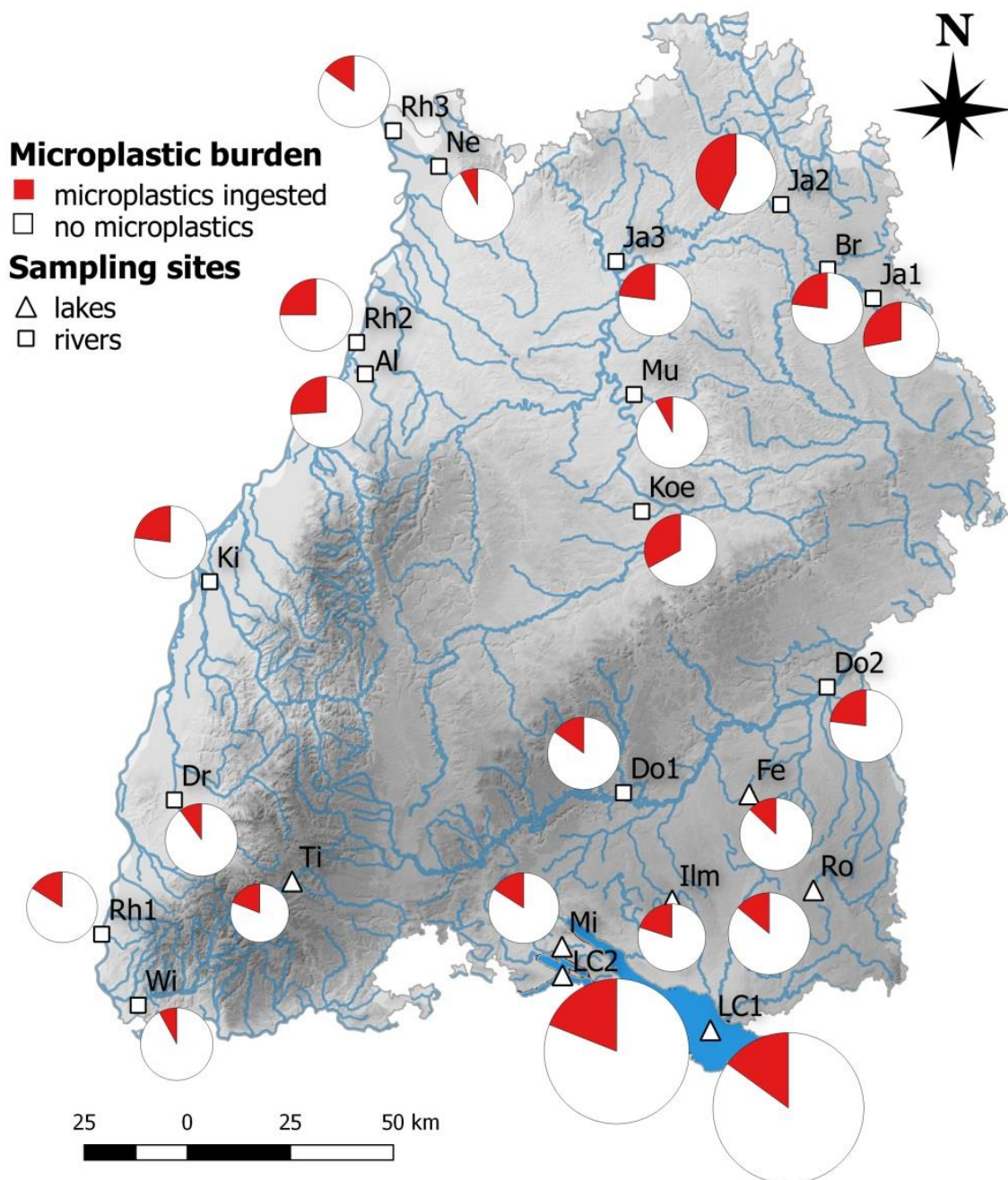


Figure 3.2. Map showing spatial prevalence of plastic occurrence in freshwater fish of Baden-Württemberg. The size of the pie chart resembles sample size. For more details, see Table S3.1.

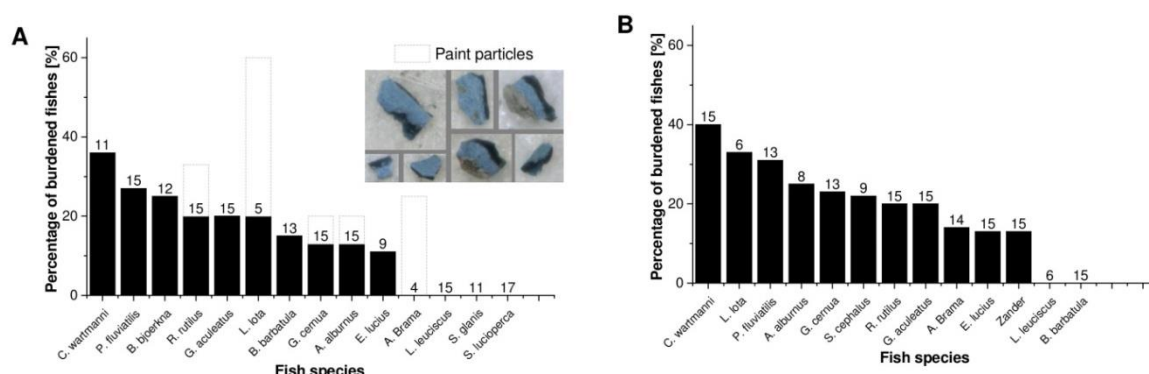


Figure 3.3. Prevalence of microplastics in different fish species from Lake Constance split into **(A)** Upper Lake and **(B)** Lower Lake. Dotted bars indicate the prevalence of so-called “paint particles” with similar shape and colour pattern.

The analysis of samples from Lake Constance revealed several locations where plastic prevalence was 50 % or higher (Fig. S3.1). However, those locations were scattered all over the lake and most comprised single individuals, with neighbouring areas showing distinctively lower prevalence (Fig. S3.1). There was no statistical difference in prevalence between the different lake sections of the Upper and Lower lake (Steel-Dwass Method, $P > 0.05$).

3.4.2. Plastic type and colour distribution of ingested microplastics

Of all the microplastics detected, the majority of particles comprised fragments, at 54 %, with fibres making up a further 39 %. Plastic films and beads were rare, accounting for 2 % and 3 %, respectively. The maximum size of identified microplastic particles was significantly larger in rivers than in lakes (Fig. 3.4; t-test, $P=0.0189$), and particle shape distribution differed significantly between these two water types (Fig. 3.4; Fisher's exact test, $P < 0.0001$). The colour distribution between particles found in lakes and rivers was statistically significant as well (Fig. 3.4; Chi-Squared test, $P < 0.0001$).

Among the microplastics detected in riverine fishes, maximum length ranged from 51 to 4986 μm (mean: $1027 \pm 1080 \mu\text{m}$), with statistically significant differences between sampling sites (Steel-Dwass-Method, $P < 0.05$; Fig. S3.2(A)). The shape and colour distributions for microplastics from each river sampling site are summarized in Figure S3.2(B) and (C). Among lake fish, the maximum length of detected particles ranged from 22 to 4715 μm (mean: $715 \pm 981 \mu\text{m}$), again with no statistically significant difference between sampling sites (Steel-Dwass-Method, $P > 0.05$; Fig. S3.3(A)). The shape and colour distributions for microplastics from each lake sampling site are summarized in Figure S3.3 (B) and (C).

The results of the MCA, illustrating potential relationships between water type, sampling site and the shape and colour of detected microplastics are shown in [Figure S3.4\(A\)](#) and [\(B\)](#) respectively. Dimensions 1 and 2 were plotted to give a cumulative percentage of adjusted inertia, *i.e.* a variance of 73.43 % for particle shape and 65.70 % for plastic colour.

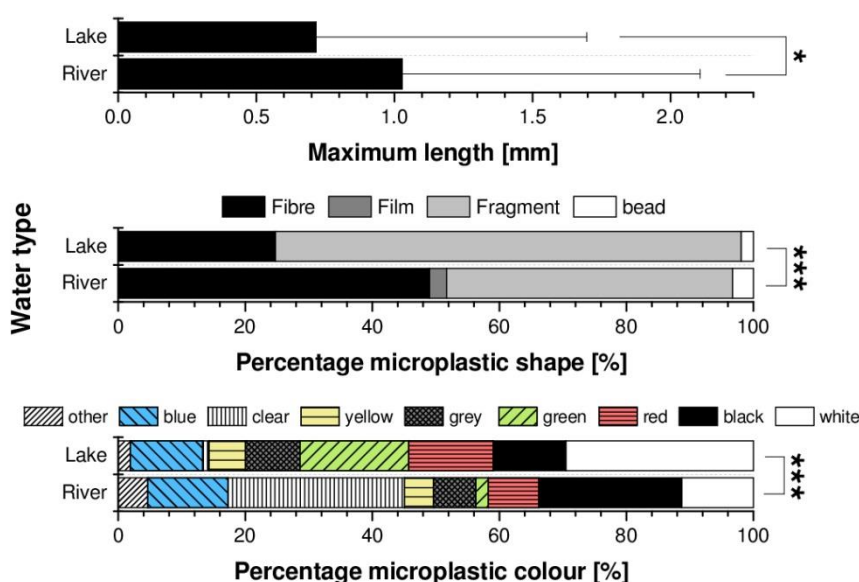


Figure 3.4. Comparison of microplastic characteristics between river and lake sampling sites. Top: mean maximum length $\pm$ standard deviation of ingested microplastics. Middle: shape distribution of ingested microplastics. Bottom: colour distribution of ingested microplastics. Asterisks indicate statistically significant differences between river and lake sites (* = $P < 0.05$, *** = $P < 0.0001$; maximum length: t-test, percentage plastic type: Fisher's exact test, percentage microplastic colour: Chi-squared test).

3.4.3. Comparison with surface water plastic pollution

Data quantifying concentrations of plastics and prevalence of plastic likely to be available for ingestion in surface waters were available for 9 sampling sites in Baden-Württemberg ([Fig. S3.5\(A\)](#)). Linear regression analysis showed no correlation between prevalence and ingestion rates (ANOVA, $P > 0.05$, $r^2_{\text{corr}} = -0.13725$; [Fig. S3.5\(B\)](#)). However the percentages of particle shape and size, summarized in [Figure S3.5\(C\)](#) and [\(D\)](#), both exhibited statistically significant differences between surface water and fish samples (percentage plastic type: Fisher's exact test, $P < 0.0001$, plastic size: Fisher's exact test, $P < 0.0001$).

3.4.4. Biotic factors influencing the microplastic burden

The GLM emerging from the combined datasets of rivers and lakes (1158 observations, d.f. = 9, $r^2(U) = 0.0177$, $P = 0.0189$) revealed statistically significant differences in plastic prevalence in fish from trophic positions

($P = 0.00356$; Fig. 3.5). Piscivorous species exhibited significantly lower levels of contamination than those with other feeding characteristics. Other biotic factors, including water type (data not shown), appeared to have no significant influence on the ingestion of microplastic (Fig. 3.5).

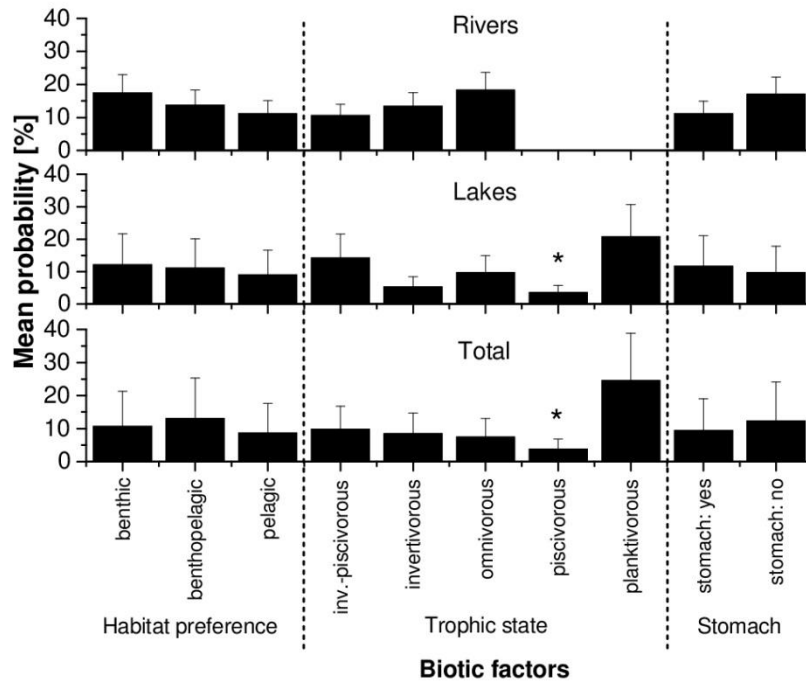


Figure 3.5. Grand marginal means of biotic factors and microplastic prevalence in fishes of Baden-Württemberg. Values indicate mean prevalences for the combined (total) dataset and individual datasets for rivers and lakes (values for fresh weight and water type are not shown, but were included as covariates in the model). Asterisks indicate statistically significant differences (*= $P < 0.05$).

No statistically significant correlation was identified between the maximum length of identified microplastics and the examined biotic factors (Fig. S3.6(A); preferred habitat: ANOVA, $P > 0.05$, diet: ANOVA, $P > 0.05$, presence or absence of stomach: t-test, $P > 0.05$). Colour distribution is summarized in Figure S3.6(B). When comparing distributions of plastic particle shape, statistically significant differences were found between stomached and stomachless fish (Fig. S3.6(C); Fisher's exact test, $P = 0.0305$), and trends were visible for fish with different preferred habitats (Fisher's exact test, $P = 0.0503$) and from different functional feeding groups (Fisher's exact test, $P = 0.0708$). However the limited sample size and weakness of significance did not allow for further statistical differentiation.

The results of the MCA, illustrating potential relationships between preferred habitat, functional feeding group, stomach presence/absence, and particle shape or plastic colour respectively are shown in Figure S3.7 (A) and (B). Dimensions 1 and 2 were plotted, resulting in a cumulative percentage of adjusted inertia, *i.e.* a variance of 64.79 % for particle shape and 69.83 % for plastic colour.

3.4.5. Particle size distribution analysis

Size classes and values for the PSDA are summarized in [Table S3.3](#). In total, for 152 particles collected from 130 fish the fitted particle size distribution showed a power law increase with declining size ([Fig. 3.6\(A\)](#)). Linearization and linear regression revealed a fairly good correlation between particle frequency and volume equivalent diameter (ANOVA, $P < 0.0001$, $r^2_{\text{corr}} = 0.91169$). The linear regression equation $\text{Log}_{10}(Y) = 3.38293 - 1.80099 * \text{Log}_{10}(x)$ revealed a power-law coefficient of $\beta = 1.8$. Potential particle numbers per size class under $40 \mu\text{m}$ were calculated using the regression function ([Fig. 3.6\(B\)](#)). Based on these calculations, a single burdened fish might be expected to carry around 23 microplastic particles between 1 and $5100 \mu\text{m}$. Of these, 17 would measure between 1 and $5 \mu\text{m}$, five between 5 and $40 \mu\text{m}$. Based on measured and extrapolated particle numbers, microplastic prevalence in fish across the state would rise to 100% .

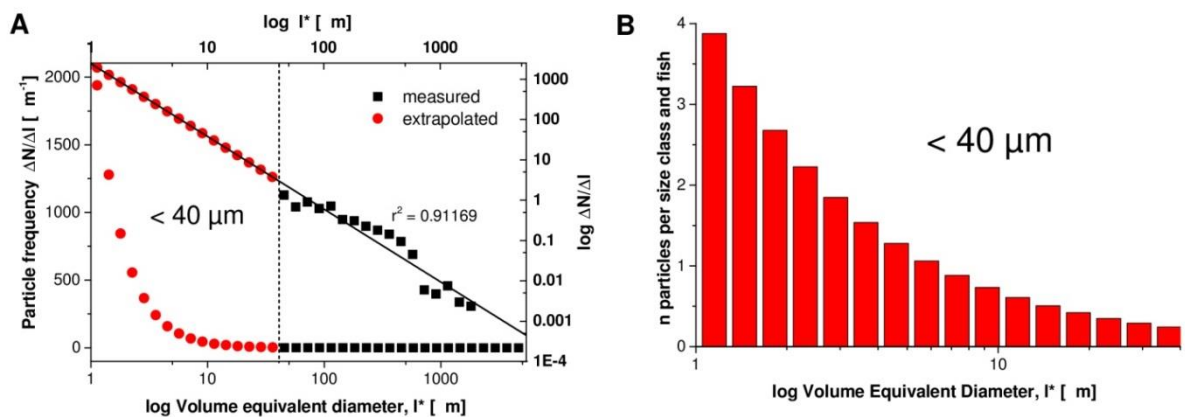


Figure 3.6. Particle size distribution analysis of microplastics found in fish. **(A)** Particle frequency per size class ($\Delta N / \Delta I$; ΔN = number of particles per size class, ΔI = size of each particle size class) versus volume equivalent particle diameter (I^*) with its log-log linear regression (top, right). **(B)** Extrapolated ($< 40 \mu\text{m}$) number of particles per size class and fish.

3.5. Discussion

3.5.1. Microplastic burden in fishes from Baden-Württemberg

Despite the importance of fish as food for humans and their pivotal role in aquatic ecosystems, the number of studies looking specifically at plastic burden in freshwater species is limited, and of these, most are restricted to case studies and/or examine only a small number of species and the majority provide only basic information about the microplastics discovered ([Tab. 3.1](#)). For this reason, findings are often contradictory and paint no clear picture of the abiotic and biotic factors influencing the prevalence and intensity of microplastics in freshwater fish. A more

holistic understanding of uptake and distribution mechanisms is urgently needed. In this context, the present study took a state-wide approach, covering both river and lake sites. This enabled the analysis of potentially relevant abiotic and biotic factors at a general level, while minimizing potential individual effects of fish species and location. Further consideration was given to prevalence and abundance of exact sizes and physical characteristics of particles.

Overall, plastic particles were detected in only one fifth of examined fishes and intensity was low, at 1 to 4 particles. Similar results have been found in other freshwater (Tab. 3.1) and marine (Jovanovic 2017) systems worldwide. In European freshwater studies, only McGoran *et al.* (2017) found significantly higher prevalence of up to 90 % in the migratory European flounder, a diadromous migrant into the river Thames. Higher prevalence of plastic contamination are found more frequently in freshwater species on a global scale, *e.g.* in USA, China and South America (McNeish *et al.* 2018; Jabeen *et al.* 2017; Silva-Cavalcanti *et al.* 2017). Microplastic intensity in the current study showed little variation between sampling sites and was considerably lower than the values reported in other studies (Tab. 3.1). However, even after several years of intensive microplastic research, there is no generally accepted or standardised method for the identification of microplastic in fishes (Hermsen *et al.* 2018) and several protocols for tissue digestion and plastic identification are used (Lusher *et al.* 2017). Thus it is still difficult to compare study results.

When looking in more detail at the current study, microplastic prevalence was higher in river fish than in fish from lakes. This is contrary to a general assumption that microplastic concentrations at the water surface are lower in rivers than lakes (Dris *et al.* 2015), because of increased sedimentation rates and rapid particle drift in the former (Tibbetts *et al.* 2018).

What is more, the present results show no increase in prevalence along the river courses and no “hot spots” were identified. The current study is to our knowledge the first attempt to comprehensively evaluate the microplastic burden in fishes across a large lake. Where previous studies have only looked at local samples (Biginagwa *et al.* 2016; Faure *et al.* 2015), we examined 15 fish species from 84 sampling sites within Lake Constance. Plastic particles were found in most species, covering all functional feeding categories and habitats. However, no clear patterns were detected in terms of the prevalence or intensity of plastic. Dace was the only species that in the current study yielded no detectable microplastics in the GIT. The exception is somewhat surprising, since ingested microplastics were found in 22 % of the closely related chub (*Squalius cephalus*).

Table 3.1. Overview of studies examining microplastic ingestion in freshwater fish. Prevalence is the percentage of fishes with at least one microplastic particle in the GIT. Percentage values for microplastics refer to the portion of particles smaller than 5 mm, compared to the total observed plastic debris.

Continent	Reference	Water type	Country	Fish species	Prevalence [%]	Percentage of microplastics	Particle intensity per fish	Mean particle size [μm]	Main plastic type
Europe	Present study	river, lake	Germany	22 fish species	18.8	97.7	$\bar{x} = 1.2$	899	fragments, fibres
	Sanchez <i>et al.</i> 2014	river	France	<i>Gobio Gobio</i>	12	n.a.	n.a.	n.a.	n.a.
	Faure <i>et al.</i> 2015	lake	Switzerland	<i>Leuciscus Leusciscus</i> , <i>Alburnus alburnus</i>	7.5	n.a.	n.a.	n.a.	n.a.
	McGoran <i>et al.</i> 2017	river	England	<i>Platichthys flesus</i> , <i>Osmerus eperlanus</i>	20 - 90	n.a.	n.a.	n.a.	fibres
	Collard <i>et al.</i> 2018	river	France	<i>Squalius cephalus</i>	15	n.a.	n.a.	2410	fibres
	Horton <i>et al.</i> 2018	river	England	<i>Rutilus rutilus</i>	33	100	0 - 6	n.a.	fibres
	Slootmaekers <i>et al.</i> 2019	river	Belgium	<i>Gobio gobio</i>	9	n.a.	n.a.	< 2000	diverse
	Phillips & Bonner 2015	river	USA	44 fish species	8	n.a.	< 12	< 500	films
	Peters & Bratton 2016	river	USA	<i>Lepomis macrochirus</i> , <i>Lepomis megalotis</i>	45	96	n.a.	n.a.	fibres
	Campbell <i>et al.</i> 2017	river	Canada	5 fish species	73.5	n.a.	1-20	n.a.	fibres, fragments
USA / Canada	McNeish <i>et al.</i> 2018	river	USA	11 fish species	85	n.a.	$\bar{x} = 13$	n.a.	fibres
	Biginagwa <i>et al.</i> 2016	lake	Africa	<i>Lates niloticus</i> , <i>Oreochromis niloticus</i>	20	n.a.	n.a.	n.a.	n.a.
	Jabeen <i>et al.</i> 2017	lake	China	6 fish species	95.7	52.2	$\bar{x} = 2.4$	n.a.	fibres
Other	Silva-Cavalcanti <i>et al.</i> 2017	river	Brasil	<i>Hoplosternum littorale</i>	83	88.6	$\bar{x} = 3.6$	n.a.	fibres
	Andrade <i>et al.</i> 2019	river	Brasil	13 fish species	26.7	29.2	n.a.	n.a.	fibres, fragments

As both these two cyprinids share the same habitat and show similar food preferences (Fischer & Eckmann 1997), it might be expected that the probability of plastic ingestion would be similar. As with the state-wide results, geographical distribution analysis in Lake Constance revealed no “hot spots” and microplastic prevalence did not differ significantly between lake areas, despite widely divergent limnologic properties, wind and current patterns and catchments (Bäuerle & Gaedke 1999).

In summary, there appears to be limited variation in the prevalence and intensity of microplastic occurrence in fish at either state-wide or local Lake Constance level. Thus it seems that abiotic factors related to the sampling location and water type may not exert a major influence on the microplastic burden in fishes from Baden-Württemberg. The lack of pattern in this extensive study suggests uptake of microplastics is passive or accidental. Some previous studies have recorded a correlation between the degree of urbanization and ingestion rates (*e.g.* Peters & Bratton 2016), suggesting a link between the concentrations of microplastics in surface waters/ sediments and prevalence in aquatic organisms, though this has rarely been investigated (see below).

3.5.2. Particle shape and colour distribution of ingested microplastics

The majority of plastic particles identified in the examined fishes came in the form of fragments and fibres, with beads and films encountered relatively rarely. However, the proportions varied distinctly between sampling sites, especially in rivers. This is in contrast with other studies, where fibres were by far the most common shape of plastic detected in fishes (Tab. 3.1).

Interestingly, however, in surface water samples from the same study areas, fragments were most prevalent and fibres played only a minor role (Heß *et al.* 2018). The present results were consistent, with fishes from different sampling sites on the same river (Danube, Jagst, and Rhine) containing very similar shapes of plastic, indicating some connection between the contaminations found in surface waters and in fish. It should be noted that the abundance of particles detected in fish was generally low, and this probably impedes the identification of patterns in particle shape and colour, with the potential for over- or underrepresentation of various characteristics.

Most studied lakes yielded similar distributions of ingested particle shapes, with fragments being most prevalent in fishes. Notable exceptions were fish from

Federsee and Mindelsee, in which over 80 % of particles were fibres. Except for Lower Lake Constance (LC2), the distribution of plastic colours showed relatively low variability within the river and lake groups. In the Lower Lake, a considerable number of green particles were found. These examples show that the data can be used in screening studies to detect outliers that deviate from commonly found characteristics distribution. While future research focussing on these areas may help to identify point sources of microplastics, current data pertaining to characteristics such as particle shape, colour or polymer type is insufficiently resolved to reliably identify sources of microplastic pollution (Duis & Coors 2016).

3.5.3. Comparison with surface water concentrations

A link between microplastic concentrations in the water and in the GIT of fish is widely implied, but rarely investigated. To test this hypothesis, the present GIT results were compared to those from the water samples of (Heß *et al.* 2018) and no correlation could be found. This is consistent with the results of another recent screening study (McNeish *et al.* 2018), in which plastic particles from surface water samples were dominated by fragments, while beads were almost absent. In contrast to the present results, large microplastics (1–5 mm) were found less often and small microplastics (20–300 µm) are even more prevalent in the surface water samples. However, it should be noted that the sampling method, using a 300 µm Manta trawl, records small size classes semi-quantitatively at best (Heß *et al.* 2018). Besides, the sampling sites in the different studies overlap imperfectly in space and time, movements of fish between samplings are unknown (Radinger & Wolter 2014), and the prevalence and abundance might be significantly influenced by biotic factors and particle egestion, as discussed below. While further studies are necessary in order to examine the relationship between concentrations in fish gut and the water at concordant sites, it is nevertheless clear that surface water samples are an insufficient proxy with which to assess fish burden. To monitor the prevalence and intensity of ingested microplastic in aquatic fauna, biotic sampling and analysis is essential.

3.5.4. Biotic factors influencing the microplastic burden

A variety of biotic factors might be used to illuminate potential uptake and transfer of pathways for microplastics in fish communities. For example, the habitats and trophic levels occupied by particular fish taxa are potentially important in defining the frequency and intensity of microplastic uptake (Lusher *et al.* 2013; Mizraji *et al.* 2017). However, statistical analysis of our data did not find any habitat effects

(benthic, benthopelagic and pelagic) and thus the influence of habitat is still poorly understood. Logic suggests that benthic fish species, which filter significant amounts of sediment while foraging, will be exposed to more microplastics (McGoran *et al.* 2017), and microplastic concentrations are thought to be higher in sediments than in the surface waters (Dris *et al.* 2015; Horton *et al.* 2017), but this theory is yet to be borne out. In fact several extensive studies in marine systems have instead found higher microplastic prevalence in pelagic fish than in demersal species (*e.g.* Rummel *et al.* 2016; Güven *et al.* 2017). In line with the present results, an earlier screening study of 44 fish species from rivers within Texas (USA) found no connection between habitat preference and microplastic prevalence (Phillips & Bonner 2015). One explanation for the ambiguous results in freshwater systems might be the spatial separation of habitats and the heterogeneous composition of fish communities. In smaller lakes and rivers especially, habitats are often closely associated and habitat choice might vary according to a number of factors, including life stage, environmental conditions and interspecific competition (Brabrand & Faafeng 1993; Heggenes *et al.* 1999). Furthermore, habitat preference, trophic position and food preference are often linked. The analysis of biotic factors and plastic characteristics in the present study reveals similar distributions of particle shape and colour between benthic invertivorous fish and omnivorous and benthopelagic types. This is understandable, as most benthic fish species feed on invertebrates and omnivorous fish species are seldom habitat specialists (Kottelat & Freyhof 2007). To properly study microplastic uptake pathways, it will be important to consider both of these biotic factors equally. The risk in examining just one factor in isolation is that patterns in linked factors might be overlooked.

The present study reveals a significantly lower microplastic burden in piscivorous fishes, than in lower trophic groups. However as with habitat preference, comparable studies have shown conflicting results: (McNeish *et al.* 2018) found highest prevalences in zoobenthivorous freshwater fish, suggesting that opportunistic feeders are more likely to ingest microplastics. In contrast, (Campbell *et al.* 2017) detected higher particle numbers in northern pike (*Esox lucius*) from a Canadian creek, and hypothesized that this might be a result of increased trophic transfer and accumulation in the GIT. The main differences between that result and the present study are in prevalence (here: 18.8 %; Campbell *et al.* 2017: 73.5 %) and mean microplastic intensity (here: 1.2 particles per fish; Campbell *et al.* 2017: around 3.5 particles per fish). A simple calculation reveals that if a piscivorous fish ingests three prey fishes, fishes from the Canadian creek might be expected to contain 5.6 particles, while only 0.7 particles might be

encountered in specimens from the present study. Egestion of particles with chime may displace microplastics from the GIT and reduce lower average burden (Grigorakis *et al.* 2017). Rates of egestion are crucial in assessing the extent of the problem. Where prevalence and intensity of microplastics in prey fish exceeds egestion rates, trophic transfer to piscivorous species may occur, but be eliminated by rapid egestion.

Finally, while the recorded distributions of microplastic particle shape and colour varied between habitats and trophic groups, they do not provide any evidence of active uptake. However previous studies have highlighted the potential for various fish species to mistake plastic debris for food. For example, Ory *et al.* (2017) demonstrated that amberstripe scad (*Decapterus muroadsi*) in the South Pacific ingested blue microplastics preferentially, presumably because of a similarity in colour to their natural copepod prey. In the present study, plastic type and colour seem to play no relevant role and the overall implication is of passive uptake, *e.g.* through the food chain or along with other food while foraging (Hoss & Settle 1990). More research is needed to evaluate potential uptake pathways, especially passive ones, as little is known about how very small, “invisible” particles might be ingested by fishes.

3.5.5. Particle size distribution analysis

Particle size is an important factor in evaluating the routes and likely effects of plastic uptake by aquatic organisms (Jovanovic 2017). In surface waters, a kind of exponential increase in microplastic abundance with smaller particle size was observed (Imhof *et al.* 2016). A nonlinear increase in particle abundance with declining size is also seen here, following a hyperbolic function – a particle frequency distribution typical of various particulates in natural freshwater and wastewater systems (Kavanaugh *et al.* 1980). A power law model can be used effectively to estimate particle number, surface area and volume contributions (Patterson *et al.* 1999).

However, in the present study, observed particle frequency decreased sharply below 40 µm and the smallest particle identified was 22 µm, indicating a progressive deterioration in detectability at the lower size range. This is a universal problem with all currently available methods for evaluating microplastic burden in freshwater fishes (Lusher *et al.* 2017). Most studies examining the microplastic burden in freshwater fishes provide no details regarding particle sizes (Tab. 3.1), and where mentioned, minimum particle size varies greatly between studies (Jabeen *et al.* 2017: 40 µm, Slootmaekers *et al.* 2019: 80 µm and Collard *et*

al. 2018: 390 μm). And yet particles smaller than 5 μm are of critical interest, as they may be able to pass the intestinal barrier and be translocated into internal tissues such as liver or muscle (Avio *et al.* 2015; Collard *et al.* 2018).

Extrapolating from the PSDA described in the current study leads to an expectation that >95 % of the total microplastic burden in fish comprises particles smaller than 40 μm . Naturally, the same extrapolation also increases the estimated prevalence of microplastic in fish to 100 %. The implication is that the majority of ingested microplastics cannot be detected with currently available methods and have thus, so far, been overlooked. This chimes with the apparent disappearance of what are known to be major sources of microplastic debris. For example, the products of tyre abrasion (Sommer *et al.* 2018) are currently not detected in the environment at all. Furthermore, the present power-law coefficient of <2 indicates that larger particles dominate surface area and volume distributions (Patterson *et al.* 1999). This might be important, given the relevance of surface area to volume ratio in determining the potential for transfer of various toxic chemicals (Lee *et al.* 2014). Since the focus of the current study was plastic particles in the range 5000–1 μm (Hartmann *et al.* 2019), the smaller nanoplastics introduced to the environment as primary particles from diverse sources including medical products and paints or formed by the degradation of microplastics (Koelmans *et al.* 2015) were not considered in our analysis. Nevertheless these must be expected to increase particle numbers even further. Several lab studies are available showing a profound detrimental effect of nano-sized particles on fish health (Chae *et al.* 2018; Mattsson *et al.* 2017), but their frequency and distribution in the water and in aquatic organisms remains unknown (Koelmans *et al.* 2015).

3.6. Conclusion

In conclusion, size-related limits on particle detection must now be recognised as the major factor influencing the recorded prevalence and intensity of microplastic burden in fishes, with significant implications for the relevance of field data. A similar concern was previously expressed by Güven *et al.* (2017) for marine fish species in the Mediterranean Sea, though in that instance without providing direct evidence. Furthermore, Covernton *et al.* (2019) could show that microplastic concentrations increased dramatically, when using tow nets with smaller mesh sizes for seawater sampling. The implication is that the most dangerous and prevalent microplastics are still a metaphorical black box. Their existence and abundance explains the lack of patterns in the present study, as pathways cannot be adequately analysed when the majority of particles are overlooked. Egestion of

microplastics will further alter prevalence and intensity and should be considered in future research. There is an urgent need to improve or innovate methods to decisively reduce the detection limit for environmental microplastics to 1 μm or lower, and to develop understanding of the detrimental effects of small size microplastics on fishes. A major challenge still is the dissolution of organic tissue in order to isolate small plastic particles (Roch & Brinker 2017). Theoretically, enzymatic digestion protocols could ensure the conservation of very small particles, but are currently too time-consuming and inefficient as they rely on a final chemical oxidation step (Lusher *et al.* 2017). Furthermore, available spectroscopic techniques need to be improved in order to effectively identify small micro- or nanoplastics (Koelmans *et al.* 2015). In the absence of adequate detection technology, a PSDA provides the most useful tool for estimating microplastic burdens in surface waters, sediments and aquatic organisms, albeit one still lacking analytical proof.

3.7. Acknowledgements

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3.8. Supplementary Material

Complementary information about the sampling sites

Lake Constance is a deep, warm-monomictic lake, located in the northern Alps with shorelines in Germany, Austria and Switzerland. There are two main basins, usually referred to as the “Upper Lake” and “Lower Lake”. The Upper Lake has a maximum depth of 254 m and a surface area of 473 km² (Petri 2006). In total, individuals analysed in the current study came from 16 river and 7 lake sampling sites, where they were caught mainly during routine surveys. Samples from Lake Constance were collected at different locations of the Upper and Lower Lake as part of the large-scale and collaborative “Projet Lac” (Alexander *et al.* 2016; Projet Lac 2016). The exact position of each sampling location in Lake Constance was noted and used to calculate a geographical distribution of microplastic ingestion, and burden and to identify possible “hot spots”. The Upper and Lower Lake was divided into a 1x1 km grid and microplastic prevalence was displayed by colour for each square. When several sampling locations occurred in one grid, a mean prevalence value calculated. Prevalence was also compared between different areas of the Upper and Lower Lake: for this purpose the Upper Lake was divided into “eastern shore” and “western shore” sections; and the Lower Lake into “Rheinsee”, “Zellersee” and “Gnadensee”.

Complimentary information about used extraction method

The extraction of microplastics was performed according to (Roch & Brinker 2017). Excised gastro-intestinal tracts (GITs) were treated in two consecutive digestions steps, using sodium hydroxide solution (NaOH 1 mol L⁻¹, Chemsolute, Th.Geyer, Renningen, Germany) at 50 °C for 15 min, and nitric acid (HNO₃ 65 %, Chemsolute, Th.Geyer, Renningen, Germany) for further 15 min, also at 50 °C (final concentration of HNO₃: 10 mol L⁻¹/ ~49 %). The temperature was then increased to 80 °C for 15 min to ensure the removal of more resistant suspended solids. The samples were then diluted with ultrapure water (0.2 µm pore size, Arium 611, Sartorius Stedim Biotech, Goettingen, Germany) and filtered on a cellulose nitrate (CN) filter (Ø 47 mm, 8 µm pore size, Sartorius Stedim Biotech, Goettingen, Germany).

When saponins derived from the first two digestion steps remained on the filters with no trace of mineral residues (see below), the CN filters were dissolved with 20 mL of 1 mol L⁻¹ NaOH at 50 °C, diluted with 100 mL of 80 °C ultrapure

water and filtered on quartz filters (Ø 25 mm, grade T293, Sartorius Stedim Biotech, Goettingen, Germany).

To reduce the quantity of mineral residue on the filters, a density separation was performed in a separation funnel after the first two digestions steps. Each CN filter was dissolved in 25 mL of 80 °C NaOH (1 mol L⁻¹) and 20 g of sodium iodide (NaI pure, Bernd Kraft, Duisburg, Germany) was added to reach a density of ~1.6 g cm⁻³. After 15 min of resting, 15 mL of the solution was drained and discarded. The supernatant was diluted 1:2 (v:v) with 80 °C ultrapure water, and samples were filtered on 25 mm quartz filters.

To evaluate the amount and type of contamination that might occur during the extraction procedure, procedural blanks were conducted according to the digestion and density separation methodologies described above, but without including any GIT samples (approximately 5 blanks for every 50 samples). The resulting quartz filters were examined for contamination under a digital microscope and, if present, identified plastic particles were used to exclude the contaminations from field samples.

Table S3.1. Summary of all sampling events. n = number of individuals sampled. Coordinates are listed as Gauß-Krueger projections (EPSG:31467).

Sampling site	Symbol	Sampling date	Fish species	Habitat preference	n	Coordinates (easting / northing)
Rivers						
Alb	Al	07.09.2017	<i>Leuciscus leuciscus</i> , <i>Barbus barbus</i> , <i>Squalius cephalus</i>	benthopelagic benthic benthopelagic	39	3453934.1 / 5429613.4
Brettach	Br	05.09.2017	<i>Squalius cephalus</i> , <i>Barbatula barbatula</i>	benthopelagic benthic	39	3566161.9 / 5455034.8
Dreisam	Dr	09.10.2017	<i>Barbus barbus</i> , <i>Squalius cephalus</i>	benthic benthopelagic	40	3407607.3 / 5326223.5
Jagst (Wei)	Ja1	13.10.2017	<i>Squalius cephalus</i> , <i>Gobio gobio</i>	benthopelagic benthic	40	3577209.4 / 5447923.6
Jagst (Ail)	Ja2	27.09.2017	<i>Squalius cephalus</i> , <i>Gobio gobio</i>	benthopelagic benthic	40	3554761.1 / 5470686.2
Jagst (Dut)	Ja3	28.09.2017	<i>Squalius cephalus</i> , <i>Gobio gobio</i>	benthopelagic benthic	43	3514829.7 / 5456905.5
Kinzig	Ki	04.10.2017	<i>Squalius cephalus</i> , <i>Gobio gobio</i>	benthopelagic benthic	49	3416178.0 / 5379194.4
Koersch	Koe	08.06.2017	<i>Gobio gobio</i> , <i>Phoxinus phoxinus</i>	benthic pelagic	40	3521032.6 / 5396249.5
Murr	Mu	12.09.2017	<i>Squalius cephalus</i> , <i>Barbus barbus</i>	benthopelagic benthic	40	3519172.5 / 5424611.4
Restrhein	Rh1	30.05.2017	<i>Squalius cephalus</i> , <i>Barbus barbus</i>	benthopelagic benthic	40	3389958.1 / 5293658.3

Rhein (Karl)	Rh2	31.07.2017	<i>Alburnus alburnus</i> , <i>Neogobius melanostomus</i>	pelagic benthic	39	3451847.6 / 5437229.6
Altrhein	Rh3	20.09.2017	<i>Cobitis taenia</i> , <i>Neogobius melanostomus</i>	benthic benthic	40	3460743.7 / 5488595.8
Wiese	Wi	12.10.2017	<i>Barbatula barbatula</i> , <i>Phoxinus phoxinus</i>	benthic pelagic	38	3398817.7 / 5276425.8
Danube (Sig)	Do1	03.05.2018	<i>Phoxinus phoxinus</i> , <i>Gobio gobio</i>	pelagic benthic	40	3516608.4 / 5327997.0
Danube (Erb)	Do2	03.05.2018	<i>Squalius cephalus</i> , <i>Phoxinus phoxinus</i>	benthopelagic pelagic	39	3566038.2 / 5353617.3
Neckar	Ne	09.05.2018	<i>Alburnus alburnus</i> , <i>Neogobius melanostomus</i>	pelagic benthic	40	3471778.5 / 5479963.5
Lakes						
Federsee	Fe	24.07.2018	<i>Rutilus rutilus</i> , <i>Scardinius erythrophthalmus</i>	benthopelagic pelagic	40	3547121.6 / 5327600.3
Ilmensee	Ilm	19.7.2017	<i>Scardinius erythrophthalmus</i> , <i>Alburnus alburnus</i>	pelagic pelagic	35	3528432.9 / 5301908.9
Mindelsee	Mi	14.07.2018	<i>Scardinius erythrophthalmus</i> , <i>Coregonus wartmanni</i>	pelagic pelagic	38	3501697.6 / 5290653.2
Rohrsee	Ro	5.07.2017	<i>Scardinius erythrophthalmus</i> , <i>Tinca tinca</i>	pelagic benthic	51	3562713.5 / 5304318.4
Titisee	Ti	10.07.2017	<i>Rutilus rutilus</i> , <i>Coregonus wartmanni</i>	benthopelagic pelagic	26	3436184.9 / 5306478.9
Lake Constance - Upper Lake	LC1	September 2014	<i>Coregonus wartmanni</i> , <i>Perca fluviatilis</i> , <i>Blicca bjoerkna</i> , <i>Rutilus rutilus</i> , <i>Gasterosteus aculeatus</i> , <i>Lota lota</i> , <i>Barbatula barbatula</i> , <i>Gymnocephalus cernua</i> , <i>Alburnus alburnus</i> , <i>Esox Lucius</i> , <i>Abramis Brama</i> , <i>Leuciscus leuciscus</i> , <i>Silurus glanis</i> , <i>Sander lucioperca</i>	pelagic benthopelagic benthic benthopelagic benthopelagic pelagic benthic benthic pelagic benthopelagic benthic benthopelagic benthic benthopelagic	172	3537713.4 / 5270367.8
Lake Constance - Lower Lake	LC2	September 2014	<i>Coregonus wartmanni</i> , <i>Lota lota</i> , <i>Perca fluviatilis</i> , <i>Alburnus alburnus</i> , <i>Gymnocephalus cernua</i> , <i>Squalius cephalus</i> , <i>Rutilus rutilus</i> , <i>Gasterosteus aculeatus</i> , <i>Abramis Brama</i> , <i>Esox Lucius</i> , <i>Sander lucioperca</i> , <i>Leuciscus leuciscus</i> , <i>Barbatula barbatula</i>	pelagic pelagic benthopelagic benthic benthic benthopelagic benthopelagic benthopelagic benthic benthopelagic benthopelagic benthopelagic benthic	159	3501805.2 / 5283704.6

Table S3.2. Summary of the microplastic burden in fishes from Baden-Württemberg. Superscript letters indicate statistical significant differences between sampling sites for each water type (abundance and intensity: Steel-Dwass-method).

Water type	Sampling site	Percentage burdened fishes [%]	Mean plastic abundance ± S.D.	Mean plastic intensity ± S.D.
Rivers	Al	25.6	0.3 ± 0.7 ^{ab}	1.3 ± 0.7 ^a
	Br	32.1	0.2 ± 0.4 ^{ab}	1.0 ± 0.0 ^a
	Do2	22.5	0.3 ± 0.6 ^{ab}	1.2 ± 0.4 ^a
	Do1	15.0	0.2 ± 0.4 ^{ab}	1.2 ± 0.4 ^a
	Dr	10.0	0.1 ± 0.3 ^a	1.0 ± 0.0 ^a
	Ja1	27.9	0.3 ± 0.6 ^{ab}	1.2 ± 0.4 ^a
	Ja2	42.9	0.5 ± 0.8 ^b	1.2 ± 0.7 ^a
	Ja3	22.5	0.4 ± 0.8 ^{ab}	1.7 ± 1.0 ^a
	Ki	22.5	0.2 ± 0.4 ^{ab}	1.0 ± 0.0 ^a
	Koe	32.5	0.4 ± 0.6 ^{ab}	1.2 ± 0.4 ^a
	Mu	7.7	0.1 ± 0.3 ^a	1.0 ± 0.0 ^a
	Ne	7.5	0.1 ± 0.3 ^a	1.0 ± 0.0 ^a
	Rh1	15.8	0.2 ± 0.4 ^{ab}	1.0 ± 0.0 ^a
	Rh2	25.0	0.3 ± 0.6 ^{ab}	1.2 ± 0.4 ^a
	Rh3	15.4	0.3 ± 0.7 ^{ab}	1.7 ± 0.8 ^a
	Wi	7.5	0.1 ± 0.3 ^a	1.0 ± 0.0 ^a
	mean	20.6	0.2 ± 0.5	1.2 ± 0.5
Lakes	Fe	12.5	0.1 ± 0.3 ^a	1.0 ± 0.0 ^a
	Ilm	20.0	0.2 ± 0.5 ^a	1.1 ± 0.4 ^a
	Mi	15.8	0.2 ± 0.4 ^a	1.0 ± 0.0 ^a
	Ro	13.7	0.2 ± 0.6 ^a	1.4 ± 0.8 ^a
	Ti	19.2	0.2 ± 0.4 ^a	1.0 ± 0.0 ^a
	LC1	14.5	0.2 ± 0.5 ^a	1.2 ± 0.6 ^a
	LC2	19.5	0.3 ± 0.6 ^a	1.3 ± 0.7 ^a
	mean	16.5	0.2 ± 0.5	1.2 ± 0.6

Table S3.3. Size classes for particle size distribution analysis. Particle size class boundaries (l_i , l_{i+1}), size (Δl_i), volume equivalent diameter (l_i^*), ratio ($\Delta l_i/l_i^*$) and number of particles per size class ($\Delta n_{\text{measured}} / \text{calculated}$).

Size class	l_i	l_{i+1}	Δl_i	l_i^*	$\Delta l_i/l_i^*$	$\Delta n_{\text{measured}}$	$\Delta n_{\text{calculated}}$
1	1,0	1,3	0,3	1,1	0,23		504
2	1,3	1,6	0,3	1,4	0,23		419
3	1,6	2,0	0,4	1,8	0,23		348
4	2,0	2,5	0,5	2,3	0,23		289
5	2,5	3,2	0,7	2,8	0,23		240
6	3,2	4,0	0,8	3,6	0,23		200
7	4,0	5,0	1,0	4,5	0,23		166
8	5,0	6,3	1,3	5,7	0,23		138
9	6,3	8,0	1,7	7,2	0,23		115
10	8,0	10,1	2,1	9,0	0,23		95
11	10,1	12,7	2,6	11,4	0,23		79
12	12,7	16,0	3,3	14,4	0,23		66
13	16,0	20,2	4,2	18,1	0,23		55
14	20,2	25,4	5,2	22,8	0,23		45
15	25,4	32,0	6,6	28,7	0,23		38
16	32,0	40,3	8,3	36,2	0,23		31
17	40,3	50,8	10,5	45,6	0,23	14	
18	50,8	64,0	13,2	57,4	0,23	9	
19	64,0	80,7	16,6	72,4	0,23	15	
20	80,7	101,7	21,0	91,2	0,23	13	
21	101,7	128,1	26,4	114,9	0,23	19	
22	128,1	161,4	33,3	144,7	0,23	11	
23	161,4	203,4	42,0	182,4	0,23	13	
24	203,4	256,2	52,9	229,8	0,23	12	
25	256,2	322,9	66,6	289,6	0,23	12	
26	322,9	406,8	83,9	364,8	0,23	12	
27	406,8	512,6	105,8	459,7	0,23	10	
28	512,6	645,8	133,3	579,2	0,23	6	
29	645,8	813,8	167,9	729,8	0,23	1	
30	813,8	1025,3	211,6	919,6	0,23	1	
31	1025,3	1291,9	266,6	1158,6	0,23	2	
32	1291,9	1627,8	335,9	1459,9	0,23	1	
33	1627,8	2051,1	423,2	1839,5	0,23	1	
34	2051,1	2584,4	533,3	2317,7	0,23	0	
35	2584,4	3256,3	671,9	2920,3	0,23	0	
36	3256,3	4102,9	846,6	3679,6	0,23	0	
37	4102,9	5169,7	1066,8	4636,3	0,23	0	

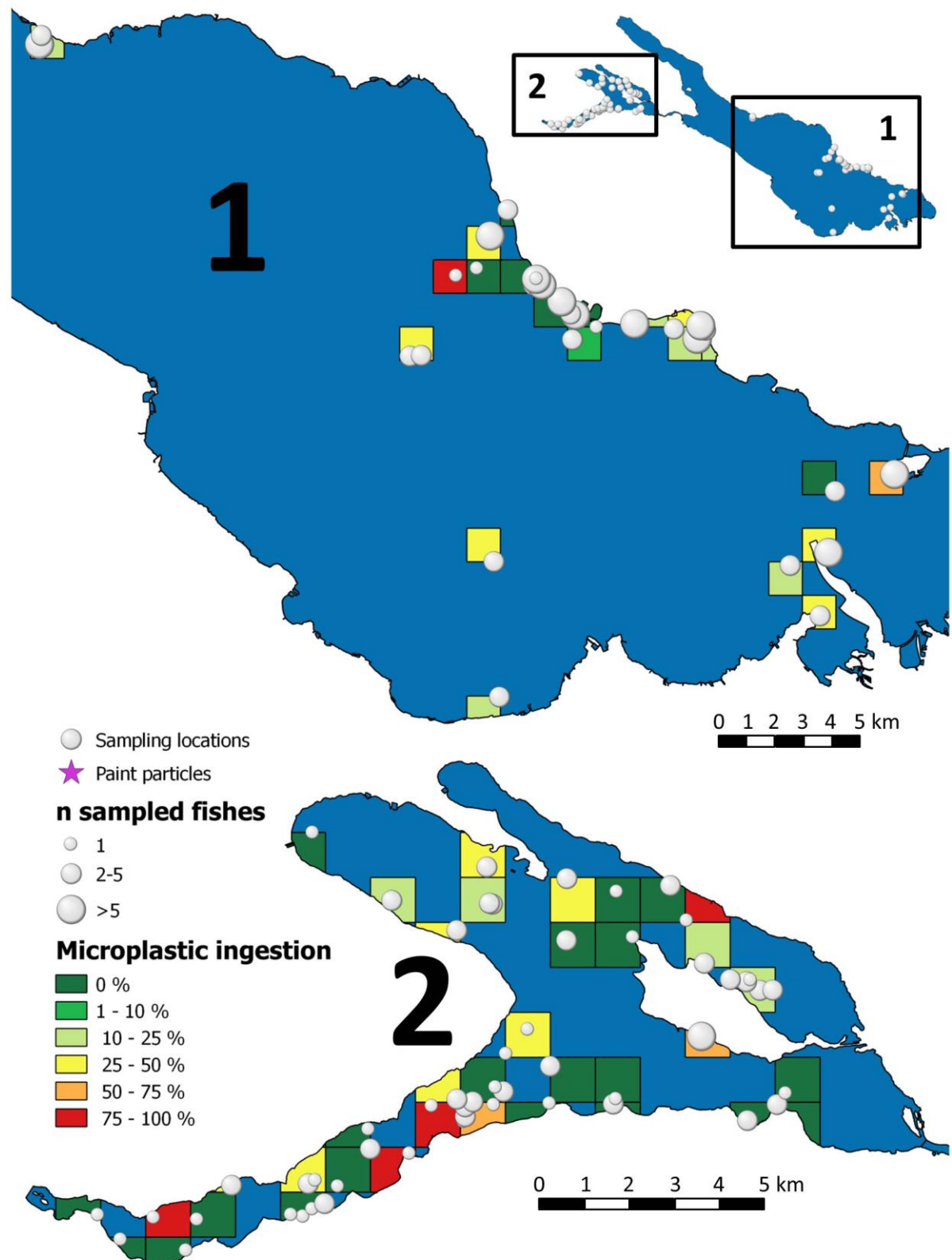


Figure S3.1. Geographical distribution of fish burdened with microplastics. Grid size is 1 km. Where several sampling site occurred in one grid, mean prevalence was calculated.

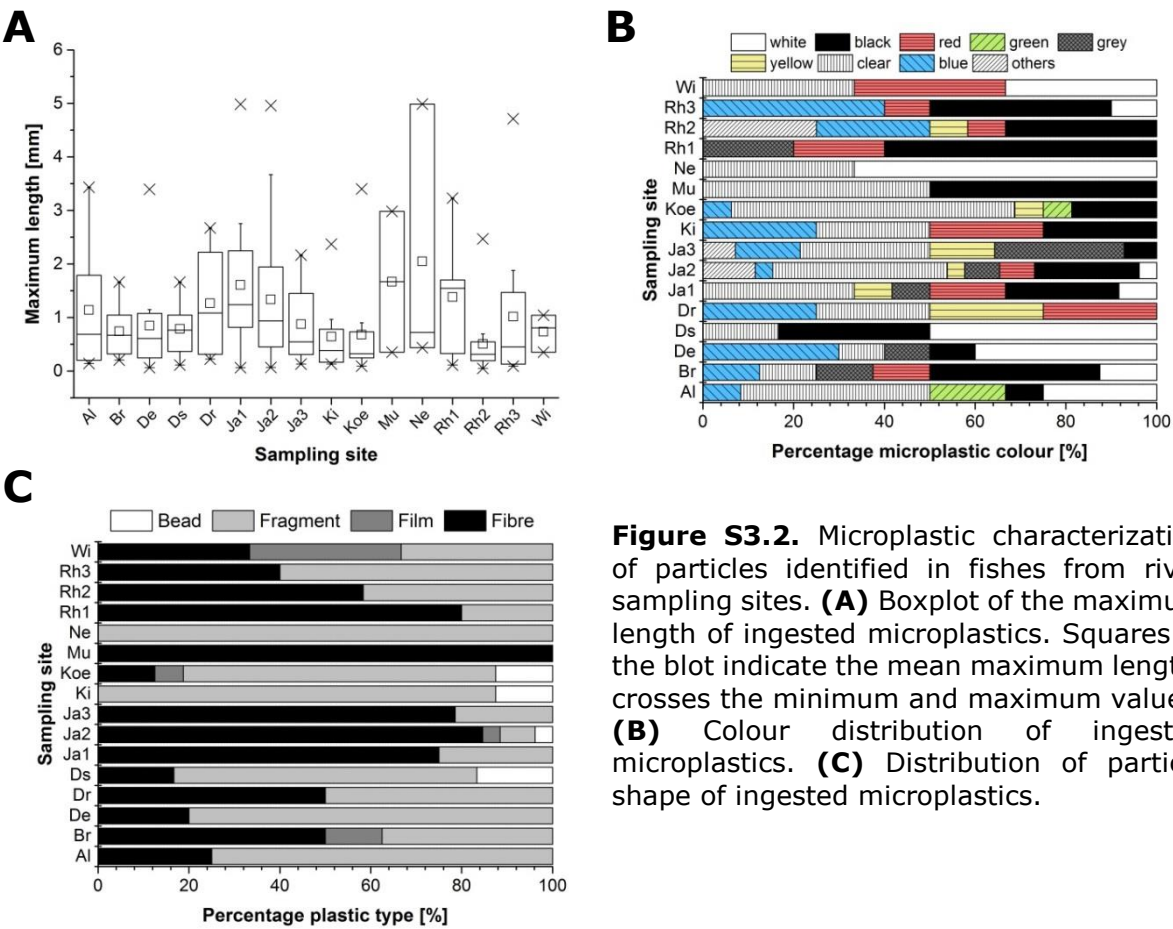


Figure S3.2. Microplastic characterization of particles identified in fishes from river sampling sites. **(A)** Boxplot of the maximum length of ingested microplastics. Squares in the blot indicate the mean maximum length, crosses the minimum and maximum values. **(B)** Colour distribution of ingested microplastics. **(C)** Distribution of particle shape of ingested microplastics.

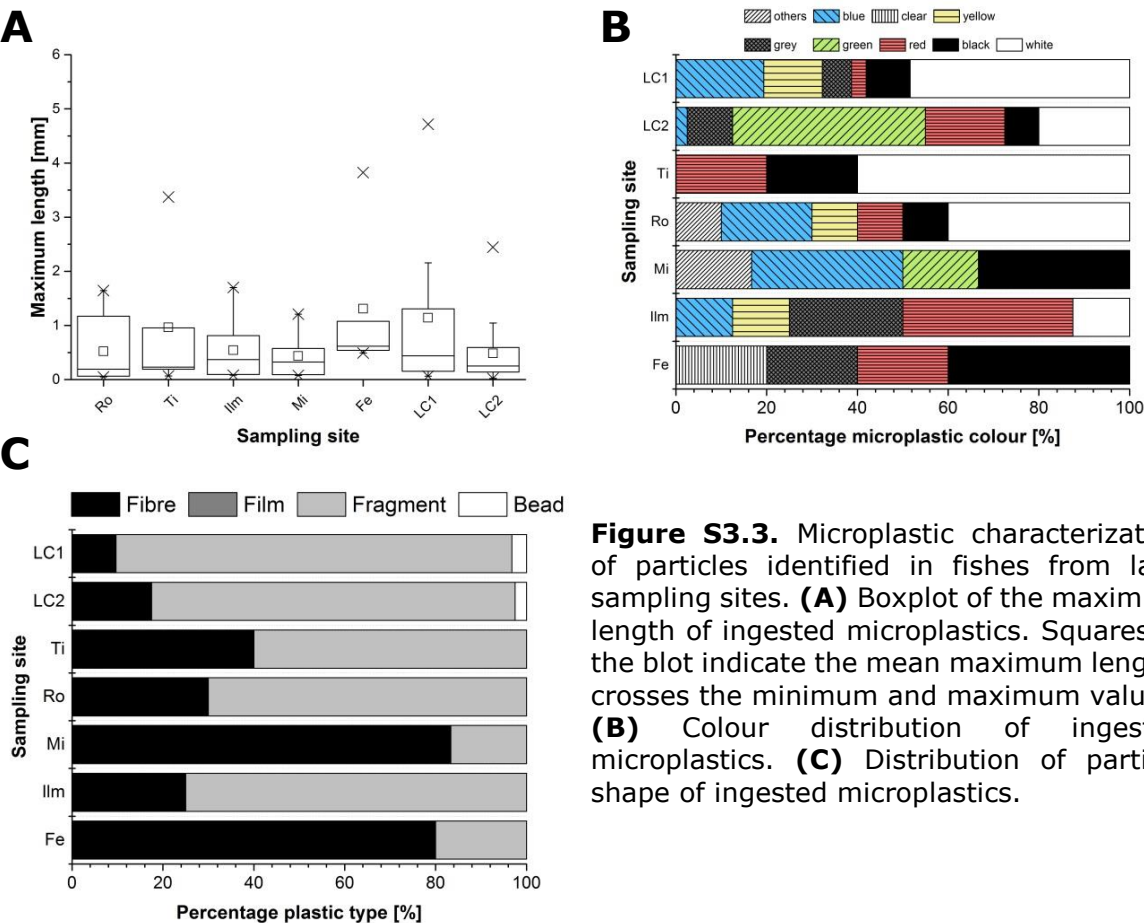


Figure S3.3. Microplastic characterization of particles identified in fishes from lake sampling sites. **(A)** Boxplot of the maximum length of ingested microplastics. Squares in the blot indicate the mean maximum length, crosses the minimum and maximum values. **(B)** Colour distribution of ingested microplastics. **(C)** Distribution of particle shape of ingested microplastics.

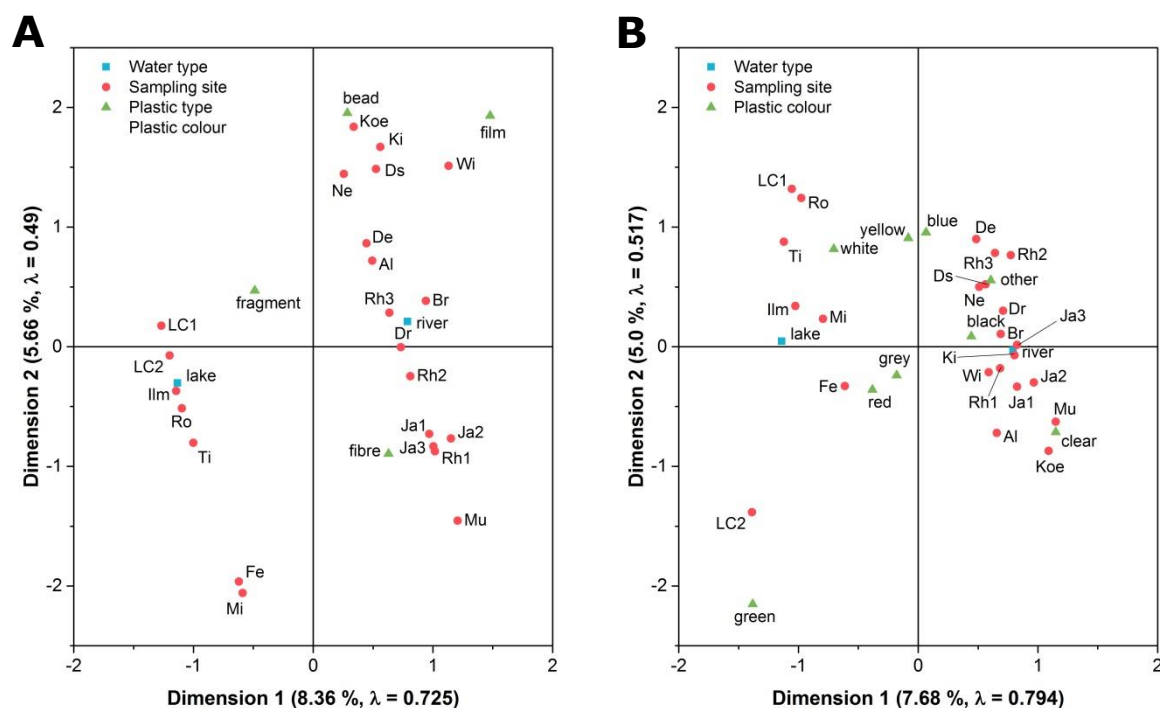


Figure S3.4. Multiple Correspondence analyses (MCA) of all identified microplastic particles, based on **(A)** particle shape and **(B)** plastic colour. Other variables that were incorporated in the MCA are: Water type and sampling site.

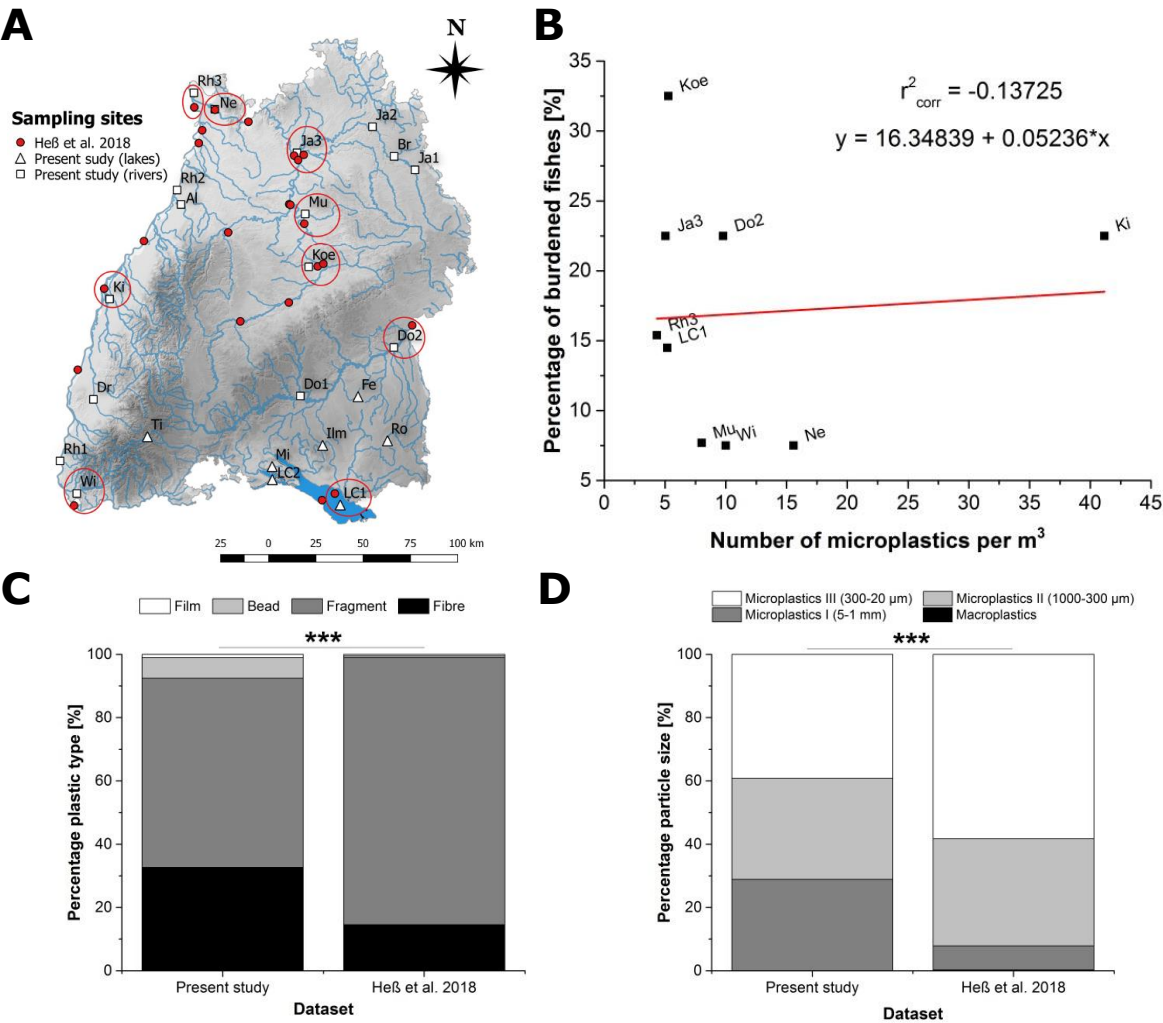


Figure S3.5. Comparison of number of burdened fish with surface water concentrations reported by Heß et al. (2018). **(A)** Overview of sampling sites that were compared. **(B)** Linear regression of burdened fishes and number of microplastics per m³. **(C)** Comparison of plastic particle form distribution between studies. **(D)** Comparison of particle size distribution between studies.

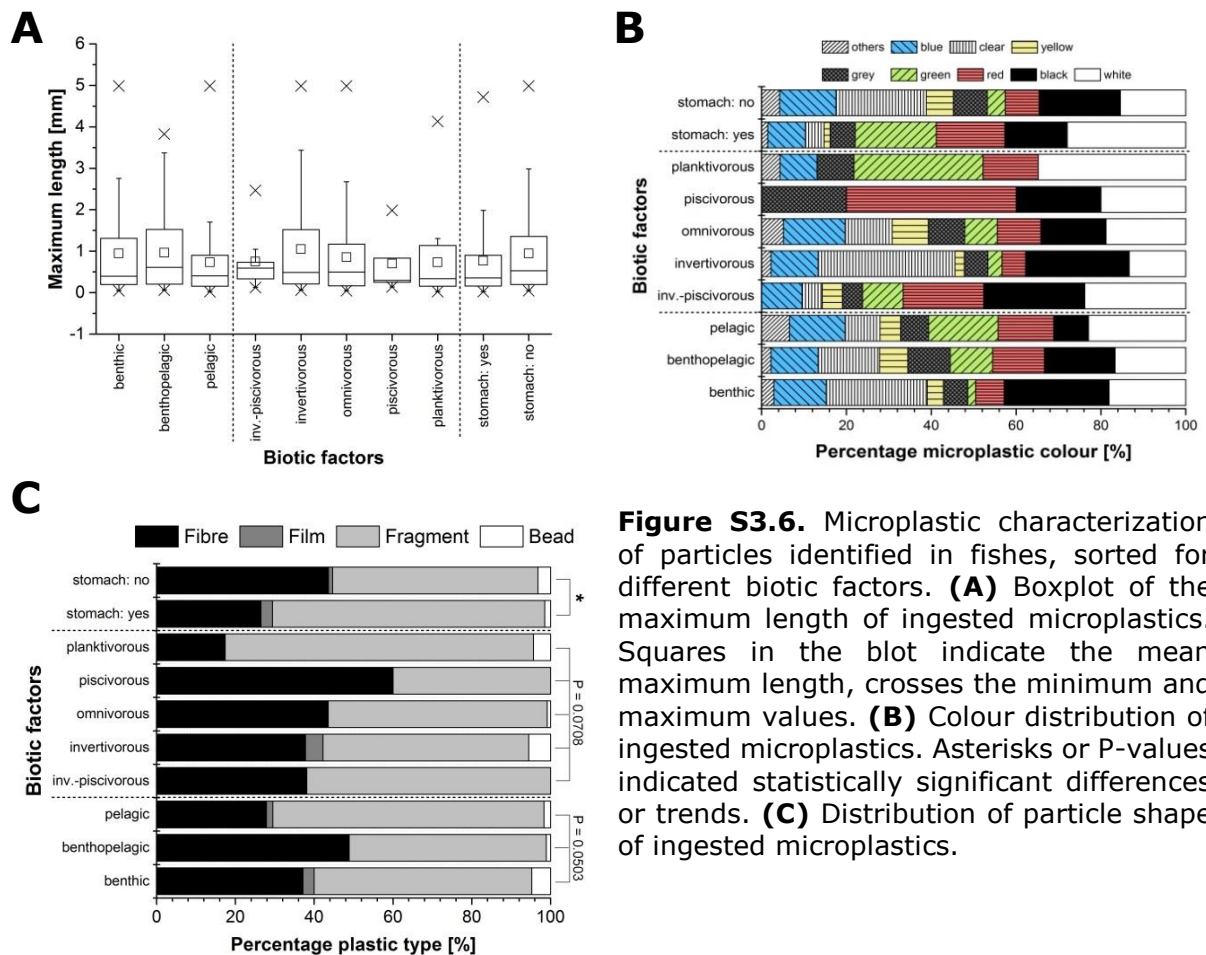


Figure S3.6. Microplastic characterization of particles identified in fishes, sorted for different biotic factors. **(A)** Boxplot of the maximum length of ingested microplastics. Squares in the blot indicate the mean maximum length, crosses the minimum and maximum values. **(B)** Colour distribution of ingested microplastics. Asterisks or P-values indicated statistically significant differences or trends. **(C)** Distribution of particle shape of ingested microplastics.

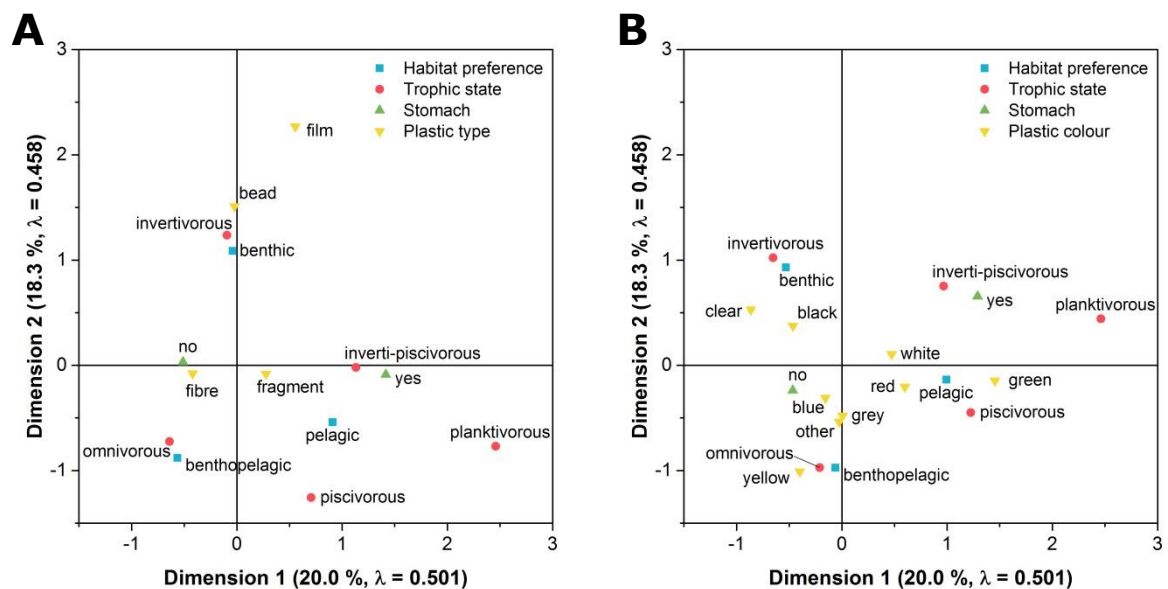


Figure S3.7: Multiple Correspondence analyses (MCA) of microplastic particles, based on **(A)** particle shape and **(B)** plastic colour. Other variables that were incorporated in the MCA are: habitat preference, trophic state and stomach presence or absence.

4. Manuscript III: Uptake routes of microplastics in fishes: practical and theoretical approaches to test existing theories

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OPEN Uptake routes of microplastics in fishes: practical and theoretical approaches to test existing theories

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4.1. Abstract

Microplastics are frequently detected in the gastrointestinal tracts of aquatic organisms worldwide. A number of active and passive pathways have been suggested for fish, including the confusion of microplastic particles with prey, accidental uptake while foraging and transfer through the food chain, but a holistic understanding of influencing factors is still lacking. The aim of the study was to investigate frequently suggested theories and identify relevant biotic factors, as well as certain plastic properties, affecting microplastic intake in fish. Four species of freshwater fish, each representing a different combination of foraging style (visual/chemosensory) and domestic status (wild/farmed) were exposed to different realistic plastic concentrations and polymer types with and without the provision of genuine food. As most previous investigations of microplastic uptake routes consider only particles large enough to be perceptible to fish, the potential for accidental intake via drinking water has been somewhat neglected. This route is evaluated in the current study using a model approach. The results show that

visually oriented fish forage actively on microplastic particles that optically resemble their usual food, while fish with a predominantly chemosensory foraging style are more able to discriminate inedible food items. Even so, the accidental uptake of microplastics while foraging is shown to be relevant pathway, occurring frequently in both visual and chemosensory foragers alike. Several factors were shown to increase plastic uptake, including microplastic concentration in the water, foraging behavior promoted by availability of genuine food, and fish size. Although both wild and farmed fish ingested microplastic particles, cultured fish showed less discernment in terms of colour and were more likely to forage actively on microplastics when no food was available. Drinking has been identified as a possible source of microplastic intake specifically for large marine fish species. Particles smaller than $<5\ \mu\text{m}$ can pass the gastrointestinal tract wall and bioaccumulation could arise when uptake exceeds release or when particles are assimilated in tissues or organs. The effects of accumulation may be significant, especially in long-living species, with implications for food web transfer and fish as food items.

4.2. Introduction

One of the first articles published on the subject of microplastics in the aquatic environment described the presence of plastic spherules in fish (Carpenter *et al.* 1972). Since then, the ingestion of microplastics by aquatic organisms has been recognized as one of the major detrimental effects of plastic pollution worldwide (Wright *et al.* 2013). Microplastics are mostly defined as synthetic polymer particles and fibres smaller than 5 mm (Law & Thompson 2014) and numerous studies suggest that they affect virtually all marine and freshwater fauna (Gall & Thompson 2015; Windsor *et al.* 2019). The origins of microplastics can be diverse, but most have been shown to derive from terrestrial sources (Jambeck *et al.* 2015).

Despite their ubiquity in aquatic organisms, knowledge of the uptake routes of microplastics remains fragmentary (Franzellitti *et al.* 2019). For fish, several pathways have been suggested (Fig. 4.1). Firstly plastic particles may be deliberately ingested having been mistaken for food. There is evidence that some fish species actively forage on microplastics that visually resemble their prey in some way (Mizraji *et al.* 2017; Ory *et al.* 2017). Second, microplastics might be ingested passively or accidentally while foraging, and thirdly they may be transferred via the food chain (Hoss & Settle 1990; Jovanovic 2017). Several examples of the latter have been described, showing the potential transfer of microplastics from prey to predator (Setälä *et al.* 2014; Santana *et al.* 2017; Welden

et al. 2018). However, a holistic understanding of all active and passive uptake routes is still lacking, controlled laboratory experiments are scarce and available studies do not generally consider factors such as habitat and related feeding preferences, the effects of particle concentration in the water or the genetic origin of fish.

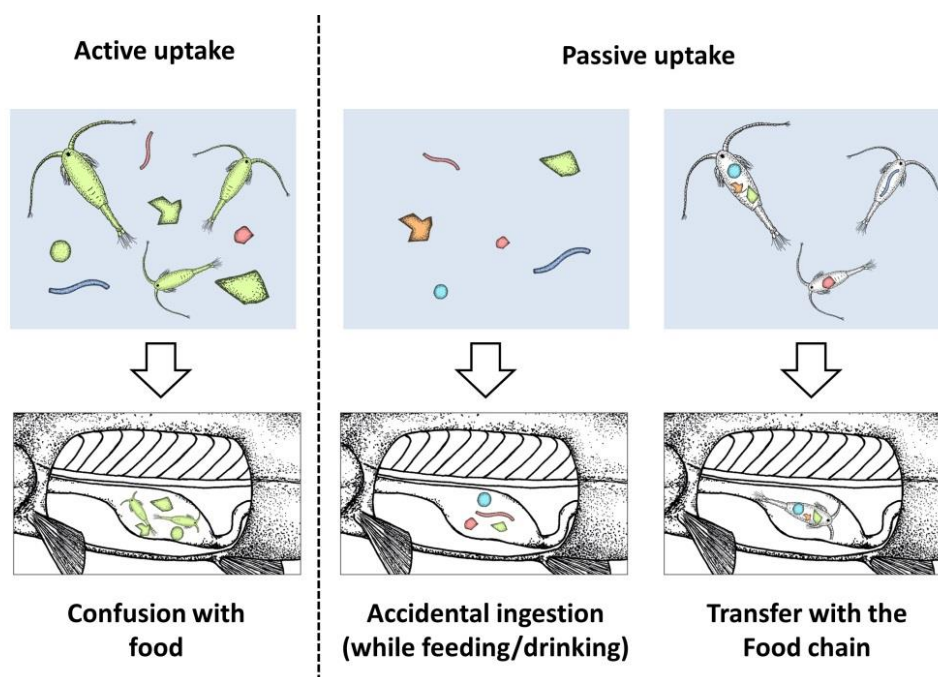


Figure 4.1. Possible uptakes routes of microplastics in fish.

Most fish species have well developed eyes (Atema *et al.* 1988) and most planktivorous and piscivorous species rely on visual cues while foraging (Lazzaro 1987; Juanes *et al.* 2002). On the other hand, most mainly benthic fish species, such as cyprinids, also possess a well-developed sense of taste, which allows them to distinguish edible and inedible food items they cannot easily see (Kotrschal & Palzenberger 1992). This chemosensory ability is especially important when searching for food on or directly in the sediment of a river-, lake- or seabed (Mauchline & Gordon 1984). Previous analyses of field data have not yet resolved possible influences of fish habitat preference or of related feeding preferences on microplastic uptake (Güven *et al.* 2017; Phillips & Bonner 2015; Roch *et al.* 2019). Nevertheless, it is assumed that chemosensory foragers should be better equipped to discriminate non-edible particles than visual foragers (Valentinčič 2004). In this context, despite the globally practiced management technique of stocking and the problem of escapees (Brown & Day 2002; Jensen *et al.* 2010), the potential effect of domestication on food selection behaviour in fish is rarely considered (Thodesen *et al.* 1999; Rikardsen & Sandring 2006; Skilbrei 2012). In freshwater environments especially, billions of fish are stocked every year to conserve or restore natural

species assemblages or to increase the abundance of favoured species (Brown & Day 2002). It is suggested that the age and experience of released fish and their origins from domesticated or wild lines, may alter or reduce their ability to discriminate plastic debris as non-food. Another previously neglected uptake route could be the ingestion of very small particles along with water. While drinking rate in freshwater fish species is comparatively low, marine fish drink almost continuously in order to maintain homeostasis (Fuentes & Eddy 1997). Thus in marine environments especially, very small particles might be passively ingested on a regular basis.

The aim of this work was to generally test the above-mentioned uptake routes (Fig. 4.1) in a laboratory setup and to examine the importance of relevant biotic factors and certain plastic properties on microplastic intake. Four fish species were selected, each representing a different combination of foraging style (visual/chemosensory) and genetic status (wild/domestic). Fish were exposed to three environmentally relevant plastic concentrations and commonly used polymer types with and without the provision of genuine food. In addition to previously investigated uptake routes, the hitherto unconsidered potential for small particles to be imbibed with drinking water was theoretically evaluated. To quantify the passive uptake of microplastics, a model was developed which took into account both uptake and egestion. Besides uptake rates and accumulation effects, the potential for long-term accumulation in tissues and organs was also considered. The results of the practical and theoretical approaches are discussed and their relevance for the field is evaluated.

4.3. Methods

4.3.1. Husbandry of experimental fish

Four representative fish species were selected to examine the effect of domestication (wild vs cultured) and foraging style (visual vs chemosensory) on microplastic uptake. The species were: rainbow trout (*Oncorhynchus mykiss*), grayling (*Thymallus thymallus*), common carp (*Cyprinus carpio*), and crucian carp (*Carassius carassius*). The rainbow trout and common carp originated from cultured lineages bred specifically for aquaculture, while the grayling and crucian carp were raised from wild offspring. All experimental fish were acclimatized for two weeks before the exposure experiments began. Fish were held in tanks (50 fish per tank, tank size: 0.5 × 0.55 × 0.55 m) and fed twice a day, six times a week with

commercial pellet food (rainbow trout, grayling: Inicio 702 (2 mm), Biomar, Denmark; common carp, crucian carp: Vital (2 mm), Alltech Coppens, The Netherlands). An overview of the relevant husbandry parameters is given in Table 4.1. The experimental tanks were cleaned daily and water parameters were constantly monitored by probes and an electronic surveillance system. All experiments were performed in triplicates.

Table 4.1. Overview of relevant husbandry parameters of experimental fish.

Fish species	System, exchange rate	Temperature [°C]	Oxygen [mg/L]	Light intensity ^a [Lux]	Feeding quantity [%] ^b
rainbow trout, grayling	flow-through, 8 L/min	8 ± 0.3	8-10	300	1.5 ^c
common carp, crucian carp	semi recirculating aquaculture system, 3 L/min	20 ± 0.5	8-10	300	2.0 ^d

^a light regime: 12 h illumination with 30 mins dawning periods, ^b percent of fish body weight, ^c food brand: Inicio 702 (2 mm), Biomar, Denmark, ^d food brand: Vital (2 mm), Alltech Coppens, The Netherlands.

4.3.2. Exposure experiments

To study the effects of plastic colouring, particle density and particle concentration on uptake by fish, fragments of six very widely used plastic polymers (PlasticsEurope 2018) with distinct properties were tested: polyethylene (PE, Falcon tube cap, blue), polypropylene (PP, storage box, grey), polystyrene (PS, plastic pellets, Glow-Side, Kretz, Germany, yellow), expanded polystyrene (EPS, packaging material, white), polyethylene terephthalate (PET, shampoo bottle, clear/purple) and polyvinyl chloride (PVC, water pipe, brown). The plastic was chopped into square particles of approximately 1–2 mm width with the help of a scalpel and stored in tap water for 24 h to minimize hydrophobicity.

During the experiments, 50 individuals of each species were exposed to three environmentally relevant particle concentrations, which were derived from available freshwater studies (Dris *et al.* 2015). The concentrations were: 100, 1000 and 5000 microplastic particles per m², or 0.19, 1.9 and 9.1 particles per litre. Corresponding particle numbers of each polymer type were evenly distributed into the tanks and remained there for two hours. No food was provided during the experiment and subsequently, all particles were removed. Ten fish were sampled from each tank directly after exposure (0 h) and a further ten after 6 and 24 hours respectively. The experiments were then repeated with the same experimental design, but the fish were also provided with genuine food after the plastic particles were added to the water. Feeding was repeated after one hour of exposure (total

amount of food: rainbow trout and grayling: 1.5 %, common carp and crucian carp: 2.0 % of fish body weight). Sampled fish were anesthetized with clove oil (0.1 mL per L water), euthanized with a cut at the gills and stored at -20 °C until required for further examination.

4.3.3. Detection of microplastic particles in the gastrointestinal tract

Sampled fish were thawed and each individual was weighed to the nearest 0.1 g and measured for total length to the nearest 0.1 cm. The stomach (rainbow trout, grayling) or the whole gastrointestinal tract (common carp, crucian carp) was dissected and placed into a 250 mL glass beaker. To digest the organic matter, 25 mL of sodium hydroxide (NaOH, 1 mol L⁻¹, Chemsolute, Th.Geyer, Germany) was added to the beaker and heated to 50 °C on a hot plate for 15 min while being mixed with a stirring bar. Each sample was diluted with 125 ml with filtered ultrapure water (0.2 µm pore size, Arium 611, Sartorius Stedim Biotech, Goettingen, Germany) and vacuum filtered through a 300 µm gauze. Recovered microplastic particles were examined under a dissecting microscope (Zeiss, Stemi SV6, 8–50x magnification) and their number and polymer type was noted. The prevalence of microplastic (percentages of burdened fishes), abundance (number of microplastic particles per fish) and the intensity of microplastic burden in contaminated fish (number of microplastic particles per fish) were calculated.

4.3.4. Model to estimate passive uptake of microplastics

In addition to the uptake routes tested above, the previously neglected possibility of a microplastic uptake via drinking was assessed. To estimate particle uptake rates, the following model was developed:

$$C_F = (Q_{dr} * W_F) * t * C_E \quad (1)$$

where C_F is the microplastic intensity in the gastrointestinal tract (GIT) of an individual (number of particles per fish), Q_{dr} is drinking rate in L per hour and kg fish weight, W_F is fish weight in kg, t is exposure time in hours and C_E is microplastic concentration in the water (number of particles per L). The distribution of particles in the water column was assumed to be random.

Three weight classes of fish (0.1 kg, 0.5 kg, 1 kg) were considered and the drinking rates of freshwater and marine fish were derived from the literature (freshwater

fish: 0.4 ml kg⁻¹ h⁻¹, marine fish: 4.1 ml kg⁻¹ h⁻¹, see Supplementary Table S1 online). Exposure time was set to 1000 h. The microplastic concentration in the water was the same as in the previous exposure experiments at 0.19, 1.9 and 9.1 particles per L. Equation (1) was extended to examine possible accumulation of microplastics imbibed through constant water uptake in the GIT, with the second term simulating the egestion of the particles over time:

$$C_F = (Q_{dr} * W_F) * t * C_E - \frac{t}{t_F} \quad (2)$$

The variable t_F accounts for the average residence time of microplastics in fish and was derived from the literature (mean: 44 h, see Supplementary Table S1 online). The model was computed with the same values as mentioned above.

The final approach evaluated the long-term accumulation of imbibed microplastic particles smaller than 5 µm in the tissue or organs of fish:

$$C_F(t) = (Q_{dr} * W_F) * t * (C_E * p * q) \quad (3)$$

As only a fraction of microplastic particles are small enough to cross the intestinal barrier, the variable p accounts for the percentage of particles in this relevant size range. Only particles smaller than 5 µm are regarded as small enough to be translocated on a regular basis (Carr *et al.* 2012; Lu *et al.* 2016). The percentage of particles of relevant size was derived from an extrapolation in a previous study of microplastic burden intensity in freshwater fish, which suggested that 70 % of particles in fish might be expected to be sub-5 µm (Roch *et al.* 2019). A particle concentration of 1.9 particles per L in the water column was assumed. The variable q considers the percentage of particles smaller than 5 µm actually crossing the intestinal barrier. While no reliable means of estimating the rate of translocation of microplastics into tissues in fish under realistic conditions has yet been reported, it should be noted that the translocation rate is likely to vary according to particle size and other factors. To account for this uncertainty, conservative rates of between 0.01 and 1 %, similar to those in rodents, were assumed (Carr *et al.* 2012), though higher values of up to 10 % have been reported (Stock *et al.* 2019; Jani *et al.* 1994; Florence *et al.* 1995). To account for weight changes and related increases in water uptake, weight for each year was calculated, using a modified von Bertalanffy growth function (von Bertalanffy 1938):

$$W_F = a * L_{\infty}^b * [1 - \exp(-K * (t - t_0))]^b \quad (4)$$

The variables a and b are length-weight relationship parameters, L_{∞} is asymptotic length (theoretical maximum length) in cm, K is the body growth coefficient and t_0 is age of zero weight. All parameters were derived from the website fishbase.org, using common carp and cod (*Gadus morhua*) as representative freshwater and marine fish species respectively. The number of particles accumulated in the tissues and organs for each year of growth up to 15 years was calculated.

4.3.5. Statistical analysis

To assess the individual effects of biotic and abiotic factors on the microplastic uptake in fish, different linear models were selected by minimizing the Akaike information criterion (AIC; Akaike 1973) as follows:

$$y_i = \beta_0 + \beta_1 x_1 + \dots + \beta_i x_i + \varepsilon_i \quad (5)$$

where y_i is the dependent variable, β_0 is the intercept, β_i is the regression coefficients, x_i is the predictor variables, and ε_i is the random residual error. The following dependent variables were defined: prevalence, abundance and intensity (for definition see above). The utilized models and associated model effects are summarized as [Supplementary Table S4.2](#) online. To examine relationships between certain relevant factors, Response Surface Methodology (RSM) graphs were plotted based on the respective linear model.

4.3.6. Ethical Statement

All methods were carried out in accordance with ethical guidelines and regulations. All experiments were conducted according to the German Animal Welfare Act (TierSchG) and approved by Referat Tierschutz of Regierungspräsidium Tübingen (LAZ 2/16, AZ 35/9185.81-4).

4.4. Results

4.4.1. Biotic factors influencing microplastic uptake

Three out of four fish species, with the exception of crucian carp, ingested microplastics during the exposure experiments. The mean total lengths and fresh weights of sampled fish are summarized as [Supplementary Table S4.3](#) online. Mean microplastic prevalences and abundances ($\pm$ standard error) for each fish species and particle concentration over time are summarized as Supplementary

Figures S4.1 and S4.2 online. The performed nominal-logistic model (whole model: 720 observations, d.f. = 5, $r^2(U) = 0.1947$, $P < 0.0001$) revealed significant factors influencing microplastic prevalence directly after exposure (Fig. 4.2). The main orientation sense used by foraging fish had a highly significant effect, with visual foragers ingesting particles more often than chemosensory foragers ($P = 0.00022$). When genuine food was available during exposure to microplastics, the portion of fish ingesting microplastics was lower than when real food was unavailable ($P = 0.01528$). Particle concentration had also a significant effect, with prevalence of burdened fish increasing with concentration ($P = 0.00036$). On the other hand, the origin and total length of fish didn't significantly influence microplastic prevalence ($P > 0.05$).

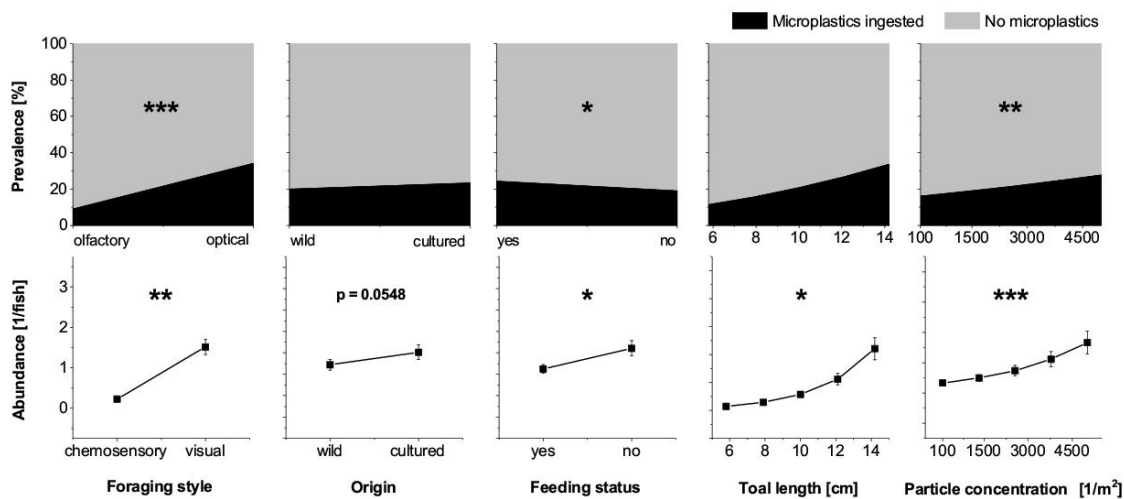


Figure 4.2. Microplastic uptake (Grand marginal means) directly after exposure. **Top:** Microplastic prevalence, **Bottom:** Microplastic abundance ($\pm$ standard error). Asterisks indicate statistically significant differences between model effects (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$). n = number of particles.

When looking at particle abundance directly after exposure, the General Linear Model (GLM, whole model: 720 observations, d.f. = 5, $P < 0.0001$) revealed several significant factors, affecting the quantity of microplastic ingested by fish (Fig. 4.2). Foraging style and particle concentration had an effect similar to that of prevalence (foraging style: $P = 0.00033$, particle concentration: $P < 0.0001$), with abundance increasing exponentially with the concentration of microplastics in the water. In contrast, abundance was higher when fish were not provided with genuine food ($P = 0.00127$). Total body length also had an exponential significant effect on the number of plastic particles ingested ($P = 0.00101$). A further trend was apparent with fish origin, where wild fish ingested fewer microplastic particles ($P = 0.05480$) than cultured ones. To explore the relationship between identified relevant biotic factors and particle concentration in the water, RSM graphs were plotted. The results for foraging style and feeding activity versus particle

concentration in the water are shown in [Figure 4.3\(a,b\)](#). In visual foragers, abundance increased exponentially with the particle concentration in water, whereas there was only a minor increase in chemosensory foragers. Abundance increased exponentially with particle concentration in water, regardless of whether genuine food was supplied or not, though the increase was lower when fish were properly fed.

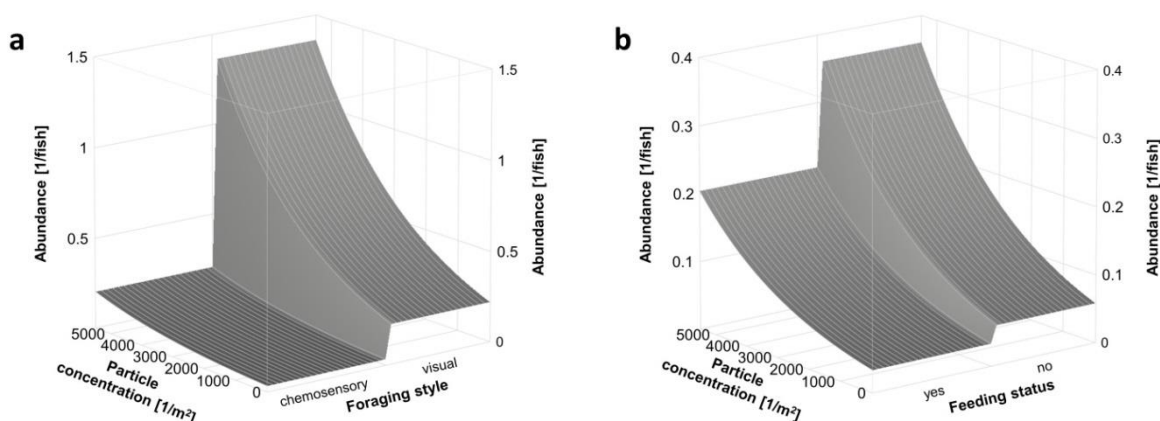


Figure 4.3. Response surface methodology graphs based on the results of a General Linear Model for particle concentration in water versus **(a)** foraging style and **(b)** availability of genuine feeding opportunity. n = number of particles.

To test if there was any egestion of microplastics during the exposure experiment, a GLM was performed for different time points (whole model: 1860 observations, d.f. = 6, $P < 0.0001$). Fish species, active feeding and particle concentration all had a significant effect on particle abundance in exposed fish (fish species: $P < 0.0001$, feeding status: $P < 0.0001$, particle concentration: $P < 0.0001$), while sampling time had no influence ($P > 0.05$).

4.4.2. Particle properties influencing microplastic uptake

When looking at particle properties, the GLM (whole model: 2616 observations, d.f. = 5, $P < 0.0001$) revealed several significant factors influencing microplastic intensity ([Fig. 4.4](#)). Time after exposure had no significant effect on the number of particles identified in fish GIT, indicating no bias resulting from microplastic excretion (see above), so all data was included in the model. Particle concentration in the water and foraging style had a significant positive effect on the number of microplastic particles in fish (particle concentration: $P < 0.0001$, foraging style: $P < 0.0001$). Furthermore, particle density and particle appearance affected particle numbers (particle density: $P = 0.00118$, particle appearance: $P = 0.04382$), with food-like particles and sinking particles being ingested most often. Carp, in contrast to the two visually foraging fish species, ingested solely sinking particles.

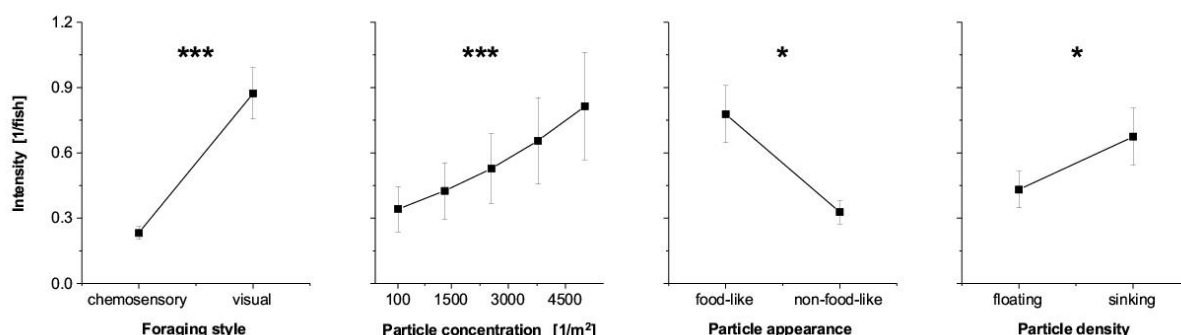


Figure 4.4. Effect of particle properties on microplastic uptake ($\pm$ standard error, all sampling points). Asterisks indicate statistically significant differences between variables (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$). n = number of particles.

Table 4.2. Results of the generalized regression model (distribution: negative binomial, model fit: elastic net, estimation method: AICc) for visually foraging fish at all time points.

Model effect	Estimates	Number of parameters	Wald chi-square	P-values
Particle appearance	1.21	1	100.42	< 0.0001
Feeding status	-0.56	1	19.60	< 0.0001
Particle Concentration	0.00017	1	18.85	< 0.0001
Fish origin	-0.36	1	8.06	0.0045
Particle appearance x particle concentration	0.00015	1	6.83	0.0090
Origin x particle concentration	-9.88×10^{-5}	1	2.75	0.0973

To further analyse the interactions between relevant factors identified in the GLM above in visually oriented fish, a generalized regression model was performed (whole model: 2400 observations, d.f. = 5, $P < 0.0001$). The results are summarized in [Table 4.2](#). All factors, except the interaction between genetic fish origin and particle concentration significantly affect the intensity of microplastic burden. An overview of the mean particle intensity of food-like and non-food-like particles, dependent on particle concentration, fish origin and feeding status is given in [Figure 4.5](#). The corresponding prevalence of ingested food-like particles in visually foraging fish is summarized as [Supplementary Figure S4.3](#) online. In wild fish, the intensity of food-like particles increased with particle concentration in the water, independent of whether fish were feeding. In contrast, particles not resembling their food were ingested in low numbers and didn't increase with particle concentration in the water. In cultured fish, microplastic intensity was generally lower, but when no food was provided, numbers of both food-like and food-unlike particles ingested increased with particle concentration in the water. This is further supported by the lower prevalence of food-like particles in visually oriented fish (see [Supplementary Fig. S4.3](#) online).

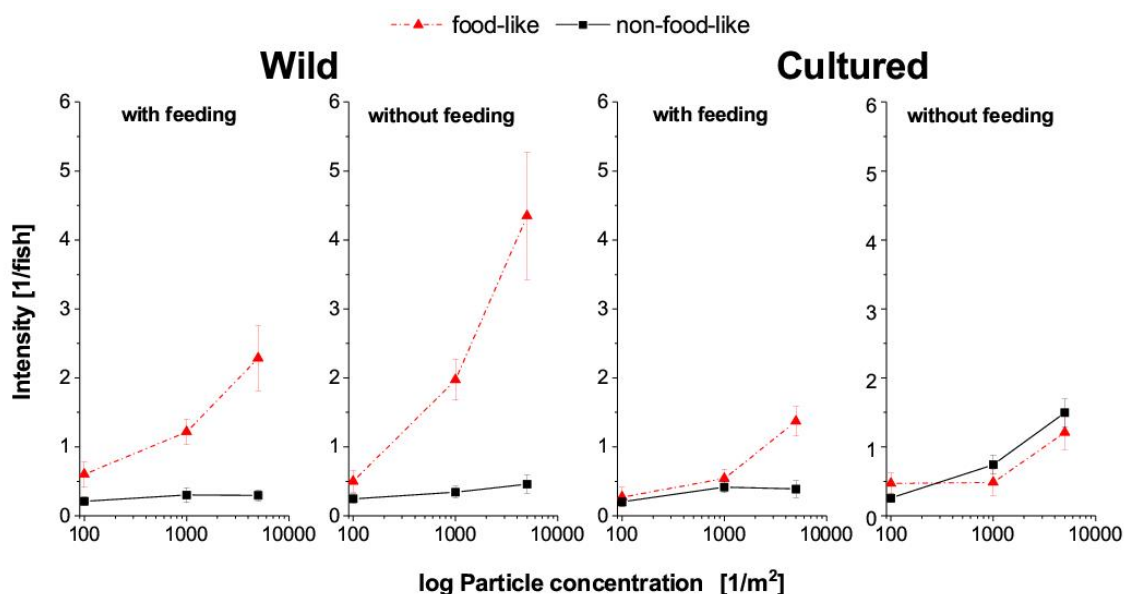


Figure 4.5. The effect of feeding and particle appearance on particle intensity (mean $\pm$ standard error) in visually oriented fishes (all time points). n = number of particles.

4.4.3. The role of drinking in the passive uptake of microplastics

The results of the modelled uptake and accumulation of microplastic in fish via drinking are shown in [Figures 4.6](#) and [4.7](#). Independent of fish weight and particle concentration in the water, the probability of freshwater fish ingesting a single microplastic particle is low, occurring approximately once every 300 h in the largest weight class at highest water particle concentrations ([Fig. 4.6\(a\)](#)). When the egestion of particles is also considered, no accumulation effects were seen to occur ([Fig. 4.6\(b\)](#)). In marine fish, the probability of ingesting microplastic particles via drinking is considerably higher at one particle approximately every 30 h in the largest fish weight class at the highest water particle concentrations ([Fig. 4.7\(a\)](#)). When egestion is considered, no accumulation effects could be observed in the two lower weight classes ([Fig. 4.7\(b\)](#)), but when looking at the largest fish exposed to highest water particle concentration, an accumulation effect was apparent, with an increase of one particle every 5 days.

To examine the potential long-term accumulation effects of small microplastics in tissues and organs of fish, particle uptake via drinking was modelled exemplarily for common carp (freshwater) and cod (marine). The results are shown in [Fig. 4.8](#). Assuming that 1 % of particles smaller than 5 μm are translocated from chyme into tissue the model predicts that around 40 microplastic particles will accumulate in the tissues of a freshwater common carp over 15 years ([Fig. 4.8\(A\)](#)). The same assumptions applied to cod, a marine species, suggest that a total of 200 particles will be accumulated ([Fig. 4.8\(B\)](#)), five times more than in the common carp.

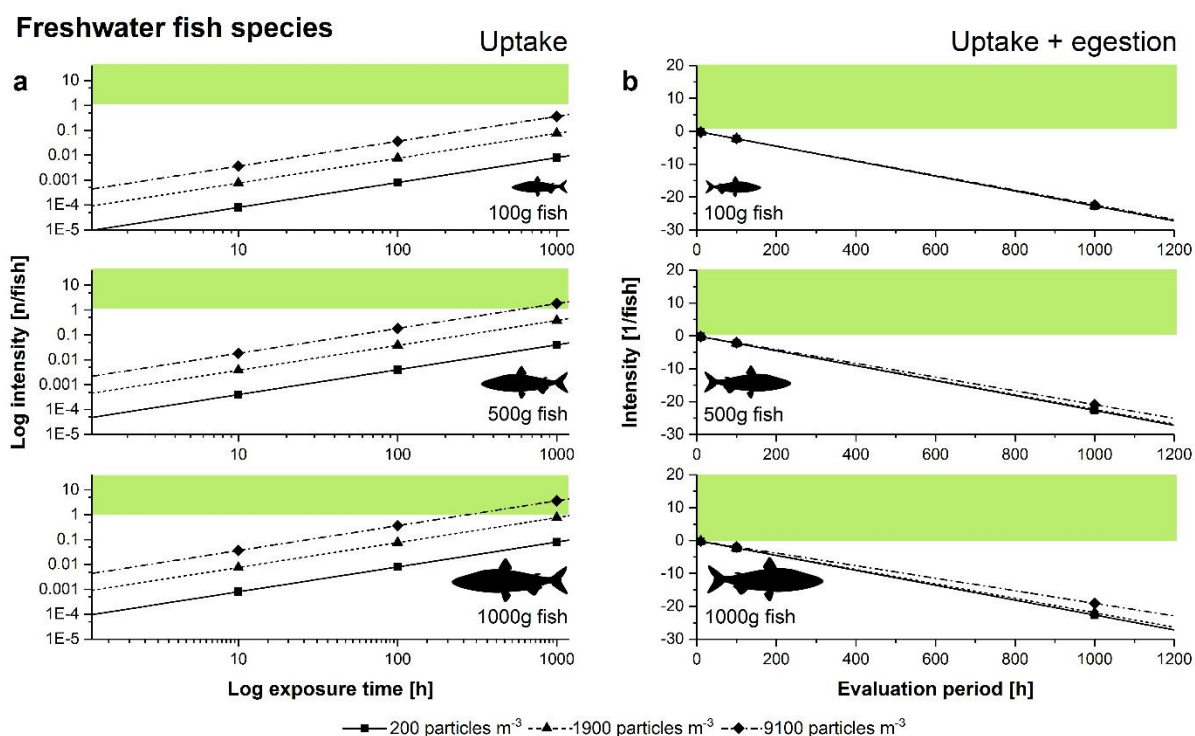


Figure 4.6. Modelled passive uptake of microplastics via drinking in freshwater fish. **(a)** Uptake rate (coloured area indicates an intensity >1) and **(b)** uptake rate combined with egestion rate for different particle concentrations in the water and fish weights respectively (coloured area indicates an accumulation effect). n = number of particles.

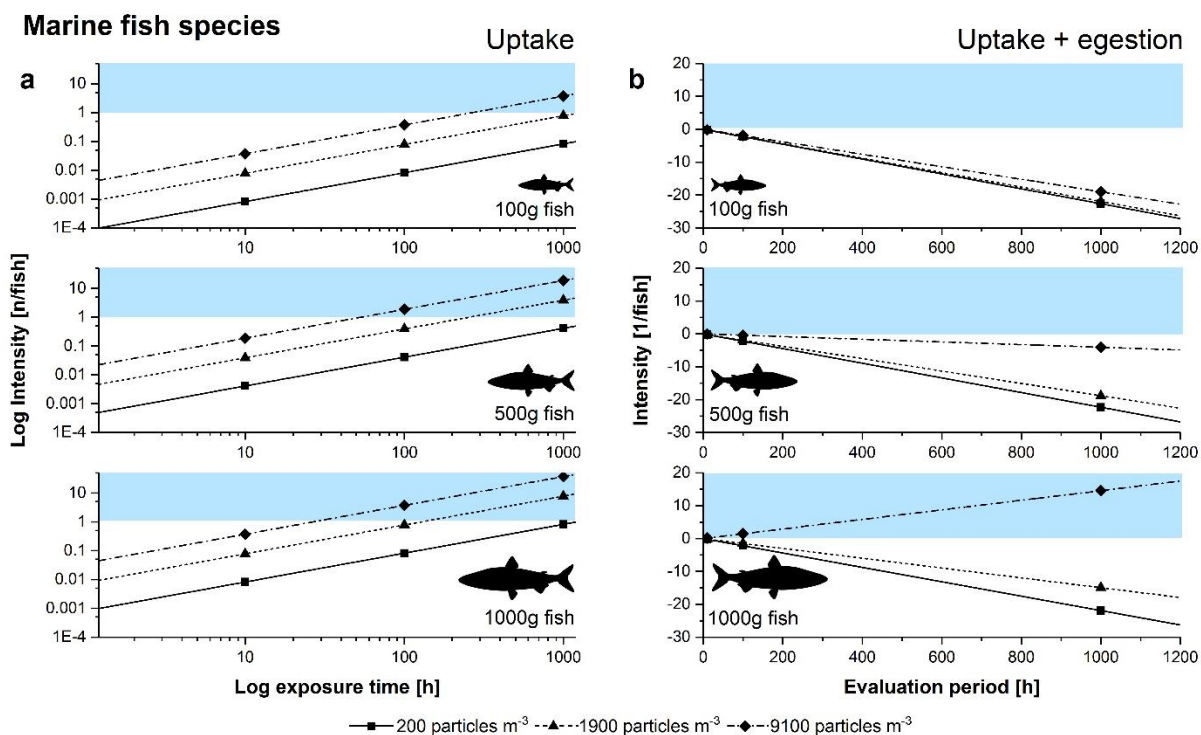


Figure 4.7. Modelled passive uptake of microplastics via drinking in marine fish. **(a)** Uptake rate (coloured area indicates an intensity >1) and **(b)** uptake rate combined with egestion rate for different particle concentrations in the water and fish weights respectively (coloured area indicates an accumulation effect). n = number of particles.

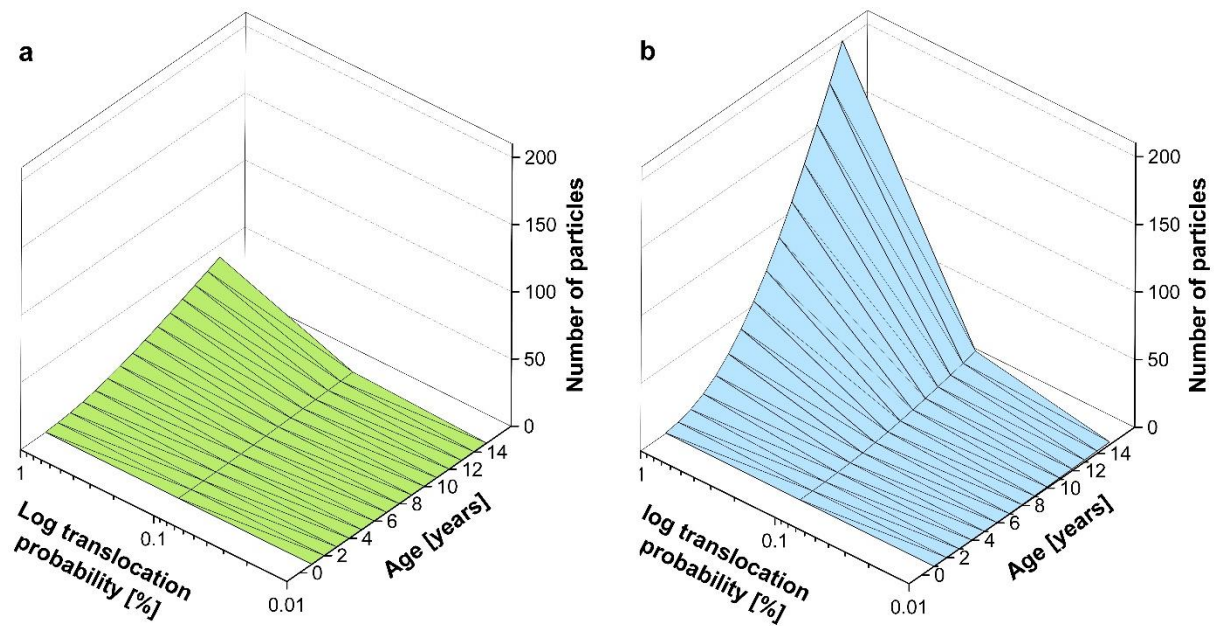


Figure 4.8. Modelled accumulation of microplastics <5 μm in fish tissue via drinking for **(a)** common carp and **(b)** cod in dependence on hypothesized translocation probability into tissue and age of fish.

4.5. Discussion

4.5.1. Biotic factors influencing the microplastic uptake

The detrimental effects of global plastic pollution on aquatic organisms have been extensively studied in recent years (Wright *et al.* 2013; Gall & Thompson 2015). In fish, microplastics have been detected in the GIT in marine and freshwater systems alike (Roch *et al.* 2019; Lusher *et al.* 2013; Rummel *et al.* 2016). However, the underlying mechanisms of how these particles are ingested are largely unclear. The results of the present study show that several biotic factors play a role. Fish that rely principally on visual foraging cues ingested microplastic particles significantly more often and in higher numbers than species that perform mainly chemosensory foraging, suggesting that a sense of taste aids discrimination of inedible food items. This is further supported by the fact that unlike visual foragers, particle concentration in the water had only a minor effect on the number of particles ingested by chemosensory foraging fish. Nonetheless, some isolated particles were ingested, indicating a potential for accidental uptake. This agrees with laboratory studies using juveniles of the common goby (*Pomatoschistus microps*), which showed an ability to distinguish food and plastic particles, but occasionally still ingested microplastics by mistake (de Sá *et al.* 2015).

The profound differences between visual and chemosensory foragers might explain why microplastics are more commonly found in marine pelagic fish species (Güven

et al. 2017; Rummel *et al.* 2016). In freshwater systems, the roles of habitat and habitat-related feeding preference are more ambiguous and vary widely between studies (Phillips & Bonner 2015; Roch *et al.* 2019). It is generally thought that chemosensory foragers feeding near the bottom are more exposed to microplastics, as concentrations are thought to be higher in the sediment than in the water column (Dris *et al.* 2015; Horton *et al.* 2017). However, the present results suggest that a developed sense of taste limits the unintentional ingestion of microplastic particles. In the field, other uptake routes, such as transfer via the food chain, might have a more profound effect on the microplastic burden of benthic fish species (Santana *et al.* 2017; Welden *et al.* 2018).

It was hypothesized that the genetic origin of fish, be it wild or cultured, has an effect on microplastic uptake. Some studies suggest that selective breeding can result in cultured fish becoming rather undiscerning in their food intake compared to wild individuals (Thodesen *et al.* 1999). For example, it has been shown that escapees of cultured fish from fish farms have lost their ability to distinguish edible and inedible items (Rikardsen & Sandring 2006; Skilbrei 2012). No differences were observed in the prevalence of ingested microplastic particles between the wild and cultures groups in this study, despite crucian carp ingesting no microplastics at all. However an abundance trend was apparent, showing that wild fish ingested fewer plastic particles. One factor that weakens the difference between wild and cultured fish might be the large number of wild grayling ingesting microplastics in the present study. The possibility that adaptation to dry pellet food prior to the exposure experiment (Marcotte & Browman 1986) or even epigenetic effects belonging to the start period of the laboratory husbandry (Gavery & Roberts 2017) had an effect on the ability of fish to differentiate between artificial and real prey cannot be excluded. Further experiments using only natural prey items will have to be conducted to further examine the effect of domestication and husbandry on the propensity for microplastic ingestion.

Finally, active feeding played an important role on the uptake of microplastic particles, where the number of fish ingesting microplastics was higher in tanks where food was being supplied. In contrast, however, the number of particles ingested was higher when no genuine food was available. Thus, feeding behaviour appears to increase the chances of ingesting a microplastic particle, but some fish were actively foraging on microplastics when no food was available. This inappropriate feeding behaviour might be linked to certain particle properties, as discussed below.

4.5.2. Particle properties influencing microplastic uptake

Previous field studies have shown that some tropical and sub-tropical percid fishes, such as *Girella laevis* and *Decapterus muroadsi*, actively ingest microplastics that visually resemble their natural prey (Mizraji *et al.* 2017; Ory *et al.* 2017), and a laboratory study of palm ruff *Seriola lalandi*, showed that particles with similar colour to artificial food pellets were ingested more often (Ory *et al.* 2018). However, it remains unclear if this was a singular case or a common phenomenon among visually foraging fish. Furthermore in this study, only one particle colour was presented at a time with food and only fish that ingested food were considered in the analysis (Ory *et al.* 2018).

In the present study, all particle colours were available simultaneously. Nonetheless, particles with food-like colours were ingested significantly more often than others, independent of whether their colour made them clearly visible or inconspicuous. Interestingly, among wild visually foraging fish, the number of ingested food-like particles increased with increasing concentrations of particles in the water, while there was no increase in the intensity of ingestion of non-food-like colours. This was independent of the availability of genuine food, which again supports the hypothesis that visual foraging fish lack an ability to accurately discriminate edible and inedible food and may even actively forage on microplastics that resembling their prey in terms of colour. With cultured, visually foraging fish, the ingestion of food-like particles also increased with particle concentration in water when food was provided, although to a lesser extent. In the absence of genuine feeding opportunities, both food-like and non-food-like particles were ingested in equal proportions. It is therefore implied that cultured fish did not distinguish between colours and therefore are more prone to active foraging on microplastics regardless of the availability of real food.

A further statistical analysis of the influence of particle colour on microplastic uptake in fish which take a chemosensory approach to foraging was not possible, due to the fact that no plastic particles were detected in crucian carp at all. As already discussed, husbandry effects, *i.e.* adaption to dry food, might have influenced the tendency to ingest particles in the present exposure experiments. Furthermore, only virgin plastic particles were used in the present exposure experiments. Over time, weathering effects may change the chemical qualities and possibly also the taste of microplastics in the environment, potentially disguising their artificial origin (Savoca *et al.* 2016).

Apart from particle colour, particle density also had an effect on the uptake of microplastics. The density of different polymer types dictates whether they float on the water surface or sink to the bottom (Andrady 2011). Plastic particles with a density greater than water, *i.e.* sinking particles, were ingested more often than floating particles. This is probably due to the fact that dry pellet food provided during acclimatization and the exposure experiment sank after around 10 seconds to the bottom of the experimental tank (data not shown). Thereby, most active feeding took place at or near the bottom and thus the probability of ingesting sinking plastic particles was increased.

4.5.3. The role of drinking on the passive uptake of microplastics

The above mentioned uptake routes require that the particle size is large enough to be perceived by fishes as potential food. However recent studies indicate that the majority of microplastics are too small to be detected either by fish or by current monitoring methods (Roch *et al.* 2019; Conkle *et al.* 2018). These smaller particles may be ingested for other reasons. As shown above, plastic particles can be ingested by accident while foraging or passed on through the food chain (Setälä *et al.* 2014; Santana *et al.* 2017; Welden *et al.* 2018), but an uptake route which has not yet been looked at is passive or accidental ingestion via drinking, especially in marine fish species (Yaacob *et al.* 2016). Regular intake of water in marine environments is essential for a number of physiological processes (Fuentes & Eddy 1997) and marine fish drink about 10 times more water than freshwater fish (see [Supplementary Table S4.1](#) online). Thus the model predicts low uptake rates in freshwater fish, balanced by frequent egestion which prevents any accumulation of particles. However because of the short duration of the present exposure experiment and the small fish sizes used, it was not possible to fully evaluate the likely effect. In marine fish species, the intake of microplastics via drinking might be more relevant, especially in larger fish, which the model indicates will ingest particles on a regular basis, with a potentially significant accumulation effect when microplastic concentrations in the water are high. Little is known about the microplastic burden in large fishes, due to the limitations of current detection methods (Lusher *et al.* 2017). Nonetheless, future research must consider impacts on larger fish and fish species and should include an evaluation of the role of drinking on microplastic burden, not least because of the relevance of many such species as food for human consumption.

Another matter that needs to be considered is the translocation of small microplastics from the GIT into tissues and organs (Browne *et al.* 2008; Collard *et*

al. 2018). For fishes, information is scarce: there are studies showing the presence of microplastics up to 600 μm in fish liver, but not in muscle tissue (Collard *et al.* 2018; Collard *et al.* 2017; Avio *et al.* 2015). On the other hand, laboratory studies by Lu *et al.* (2016) found that only particles smaller than 5 μm were translocated into the liver. This is in accordance with a number of *in vivo* and *in vitro* studies in mammals, showing that translocation rates increase considerably with decreasing particle size (Carr *et al.* 2012; Stock *et al.* 2019). The accumulation of microplastics modelled in the present study could show that even when other uptake routes are not considered, and assuming that only 0.01 % to 1 % of very small particles are regularly translocated, the number of accumulated microplastics might be significant in fishes with a long lifespan, up to 200 particles over 15 years in the modelled cod. The issue is of particular concern for marine species due to their increased water uptake and there are of course important implications for transmission within the food web. The risks of accumulated particles could increase further, if we assume a higher translocation rate of microplastics through the intestinal barrier. Several studies have reported rates over 1% in mammals (Stock *et al.* 2019; Jani *et al.* 1994; Florence *et al.* 1995), but rates vary, with particle sizes, properties and experimental setups all seeming to play an important role (Lusher *et al.* 2017). Regardless of these uncertainties, there is no information available on how these translocated particles affect might affect fish health and it is not known, if microplastics can be removed from the blood or tissues/organs via natural excretion pathways (Yoo *et al.* 2011). It is important that further research considers the uptake of microplastic via drinking, the translocation pathways of micro- and nanoplastics from the GIT of fish, and the potential implications of these phenomena on fish as human food (Rist *et al.* 2018).

4.6. Acknowledgements

We would like to thank Amy-Jane Beer for the language correction and improvement of the manuscript. This work was supported by the “Fischereiabgabe” of the federal state Baden-Württemberg, Germany.

4.7. Supplementary information

Table S4.1. Drinking rates of marine and freshwater fish and residence time of microplastics in the gastrointestinal tract for the model approaches to calculate the passive uptake of microplastics via drinking.

Variable	Habitat	fish size	Value	Reference
Drinking rate	freshwater	20-30 g	$0,15 \pm 0,03 \text{ ml kg}^{-1} \text{ h}^{-1}$	Fuentes & Eddy 1997
		20-30 g	$0,25 \text{ ml kg}^{-1} \text{ h}^{-1}$	Fuentes <i>et al.</i> 1996
		50 g	$1,43 \pm 0,31 \text{ ml kg}^{-1} \text{ h}^{-1}$	Lovegrove & Eddy 1982
		150 - 250 g	$0 \text{ ml kg}^{-1} \text{ h}^{-1}$	Shehadeh & Gordon 1969
		n.a.	$0,03 \pm 0,01 \text{ ml kg}^{-1} \text{ h}^{-1}$	Perrott <i>et al.</i> 1992
		mean	$0.4 \text{ ml kg}^{-1} \text{ h}^{-1}$	
	marine	20-30 g	$3,89 \pm 0,28 \text{ ml kg}^{-1} \text{ h}^{-1}$	Fuentes & Eddy 1997
		20-30 g	$2,4 \text{ ml kg}^{-1} \text{ h}^{-1}$	Fuentes <i>et al.</i> 1996
		50 g	$7,40 \pm 1,63 \text{ ml kg}^{-1} \text{ h}^{-1}$	Lovegrove & Eddy 1982
		150 - 250 g	$5,4 \pm 0,5 \text{ ml kg}^{-1} \text{ h}^{-1}$	Shehadeh & Gordon 1969
		n.a.	$1,42 \pm 0,23 \text{ ml kg}^{-1} \text{ h}^{-1}$	Perrott <i>et al.</i> 1992
		mean	$4.1 \text{ ml kg}^{-1} \text{ h}^{-1}$	
Residence time	freshwater, marine	-	51 h	Roch <i>et al.</i> (in preparation)
		-	33 h	Grigorakis <i>et al.</i> 2017
		-	48 h	Lu <i>et al.</i> 2016
		mean	44 h	

Table S4.2. Model specifications for each performed model to examine the microplastic uptake of fish in the exposure experiments.

Model	Dependent variable	Fish species	Sampling points ^d	Model effects
Nominal-logistic regression model	prevalence	rainbow trout, grayling, common carp, crucian carp	0 h	origin, foraging style, feeding status, particle concentration, total length, foraging style x feeding
	abundance	rainbow trout, grayling, common carp, crucian carp	0 h	origin, foraging style, feeding status, particle concentration, total length
Generalized linear model ^a	abundance	rainbow trout, grayling, common carp, crucian carp	0 h, 6 h, 24 h	sampling time, fish species, feeding status, particle concentration
	intensity	rainbow trout, grayling, common carp, crucian carp	0 h, 6 h, 24 h	feeding status, particle concentration, particle appearance, particle density
Generalized regression model ^b	intensity	rainbow trout, grayling	0 h, 6 h, 24 h	particle concentration, origin, feeding status, particle appearance, particle appearance x particle concentration, origin x particle concentration

^a A Poisson distribution with a logarithmic link function was assumed for each GLM. Furthermore, an overdispersion parameter was included in each model to adjust variance independent of the mean (McCullagh & Nelder 1983). ^b A negative binomial distribution was assumed and the model was fitted with help of an elastic net and AIC as the model evaluation method (Sokal & Rohlf 2003).

Table S4.3. Summary of the total length and fresh weight of experimental fish used in the exposure experiments. SD = Standard deviation.

Fish species	Total length [cm]		Fresh weight [g]	
	mean	SD	mean	SD
rainbow trout	11,3	0,8	17,0	3,8
grayling	11,8	1,0	9,1	2,3
common carp	8,0	0,7	7,3	2,9
crucian carp	7,0	0,5	5,3	1,4

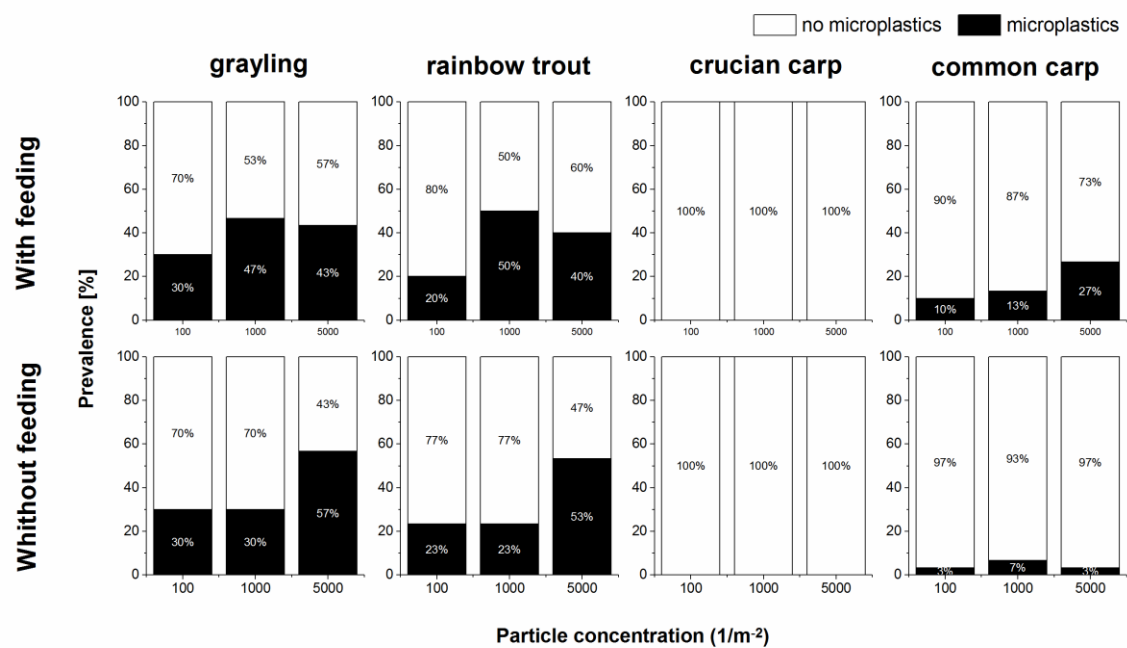


Figure S4.1. Mean microplastic prevalence for each fish species, particle concentration and feeding status, directly after exposure.

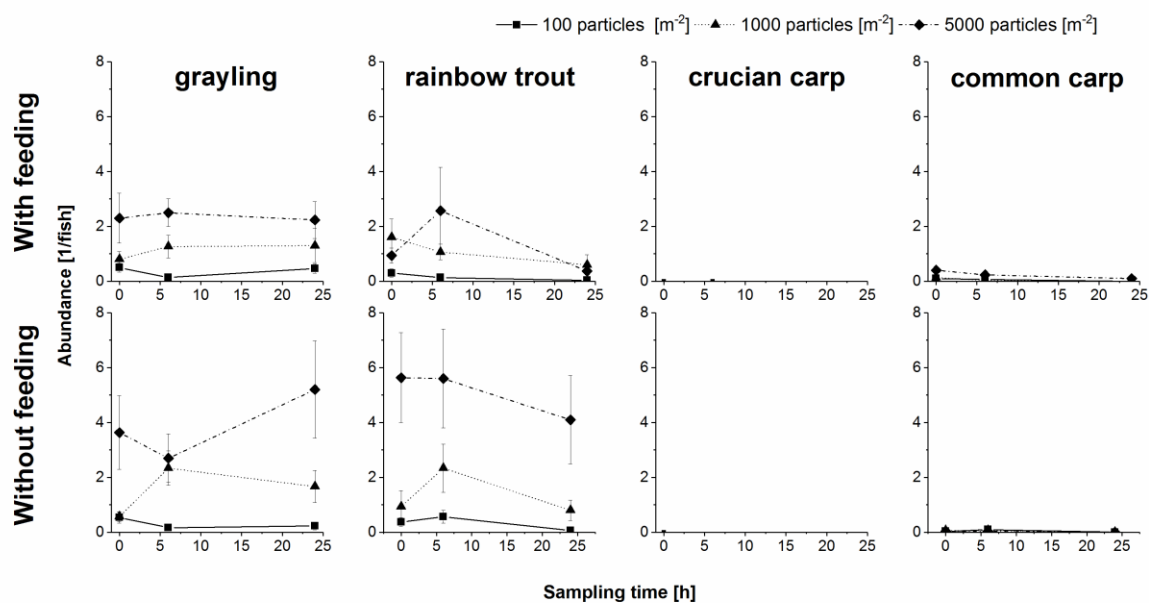


Figure S4.2. Mean microplastic abundance [$\pm$ standard error] for each fish species, particle concentration, sampling point and feeding status.

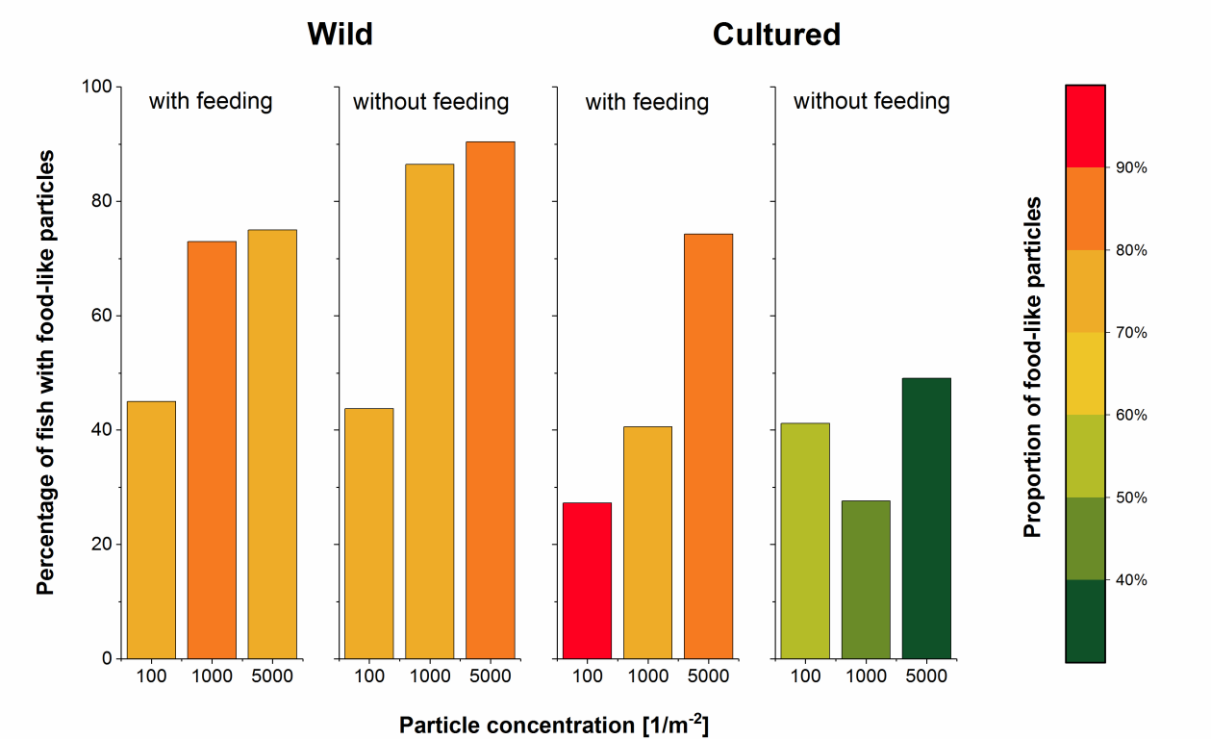


Figure S4.3. Percentage of fishes with microplastics that ingested at least one food-like particle (all time points). Colours indicate the proportion of food-like particles, compared to all particles ingested.

5. Manuscript IV: Microplastic evacuation in fish is particle size-dependent

ORIGINAL ARTICLE

Freshwater Biology WILEY

Microplastic evacuation in fish is particle size-dependent

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5.1. Abstract

1. The pollution of aquatic systems with microplastics (MPs) affects marine and freshwater fish species worldwide. However, little is known about the size-dependent retention time of these inert particles. To approach this question, the retention time of MP particles was examined in two freshwater fish species with distinct differences in gastrointestinal morphology: rainbow trout (*Oncorhynchus mykiss*) with a true stomach, and stomachless common carp (*Cyprinus carpio*).

2. A special diet was developed that contained environmentally relevant concentrations of MPs with sizes ranging from 20 to 1,000 μm . The two species were exposed to three different concentrations and numbers of retained particle were determined up to 72 hr after administration.

3. The results revealed significant differences in retention time between large and small MP particles in rainbow trout, in which the T_{50} value (time for 50 % of particles to be evacuated) ranged from 12.1 hr for 42.7 μm particles to 4.0 hr for 1,086 μm particles. In contrast, the differences observed between sizes in common carp were considerably smaller, with T_{50} ranging from 7.3 hr for 42.7 μm particles to 4.6 hr for 1,086 μm particles.

4. It is therefore concluded that large particles in rainbow trout must be actively transported out of the stomach, as the evacuation times are significantly shorter than those for food. Small particles, however, are passively excreted with the chyme. In common carp, the evacuation rates of all particle sizes were in the range of that for food, suggesting a passive excretion.

5. The results are particularly relevant in the contexts of particle transfer through the food chain, the release of toxic chemicals, and the translocation into tissues and organs. For this reason, it is essential that the particle size is given more consideration in future studies of microplastics in aquatic environments.

5.2. Introduction

The ingestion of indigestible particles by fish is an issue that has been of limited relevance for a long time. It is known, for example, that fishes swallow stones, sediment, or indigestible organic material either accidentally or deliberately without suffering major negative effects (Brewer *et al.* 2001; German 2009; Mauchline & Gordon 1984). Furthermore, there is some evidence that escapees of cultured fish may have a reduced ability to discriminate between edible and non-edible food items (Rikardsen & Sandring 2006), although it remains largely unclear how exactly these indigestible particles behave in the gastrointestinal (GI) tract. However, in the light of the worldwide contamination of aquatic systems and their inhabitants with microplastics (MPs), the topic takes on new importance.

Microplastics include fragments, beads, films, and fibres smaller than 5 mm, which are introduced directly into the environment or are produced by weathering and fragmentation from larger plastic waste (Barnes *et al.* 2009; Lambert *et al.* 2014). In fish, there are several routes by which MPs might be ingested: they may be transferred directly through the food chain (Santana *et al.* 2017); accidentally ingested while eating or drinking (Roch *et al.* 2020); or in some cases, can be confused with food, leading to active ingestion (Ory *et al.* 2017).

Previous studies have shown that MP particles ingested by fish are evacuated after a certain time with the chyme (Cong *et al.* 2019; Grigorakis *et al.* 2017; Mazurais *et al.* 2015). However, the influence of GI tract morphology on the retention time of those particles has not been considered so far. The structure of the GI tract is closely related to eating habits. Carnivorous fish, for example, possess a true stomach, whereas herbivorous fish have an elongated intestine without any distinct morphological subdivisions (German 2011; Wilson & Castro 2010).

Another factor that may influence MP retention time is the size of the individual ingested MP particles (Grigorakis *et al.* 2017). Particle concentrations in fish and in the water column exponentially increase with smaller size (Imhof *et al.* 2016; Roch *et al.* 2019). In addition, particle size has an influence on the transfer of toxic chemicals, as the surface area to volume ratio exponentially increases with smaller size (Lee *et al.* 2014). Small particles also can pass through the intestinal wall and

migrate into organs such as the liver (Collard *et al.* 2018; Lu *et al.* 2016). For these reasons and more, the residence time of particles of different size in the GI tract is of crucial importance. With regard to food particles, retention time is affected by a number of factors, including fish size, water temperature, meal size, and surface-to-volume ratio of food (Guillaume *et al.* 1999; Jobling 1987). For small inert particles, such as MPs, there is no reliable information concerning retention time, especially regarding the effect of particle size.

In the present study, the size-dependent retention time of MP particles was evaluated in two fish species using a specially developed diet containing MPs in a wide range of sizes. The selected range and progression of the size distribution was based on analyses of environmental fish samples, showing that particle numbers increase exponentially with lower size (Roch *et al.* 2019). Rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) were selected due to their archetypal GI tract morphologies. In rainbow trout, the oesophagus is connected to a j-shaped stomach, which is again separated from the intestinal tract by a pyloric valve (Weinreb & Bilstad 1955). The intestinal tract itself can be separated into an anterior and posterior section, of which the former is associated with pyloric caeca (blind worm-like ducts that are similar in structure to the intestinal tract itself; Wilson & Castro 2010). In contrast, like all cyprinids, the common carp possesses no anatomical stomach, but rather a prolonged foregut in which the main important digestion processes take place (Hofer 1991).

Sampling at different time intervals allowed an assessment of the retention time of MPs according to particle burden and particle size. The results were also used to calculate the time required for 50 % of particles of a certain size to be evacuated (T_{50}). Finally, a deterministic approach was developed to calculate average retention time of MP particles, independent of size. The results are discussed in the context of the current state of knowledge and the possible consequences for fish health, as well as future research priorities, are evaluated.

5.3. Methods

5.3.1. Experimental diet

For the exposure experiments, an experimental diet was developed (pellet size: 2 mm). An overview of the general formulation is given in [Table 5.1](#). To produce MP particles of different sizes, fluorescent polymethylmethacrylate (Glow-Side) granules were crushed in a mortar grinder (Pulverisette 2, Fritsch), then wet

sieved to exclude particles smaller than 20 µm and larger than 1,000 µm. Three grams of MP particles were added to 1 kg of the experimental diet (hereafter: MP diet). Particle concentration and size distribution were assessed by digesting 10 pellets (Roch & Brinker 2017). To generate different MP concentrations for the exposure experiments, the MP diet was mixed with the untreated diet (ratio by weight of MP diet to untreated diet in three different proportions: 10:90 [low], 25:75 [medium], and 50:50 [high]).

5.3.2. Husbandry of fish

All experimental fish were acclimatised for 2 weeks in tanks (50 fish per tank, tank size: 50 × 55 × 55 cm) and fed twice a day with untreated food (see above). Rainbow trout were held in a flow-through system (8 L/min water exchange rate) with an average water temperature of 8°C and oxygen levels between 8 and 10 mg/L. Common carp were held in a semi-recirculating aquaculture system (3 L/min exchange rate) with an average water temperature of 20°C and oxygen levels between 8 and 10 mg/L. For both species, light intensity was around 300 Lux, with 12 hr illumination and 30 min transition periods approximating dawn and dusk. Water parameters were monitored continuously, and tanks were cleaned on a daily basis.

Table 5.1. Formulation of the experimental diets supplied to rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*).

Raw materials	Rainbow trout	Common carp
	[g/kg]	[g/kg]
Fish meal (<i>Clupea</i> sp.)	502.1	127.4
Blood meal (III Sonac)	48.9	47.7
Wheat gluten	153.4	11.7
Soy protein concentrate	0	149.7
Wheat starch	154.6	271.6
Wheat meal	0	210.8
Biolys (Lysin)	0	4.6
Metamino (Methionin)	0	9.2
Fish oil	94.0	55.1
Vitamin / Mineral premix	9.4	9.2
Carboxymethylcellulose	37.6	0
Sum [g]	1000	1000

Manufactured by: GMA – Gesellschaft für marine Aquakultur mbH, Büsum, Germany

5.3.3. Experimental setup

All experiments were performed in triplicate, resulting in nine replicates for each species. In each replicate, 50 individuals of each species were fed with the three prepared MP diets. The quantity of food supplied was equivalent to 1.5 and 3 % of fish body weight for rainbow trout and common carp, respectively, and was delivered within an hour. Sampling took place directly after feeding (0 hr) and after 8, 24, and 48 hr, respectively. Five individuals per time point and MP concentration were anaesthetised with clove oil (0.1 ml/L water, Euro OTC Pharma), euthanised with a cut at the gills, and stored at -20 °C until further examination. As MP particles were still found in the GI tract of the fishes on the last sampling point, the experiment was repeated over an extended period (rainbow trout: 56 and 72 hr, common carp: 64 hr). In total, 270 individual rainbow trout and 224 common carp were examined for MP content in the GI tract. All experiments were conducted according to the German Animal Welfare Act (TierSchG) and approved by Referat Tierschutz of Regierungspräsidium Tübingen (LAZ 2/16, AZ 35/9185.81-4).

5.3.4. Particle detection in the GI tract

To determine the MP concentration and particle size distribution within the fish, each individual was thawed, the total length (to the nearest 0.1 cm) and wet weight (to the nearest 0.1 g) were determined and the GI tract was removed. The dissection of rainbow trout was restricted to the stomach to ensure that potential retention processes were attributed to the stomach itself. An additional analysis of the intestine in the present study was not possible, as this would have resulted in a significant increase in the number of samples and therefore an increased workload. In contrast, the whole GI tract was dissected for examination in common carp. The stomachs or GI tracts were digested using a protocol described by Roch & Brinker (2017). Briefly, the tissue samples were placed in glass beakers containing 10 ml sodium hydroxide (NaOH, 1 mol/L, Chemsolute, Th.Geyer) and the solution was heated for 15 min at 50 °C. Next, 36 ml of nitric acid (HNO₃, 65 %, Chemsolute) and 4 ml of ultrapure water (0.2 µm pore size, Arium 611, Sartorius Stedim Biotech) were added and the samples were heated for a further 15 min at 50°C. The temperature was then increased to 80 °C for 15 min and the samples were subsequently diluted with 100 ml of ultrapure water. Finally, the solutions were filtered on a cellulose-nitrate filter (47 mm, 8 µm pore size, Sartorius Stedim Biotech) and dried at room temperature overnight.

MP particles recovered on the filter were counted and measured using a digital microscope (Keyence VHX700), a black-light lamp and the on-board software of the microscope. Where more than 20 particles were present on the filter, particle numbers were only counted in five random squares with an area of 56.25 mm² each and then extrapolated to give an estimated value for the whole filter. To determine the size distribution of recovered MP particles, the area of each particle was calculated (to the nearest µm) using the Equivalent Circular Area Diameter (ECAD) according to Li *et al.* (2005), to ensure the comparability of the particles. The particles were then grouped into 18 size classes, ranging from 21.7 to 1,086 µm. To satisfy the ratio conditions stated by Kavanaugh *et al.* (1980), each size class range was increased by a factor of 1.26.

5.3.5. Data analysis

To assess the concentration and size distribution of the MP particles in the diet, mean particle abundance per pellet was calculated and the size distributions were plotted for both rainbow trout and common carp diet. Furthermore, final particle concentrations per gram for each size class were calculated for the blended MP diets applied in the exposure experiments. Particle counts from the exposure experiments were used to calculate mean particle abundances ($\pm$ SE) for each sampling time point and particle concentration in the diet (particle burden). In addition, mean particle prevalence was calculated for each sampling time point (independent of particle burden).

The experimental fish were supplied with three different MP concentrations in their diet. To test whether particle density had an effect on the retention time of these plastics in the stomach and GI tract, the following statistical procedure was used: first particle abundance was expressed as a relative value for each size class in comparison with the overall abundance of MP particles in the fish. These relative values (ranging from 0 to 1) were tested using a generalised linear mixed model with a binomial error distribution and probit link (see Supplemental information for more details). These model calculations, performed separately for rainbow trout and carp, indicated no statistically significant contribution of initial particle density to changes in the relative abundance of particles ([Table S5.1](#) and [S5.2](#)). Therefore, the original count data were used for further analysis and particle burden was entered as a model offset.

Particle counts showed an excess of zero values, resulting in poor fit and invalidating the use of standard statistical models (*e.g.* generalised linear mixed models; Perumean-Chaney *et al.* 2013). To deal with these data statistically, zero-

inflated models were developed to partition zero values into two categories, one originating from the sampling distribution, and one representing a structural category attributable to other origins (Brooks *et al.* 2017). For example, structural excess zeros in particle abundance in the present experiment could be produced by individuals that did not consume the experimental pellets containing MPs. In addition to the excess zeros, particle counts also showed highly overdispersed variation (overdispersion parameter: rainbow trout, 4.61 and carp, 5.56). To account for both excess zeros and overdispersion, a zero-inflated model based on the negative binomial distribution was fitted to the particle count data. The models were calculated using the glmmTMB package in R Version 3.6.3 (R Core Team 2020). The model was corrected for individual (ind) and fish tank (tank) as random variables. Particle size class mean (Ps) and sampling time after exposure (T) were added as independent predictor variables, and the particle burden (Pb) was corrected for as offset. The models were specified as follows: glmmTMB (Particle abundance (N) ~ Ps * T + (1 | ind) + (1 | tank) + offset (Pb), data = rainbow trout or common carp, ziformula = ~1, family = nbinom1). Diagnostics for Hierarchical Regression Models (DHARMa; Hartig 2019) was then used to validate assumptions of normality for the model and to produce predicted response values that were fitted on the glmmTMB model. These predicted responses were used to produce a graphical representation of the model outcome. The T₅₀ values of particles in the fish represent the negative inverse of the slope of a linear regression between the log₂ value of the predicted response and the time after exposure:

$$\log_2 N_{Ps} = \log_2 N_{0Ps} - \frac{1}{t_{50,Ps}} \cdot T \quad (1)$$

with

$$\frac{1}{T_{50,Ps}} = a + b \cdot Ps, \quad (2)$$

where N_{Ps} = (model) particle abundance of a given particle size, N_{0Ps} = (model) particle abundance of a given particle size at the start of the experiment, T_{50,Ps} = time for 50% of particles of a given particle size to be evacuated (hr), T = sampling time after exposure (hr), a = constant (inverse of T₅₀ of a particle with size = 0 µm; 1/hr), b = particle size coefficient (1/[µm hr]) and Ps = particle size class mean (µm). Finally, the results of the model were also used in a deterministic approach to develop a formula that allows the calculation of a size-independent mean of T₅₀ values for MP particles in the two species studied (for more details, see Supplemental Information). For both species, the T₅₀ values obtained from the models were then compared with reported food evacuation times (see [Table S5.4](#)

and S5.5). All statistical analyses were performed using JMP Pro 14 (Vers. 14.0, SAS Institute Inc.) and R Version 3.6.3 (R Core Team 2020).

5.4. Results

5.4.1. Experimental diet

In the experimental MP diets, mean particle numbers ($\pm$ SD) per pellet were 81 ± 24 particles for the rainbow trout diet and 85 ± 39 particles for the common carp diet. Particle sizes ranged from 27 to 958 μm in both diets. Corresponding histograms of size distribution and particle prevalence for each size class are shown in Figure 5.1. Mean total particle abundance per gram of the MP diets used in the exposure experiments were 356 (low), 891 (medium) and 1,782 (high) for the rainbow trout diet and 373 (low), 933 (medium), and 1,866 (high) for the common carp diet.

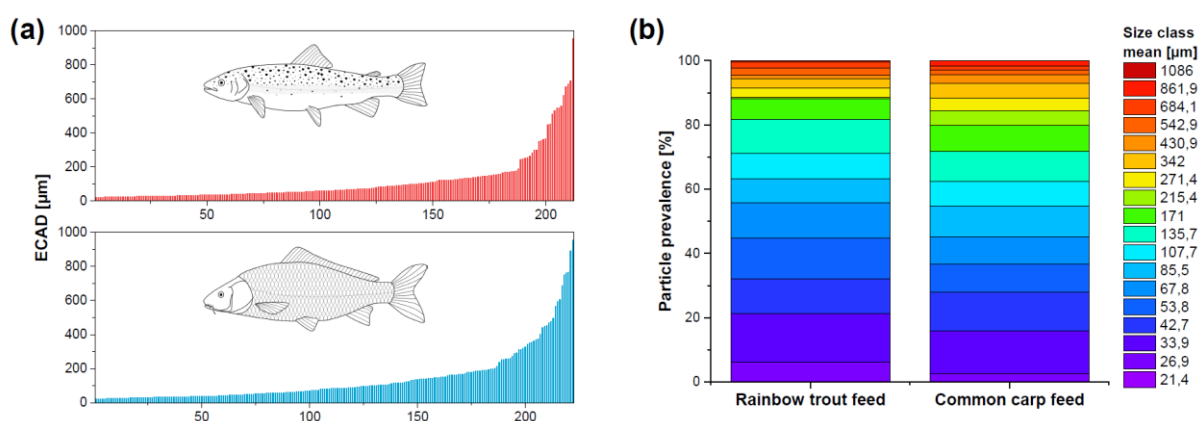


Figure 5.1. Microplastic (MP) particle size distribution in the experimental diets fed to rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). **(a)** Accumulative histogram of the ECAD (Equivalent Circular Area Diameter) of all MP particles detected in 10 Pellets (0.2 g). **(b)** Mean MP particle prevalence per pellet and size class.

5.4.2. Exposure experiments

Age, total length, and fresh weight of experimental fish are summarised in Table S5.3. Neither length nor weight differed within rainbow trout and common carp, when comparing the three treatments (ANOVA, $p > 0.05$). However, differences in length and weight were statistically significant between the two species (total length: t-test, $p < 0.0001$; fresh weight: t-test, $p < 0.0001$).

Both fish species ingested the MP diet without any problems. Particle numbers per fish directly after exposure (0 hr) ranged from 0 to 309 particles in rainbow trout and 0 to 424 particles in common carp. Particle numbers subsequently decreased

over time (Fig. 5.2). By the last sampling point (rainbow trout 72 hr, common carp 64 hr), 99% of ingested particles had been evacuated. The particle size distribution observed in fish directly after exposure (0 hr) was similar to that in the MP diet (Fig. 5.3). In rainbow trout and common carp, particles with a size class mean of 171 μm and larger appeared to have cleared the stomach or GI tract after 56 and 64 hr respectively. In common carp, particles smaller than a size class mean of 42.7 μm were completely evacuated after 48 hr (Fig. 5.3). However, it must be noted that the number of particles per fish was very low at the later time points. These particles exclusively belonged to the size classes that were most abundant in the experiment (Fig. S5.1). For this reason, only the first four time points (0 - 48 hr) were used in the subsequent model analysis. Furthermore, particle size classes smaller than 42.7 μm were omitted, as particle numbers decreased with lower size classes (Fig. S5.1).

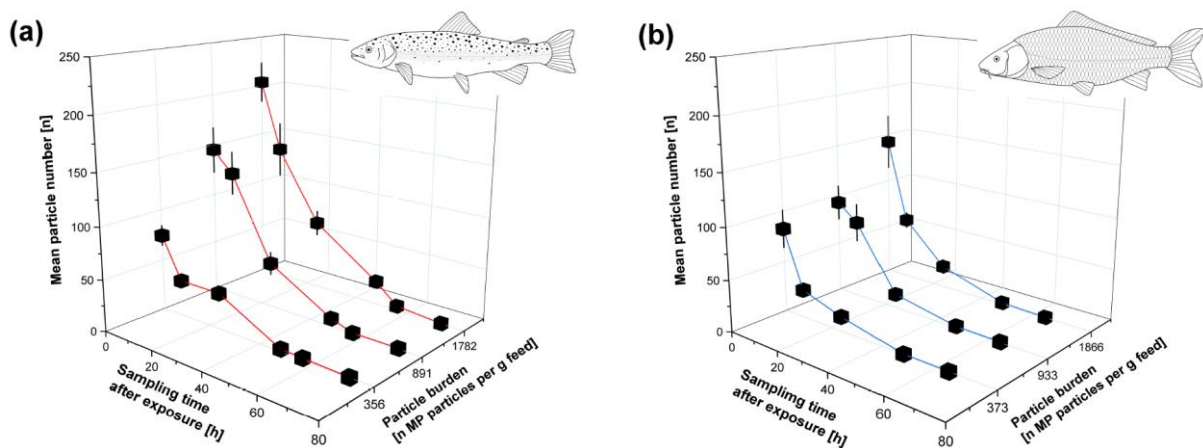


Figure 5.2. Mean microplastic particle numbers ($\pm$ standard error) in the experimental fish in relation to time after exposure and particle concentration in the diet (particle burden). **(a)** Mean particle numbers in the stomach of rainbow trout (*Oncorhynchus mykiss*). **(b)** Mean particle numbers in the GI tract of common carp (*Cyprinus carpio*).

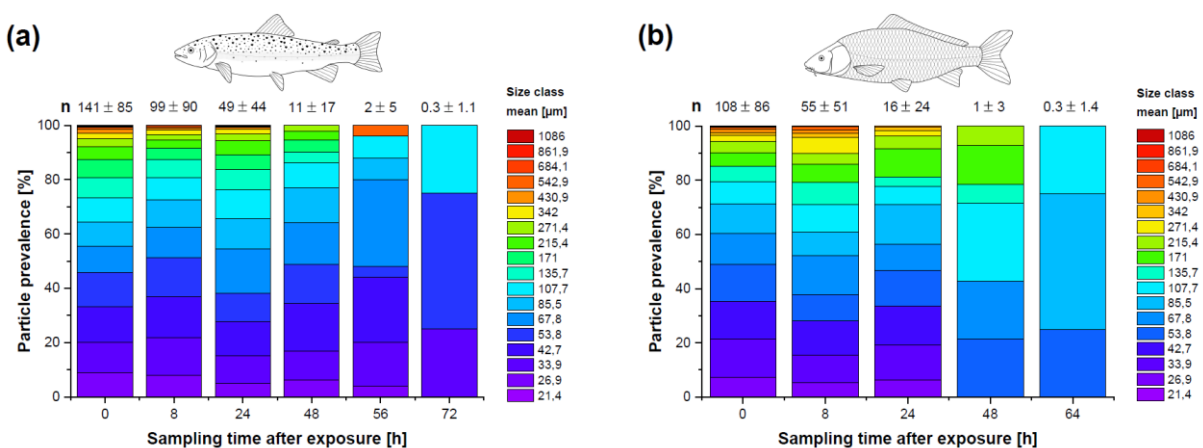


Figure 5.3. Mean microplastic particle prevalence per sampling time after exposure and size class. **(a)** Mean particle prevalence in the stomach of rainbow trout (*Oncorhynchus mykiss*). **(b)** Mean Particle prevalence in the gastrointestinal tract of common carp (*Cyprinus carpio*).

The results of the zero-inflated model based on the negative binomial distribution for rainbow trout (whole model: 2,625 observations, $df = 8$, $r^2 = 0.884$, $p < 0.0001$) revealed that particle size (estimate = -2.6234 , $p < 0.0001$), time after exposure (estimate = -1.4470 , $p < 0.0001$) and the interaction of particle size and time (estimate = -1.4375 , $p < 0.0001$) had a significant effect on particle abundance in the stomach. For common carp (whole model: 2,625 observations, $df = 8$, $r^2 = 0.813$, $p < 0.001$), the results were comparable (particle size: estimate = -2.9372 , $p < 0.001$; sampling time: estimate = -2.6693 , $p < 0.001$) with the exception that the interaction between the two factors was not statistically significant (estimate = -0.7150 , $p > 0.05$). [Figure 5.4](#) shows a graphical representation of the model based on predicted responses, with abundance expressed as a relative value in comparison to the maximum abundance of the MP particles at the start of the experiment.

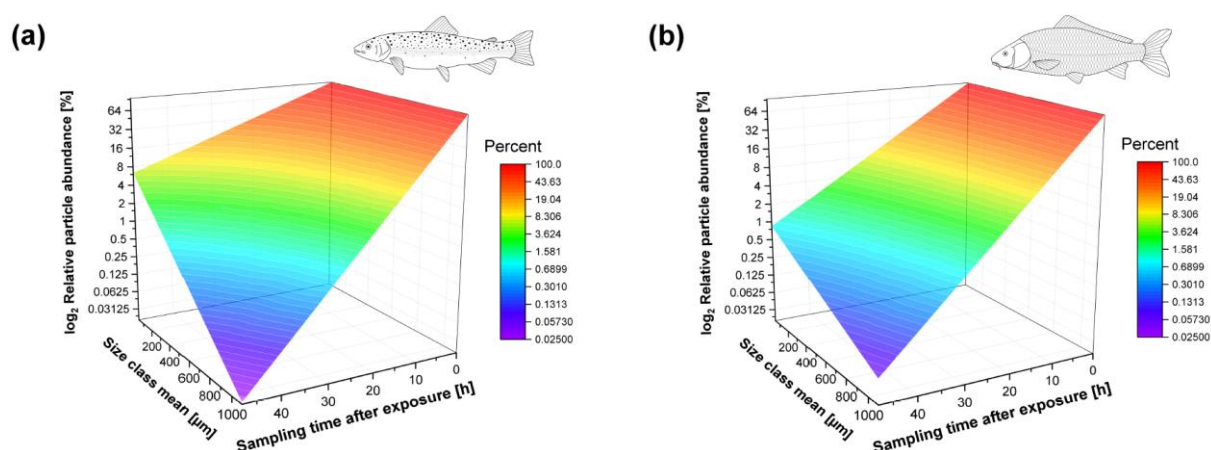


Figure 5.4. Relative particle abundance (a relative value in comparison to the maximum abundance of the MP particles at the start of the experiment) of MP particles based on the predicted responses of the negative binomial model by size class and time after exposure in **(a)** the stomach of rainbow trout (*Oncorhynchus mykiss*), and **(b)** the gastrointestinal tract of common carp (*Cyprinus carpio*).

The predicted responses were also used to calculate the T_{50} value of MP particles by particle size for both species ([Fig. 5.5](#)). Distinct differences in particle T_{50} values were found between the two fish species. In rainbow trout, values ranged from 12.1 hr (42.7 μm) to 4.0 hr (1,086 μm). In contrast, values in common carp ranged from 7.3 hr (42.7 μm) to 4.6 hr (1,086 μm). These values were within the range of previously published food evacuation times ([Table S5.4](#) and [S5.5](#)). To obtain a formula allowing the calculation T_{50} values, a size-independent mean value was assigned using Equation (2) to each particle fraction enclosed by the sizes Ps_l (lower size boundary) and Ps_u (upper size boundary; for more details, see Supplemental Information):

$$T_{50} = \frac{\ln\left(\frac{a+b \cdot P_{S1} \cdot P_{Su}}{a+b \cdot P_{Su} \cdot P_{S1}}\right)}{a \cdot \ln\left(\frac{P_{Su}}{P_{S1}}\right)}. \quad (3)$$

The constants a and b were determined for both species by fitting Equation S2 (see Supplemental Information) to the data given in Figure 5.5: $a = 0.07587$ 1/hr and $b = 0.00016$ 1/($\mu\text{m} \cdot \text{hr}$) for rainbow trout, $a = 0.133280$ 1/hr and $b = 0.000077$ 1/($\mu\text{m} \cdot \text{hr}$) for common carp. With the help of Equation (3), mean retention times (including all particles sizes: $P_{S1} = 42.7 \mu\text{m}$, $P_{Su} = 1,086 \mu\text{m}$) of 8.7 hr for rainbow trout and 6.4 hr for common carp were identified.

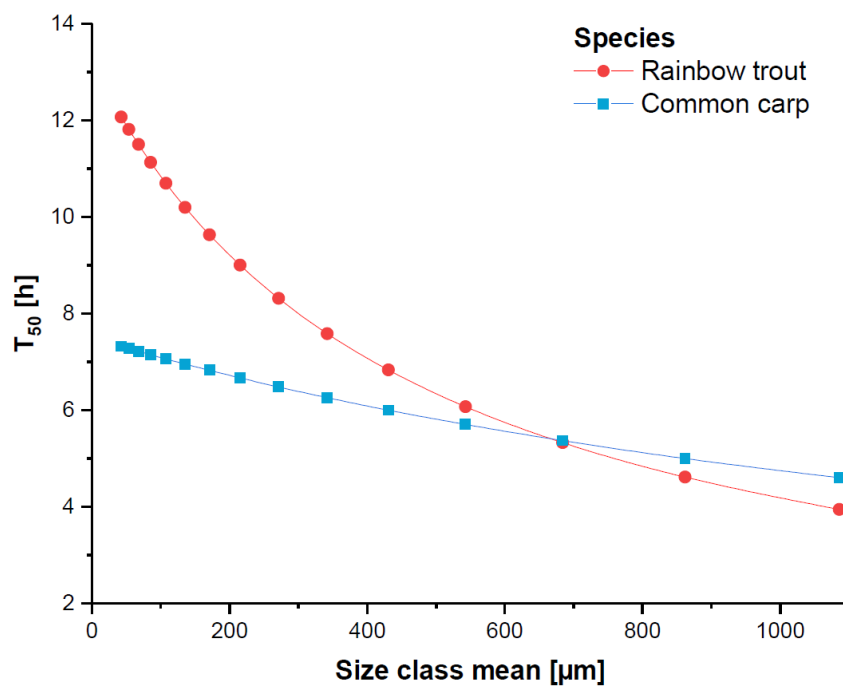


Figure 5.5. Modelled T50 value (time for 50 % of MP particles to be evacuated) by particle size in the stomach of rainbow trout (*Oncorhynchus mykiss*) and the gastrointestinal tract of common carp (*Cyprinus carpio*).

5.5. Discussion

The size-dependent retention time of indigestible particles swallowed by fish has gained new relevance in the light of the escalating plastic burden on the environment. Microplastics can be detected in freshwater and marine fish species worldwide (Barboza *et al.* 2018; Collard *et al.* 2019), but information regarding the behaviour of the particles, once ingested, remains scarce. Investigations with other aquatic organisms have already indicated that the size and shape of particles can have an influence on evacuation rate (Jeong *et al.* 2016; Rist *et al.* 2017). Similar results have been suggested for fish (Grigorakis *et al.* 2017; Hoang & Felix-Kim 2020), but so far only individual species or MP sizes have been investigated and results were inconclusive.

The present results show a strong negative correlation between particle size and retention time in rainbow trout, a species with a stomach. In stomachless common carp, by contrast, while a similar trend is visible, the magnitude is marginal. Although the mean T_{50} values did not differ strongly (9 hr for rainbow trout vs. 6 hr for common carp), a comparison of T_{50} values for the smallest (42.7 μm) and largest (1,086 μm) size class revealed a difference exceeding 8 hr for rainbow trout, but less than 3 hr for common carp. This difference is most likely to be due to the fundamental differences in GI tract structure of the two species. For fish with a true stomach, transit time is largely dependent on the time the food remains in the stomach itself (German 2011). In herbivorous fish, food transit time can vary and also depends on whether hindgut fermentation is involved (Clements *et al.* 2009). The mechanisms causing size-selective evacuation of indigestible particles from the stomach are not yet clear.

According to the Stokes–Einstein equation, small spherical particles diffuse more quickly through a liquid than large ones (Miller 1924). For food particles in the stomach of fish, a prolonged retention time with increasing particle size was likewise found (He & Wurtsbaugh 1993; Sveier *et al.* 1999). This trend was attributed to slower mechanical and chemical breakdown associated with the lower surface-to-volume ratio of larger particles (Jobling 1987; Sveier *et al.* 1999). Reported T_{50} values for food particles in common carp and other closely related stomachless fish species average around 6 hr, under similar experimental conditions (*e.g.* Opuszynski & Shireman 1991). These values coincide with the T_{50} value for the MP particles investigated in the present study, implying an exclusively passive excretion of these particles from the GI, and no accumulation, independent of particle size. Similar correlations with T_{50} values for food were also found in the rainbow trout (*e.g.* Windell *et al.* 1976), but only for the smallest particle sizes. Larger particles, however, seem to be actively evacuated more quickly in fish possessing a stomach. In mammals, it has been shown that the stomach has an ability to discriminate solid food by size and therefore to only release small particles (Becker & Kelly 1983). Similar size-dependent separating mechanisms appear to exist in fish to some extent, but the exact mechanisms of action are unknown (German 2011). In a study by dos Santos & Jobling (1991), 5-mm plastic beads were retained in the stomach of cod (*Gadus morhua*) significantly longer than 2-mm beads but in this case, the difference was attributed mainly to the maximum size of particles that could pass through the pylorus. Further studies are required to examine the size-sorting capabilities of the pyloric region in various fish species with stomachs. Future work should also investigate the possibility that

properties other than particle size (*e.g.* density, shape) might have an influence on the retention time (Grigorakis *et al.* 2017; dos Santos & Jobling 1991).

Generally, the results of the present study mostly confirm the scarce previous data for MP retention. In fathead minnow larvae (*Pimephales promelas*) exposed to MP beads 125–150 µm in diameter, all ingested beads were evacuated within 12 hr, while beads with measuring 63–75 µm were still present in the GI tract after 24 hr (Hoang & Felix-Kim 2020). Based on the present results, one would expect that for a species without a stomach, the differences in retention time between those size classes would not be so great, but comparability is severely limited because the larvae used by Hoang & Felix-Kim (2020) were only 2 days old and exhibited significant differences in the length and developmental stage of the intestine (Rønnestad *et al.* 2013). In a study with goldfish (*Carassius auratus*) the T₅₀ value of MP particles in the GI tract was found to be 10 hr (Grigorakis *et al.* 2017). This is comparable to the results in the present study for the closely related common carp, when exposed to particles of 50 µm.

The time taken for complete elimination of MP particles appears to vary, according to previously published reports. Mazurais *et al.* (2015) documented complete excretion of small microbeads (10–45 µm) from sea bass larvae (*Dicentrarchus labrax*) after 48 hr, but further studies reported rapid excretion within the first 24 hr, but found some particles were still present up to 7 days after exposure (Cong *et al.* 2019; Grigorakis *et al.* 2017). In the present study, 99% of all particles were evacuated after 72 hr in rainbow trout and 64 hr in common carp. Furthermore, the particle concentration did not appear to influence the retention time. These results support the hypothesis that the majority of MP particles are excreted with the chyme, but also show that individual particles are retained in the GI tract for longer periods, presumably with remaining food residues. The particles that remained longest in the intestine in the current study were among the size classes with the highest abundance. This was likely to increase the probability that single particles of those size classes were retained in the fish longer. It should be noted that the overall retention time for rainbow trout in the present study might vary from that reported, as only the stomach was investigated. When considering the entire GI tract, the complete elimination of the particles could be prolonged because the particles have to pass through the intestine. However, the time differences are estimated to be small, since the transit time of food through the body is largely dependent on the time it spends in the stomach itself (German 2011). Furthermore, it may be that individual differences between fish (*e.g.* length of an initial lag phase until the stomach begins to empty) and husbandry (*e.g.*

temperature, ration size, food quality) have an influence on the retention time of chyme (Guillaume *et al.* 1999; Jobling 1987) and thereby on the evacuation of MP particles.

The size-dependent retention time of MP particles has particular relevance in the contexts of translocation into tissues and organs, particle transfer through the food chain and the release of toxic chemicals (Collard *et al.* 2019). While MP transmission via the food chain has been repeatedly demonstrated in laboratory experiments Santana *et al.* (2017), almost no studies have attempted to show it happening in the field (Provencher *et al.* 2019). The model calculations by Diepens & Koelmans (2018) were unable to establish any biomagnification for MP particles along the food chain. The study considered nine different species but did not take particles size or related retention times into account. After investigating a large number of freshwater fish species from different trophic levels, Roch *et al.* (2019) came to the conclusion that MP exposure was significantly lower in piscivorous fish, indicating evacuation rates exceeding uptake of MP even at the top of the food chain. Since the present study also failed to find any evidence of accumulation, it can be assumed that the MP particle retention times in fish are too short to facilitate bioaccumulation along the food chain. However, it must also be taken into account that the microplastic burden in fish to date appears to have been severely underestimated (Roch *et al.* 2019), due mainly to the lack of suitable methods for reliably detecting MP particles below a certain size (Lusher *et al.* 2017). Furthermore, the assumption holds only for particles not passing into body tissues of the fish. A more reliable assessment of MP particle transfer along the food chain can only be made when detection methods have improved and factors such as the size-dependent retention time are taken into account.

Besides the potential for physical damage, other well-known detrimental impacts of MPs include the transfer to aquatic organisms of toxic chemicals, such as hydrophobic organic contaminants and plastic additives (Koelmans *et al.* 2013; Teuten *et al.* 2009). While the probability of adsorption of harmful substances increases with smaller size due to the exponential increase of surface area to volume ratio (Lee *et al.* 2014), current knowledge suggests that the overall influence of MP particles on aquatic organisms' exposure to such substances is marginal. Koelmans *et al.* (2014) used a biodynamic model to estimate the contribution of MP particles to the total exposure of cod (*Gadus morhua*) to plastic additives. Despite an assumption of long retention time (7 days) for MP particles in the GI tract, the contribution was found to be negligible, compared to other sources. Other studies have supported these results for hydrophobic organic

contaminants and even suggested that MP particles in the stomach can act as passive samplers for toxic chemicals (Beckingham & Ghosh 2017; Herzke *et al.* 2016; Koelmans *et al.* 2016).

Finally, several studies in recent years have documented the translocation of MPs through the intestinal wall (Collard *et al.* 2018; Lu *et al.* 2016; Zeytin *et al.* 2020). While it has not yet been established which particle sizes are able to migrate and which tissues and organs are exposed in this way, it stands that an extended residence time will increase the probability of such translocations. While some laboratory studies suggest that only particles smaller than 5 µm can translocate into organs like the liver (Lu *et al.* 2016; Zeytin *et al.* 2020), other authors report that larger particles can reach different organs including muscle tissues and even the brain (Avio *et al.* 2015; Collard *et al.* 2017; Collard *et al.* 2018). While one must be careful when interpreting these results due to limited transferability, the possibility of contamination and the overall low translocation rates (Cunningham *et al.* 2020; O'Connor *et al.* 2020), these studies nevertheless emphasise that particle size is highly pertinent to the potential impacts of MPs on aquatic organisms. Information on the size distribution of MPs in the environment is still insufficient, as reliable methods for the non-destructive isolation of very small particles and suitable detection techniques are still lacking (Roch *et al.* 2019). For this reason, there is an urgent need to consider particle size in future studies and to focus on small particles, including nanoplastics.

5.6. Acknowledgements

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5.7. Supplemental information

Model to test the effect of particle burden on microplastic (MP) retention time in the stomach of rainbow trout (*Oncorhynchus mykiss*) and the gastrointestinal tract of common carp (*Cyprinus carpio*).

MP particle abundance was expressed as a relative value for each size class in comparison to the overall abundance of MP particles in the fish. The relative values were expressed as a binomial output (for each individual: number of particle of a particular size class (Mps) / number of all particles (MP)) and were analyzed using a generalized linear mixed model with a binomial error distribution and probit link. Individuals (ind) and fish tanks (tank) were added as random factors. Particle size class mean (Ps), sampling time after exposure (T) and particle burden (Pb) were added as independent predictor variables. The models were specified as follows: glmmTMB ($y \sim P_s * T + P_b + (1 | ind) + (1 | tank)$), data = rainbow trout and common carp, family = binomial (link = probit), where $y = (Mps, Mp)$.

The results of the model indicated no statistically significant effect of particle burden on retention time for both rainbow trout (whole model: 2625 observations, d.f. = 8, $r_m^2 = 0.675$; Tab. S5.1) and common carp (whole model: 2625 observations, d.f. = 8, $r_m^2 = 0.591$; Tab. S5.2).

Table S5.1. Results of the model to test the effect of particle burden on MP retention time in rainbow trout (*Oncorhynchus mykiss*).

	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	-0.935229	0.015439	-60.58	< 0.0001*
size class	-1.224675	0.024909	-49.17	< 0.0001*
time	0.165556	0.020634	8.02	< 0.0001*
burden	-0.003376	0.010917	-0.31	0.757
size class : burden	-0.608254	0.060679	-10.02	< 0.0001*

* = statistically significant effect ($\alpha = 0.05$)

Table S5.2. Results of the model to test the effect of particle burden on MP retention time in common carp (*Cyprinus carpio*).

	Estimate	Std. Error	z value	Pr (> z)
(Intercept)	-0.90235	0.01942	-46.46	<0.0001*
size class	-1.36346	0.03256	-41.87	<0.0001*
time	0.06407	0.03722	1.72	0.0852
burden	0.00471	0.01450	0.32	0.7452
size class : burden	-0.20603	0.10134	-2.03	< 0.05*

* = statistically significant effect ($\alpha = 0.05$)

Deterministic approach to calculate the size-independent T_{50} value (50 % of particles evacuated) of microplastic particles in the stomach of rainbow trout (*Oncorhynchus mykiss*) and the gastrointestinal tract of common carp (*Cyprinus carpio*).

The deterministic part of eq. (1) in the text can be represented as follows:

$$\log_2 N(T, P_s) = \log_2 N_0 - (a \cdot T + b \cdot P_s \cdot T), \quad (S1)$$

where $N(T, P_s)$ = Particle abundance of a given particle size, N_0 = Particle abundance at the start of the experiment, a and b = constants, T = sampling time after exposure (h), P_s = Particle size class mean (μm).

Due to the fact that $N(T, P_s)$ depends for a given T explicitly on P_s (but not on particle burden), equation (S1) has to be extended by a corresponding summand with a constant c yielding the following equation:

$$\log_2 N(T, x) = \log_2 N_0 - (a \cdot T + b \cdot x \cdot T + c \cdot x). \quad (S2)$$

This equation describes exactly the dependencies of relative particle abundance on the size class mean and sampling time after exposure shown in Fig. 5.4. In this picture, the starting conditions are $\log_2 N_0 - c \cdot x = 1 = N_0 x (= 100\%)$ for all particle size fractions. For each particle fraction, the corresponding equation meets the T_{50} conditions ($N(T_{50}, x) = N_0 x / 2$), when:

$$\log_2 N(T_{50}, P_s) = \log_2 N_0 x - T_{50} \cdot (a + b \cdot P_s) = \log_2 N_0 x - 1. \quad (S3)$$

This immediately results in eq. (2) given in the text.

Using the following definition of a size independent T_{50} value:

$$T_{50} = \frac{\int_{P_{s_l}}^{P_{s_u}} T_{50}(Ps) d \log Ps}{\int_{P_{s_l}}^{P_{s_u}} d \log Ps}, \quad (S4)$$

eq. (3) is obtained.

Table S5.3. Overview of age, length and weight parameters of rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*) used in the exposure experiments.

Fish species	Particle burden	Age	Mean total length ± SD ^a [cm]	Mean fresh weight ± SD ^a [g]
Rainbow trout	low	0+	11.6 ± 0.9	18.4 ± 4.3
	medium		11.6 ± 1.0	18.6 ± 4.8
	high		11.6 ± 1.0	18.2 ± 4.7
	overall mean:		11.6 ± 0.9	18.4 ± 4.6
Common carp	low	0+	8.4 ± 0.7	7.0 ± 2.2
	medium		8.6 ± 0.8	8.4 ± 2.1
	high		8.5 ± 1.0	8.3 ± 2.6
	overall mean:		8.5 ± 0.8	8.2 ± 2.3

^a SD = Standard deviation

Table S5.4. Overview of reported food evacuation times in different stomachless fish. T_{50} = time for 50 % of food to be evacuated. T_{90} = time for > 90 % of food to be evacuated and gastrointestinal tract is considered as empty.

Reference	Fish species	Temperature [°C]	Mean fish weight [g]	T_{50} [h]	T_{90} [h]
Schade & Kausch 1978	<i>Cyprinus carpio</i>	25	23	13	20
Grove & Crawford 1980	<i>Blennius pholis</i>	18	15	n.a.	26
Białokoz & Krzywosz 1981	<i>Hypophthalmichthys molitrix</i>	20	1049	n.a.	16
Persson 1982	<i>Rutilus rutilus</i>	20	93	2	7
Białokoz 1990	<i>Alburnus alburnus</i>	24	4.8	3	8
Opuszynski & Shireman 1991	<i>Aristichthys nobilis</i>	21	29	6	10
Mean ± standard deviation:				6 ± 5	15 ± 8

Table S5.5. Overview of reported food evacuation times in different fish species with a real stomach. T_{50} = time for 50 % of food to be evacuated. T_{90} = time for > 90 % of food to be evacuated and stomach is considered as empty. $\bar{x}$ = arithmetic mean.

Reference	Fish species	Temperature [°C]	Fish weight [g]	T_{50} [h]	T_{90} [h]
Windell <i>et al.</i> 1976	<i>Oncorhynchus mykiss</i>	10	$\bar{x}$ = 35	15	38
Grove <i>et al.</i> 1978	<i>Oncorhynchus mykiss</i>	8.5	60-80	n.a.	34
Fauconneau <i>et al.</i> 1983	<i>Oncorhynchus mykiss</i>	9	$\bar{x}$ = 80	n.a.	34
From and Rasmussen 1984	<i>Oncorhynchus mykiss</i>	10	$\bar{x}$ = 72	25	n.a.
He & Wurtsbaugh 1999	<i>Salmo trutta</i>	10	$\bar{x}$ = 1150	7	41
Olsson <i>et al.</i> 1999	<i>Oncorhynchus mykiss</i>	10	700-1600	22	n.a.
Storebakken <i>et al.</i> 1999	<i>Salmo salar</i>	9	150-200	18	27
Sveier <i>et al.</i> 1999	<i>Salmo salar</i>	7.1	$\bar{x}$ = 659	13	n.a.
Mean $\pm$ standard deviation:				17 $\pm$ 6	35 $\pm$ 5

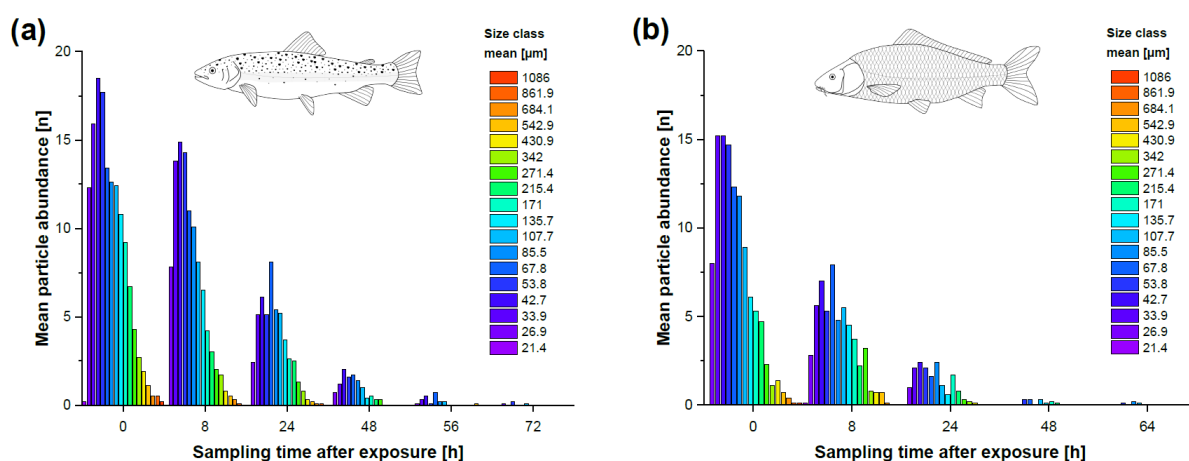


Fig. S5.1. Mean microplastic particle abundance per sampling time after exposure and size class. **(a)** Mean particle abundance in the stomach of rainbow trout (*Oncorhynchus mykiss*). **(b)** Mean Particle abundance in the gastrointestinal tract of common carp (*Cyprinus carpio*).

6. Manuscript V: Combined proteomic and gene expression analysis to investigate reduced performance in rainbow trout (*Oncorhynchus mykiss*) caused by environmentally relevant microplastic exposure

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This manuscript is currently being prepared for publication.

6.1. Abstract

The pollution of the environment with microplastics (MPs) is affecting aquatic organisms worldwide, and yet intensive research, has thus far failed to deliver adequate understanding of the detrimental effects MP ingestion by fish. Investigations using established health and performance parameters are often insufficient to determine MP toxicity, especially when considering MPs in environmentally relevant concentrations. In the present study, label-free quantitative proteomics (LFQ) of liver tissue was combined with gene expression analysis in order to investigate the long-term effects of MP exposure on rainbow trout (*Oncorhynchus mykiss*). With the help of a specially designed diet, two groups of fish were exposed for 120 days to environmentally relevant concentrations of MPs (around 14 particles per fish, every two days) and to slightly increased levels representing those expected in the near future (around 75 MP particles per fish, every two days). Both groups were compared to a control. The results provide evidence that long-term exposure to MPs has a dose-dependent negative effect on the performance of rainbow trout. No differences in blood glucose level, hematocrit level or lipid peroxidation were observed between treatments. The proteomic

analysis revealed 6071 unique proteins, but no significant change in hepatic protein concentrations compared to their matching controls, although certain proteins appear to have been up- or down-regulated multifold. When comparing highly regulated proteins with the levels of their respective mRNA transcripts, a good correlation was observed in just one – trout protein 1, encoded by *drtp1*. This may therefore be a suitable biomarker for future studies with trout. Several hypotheses were put forward to explain the observed differences in growth and compared with the results of the current study. Recommendations were made for future research into the detrimental effects of MP on fish. Direct evidence of a significant negative influence of long-term exposure to realistic and near-future MP concentrations on fish, highlight the importance of measures to prevent a further increase of MPs in the environment.

6.2. Introduction

Pollution with microplastics (MPs) are widely recognized as an emerging threat to the global environment (Rochman *et al.* 2013), with longevity in the environment and an exponential increase in plastic production over the last eight decades combining (Andrady 2011), resulting in a rapid accumulation of MPs in rivers, lakes and the ocean (Everaert *et al.* 2020; Li *et al.* 2018). The myriad pathways by which these pollutants enter the environment are incompletely understood but are known to include direct inputs via wastewater (Schmidt *et al.* 2020) and via the mechanical and chemical degradation of larger debris (Barnes *et al.* 2009; Lambert *et al.* 2014). MPs are usually defined as particles of plastic smaller than 5 mm (Hartmann *et al.* 2019), and are therefore small enough to be ingested by a wide range of aquatic organisms (Gall & Thompson 2015). Numerous studies have shown that MPs are taken up by a variety of fish species in both freshwater and marine systems (Parker *et al.* 2021; Hoss & Settle 1990) mostly by ingestion into the gastrointestinal tract. MPs are ingested either accidentally when foraging or indirectly with other food already containing plastic particles (Roch *et al.* 2020). In some cases, MPs are also mistaken for natural food (Ory *et al.* 2017). Research has addressed the detrimental effects of MPs on fish early in the history of MP pollution studies. However, the results obtained so far are often contradictory, with some studies failing to demonstrate any negative impact, while others highlight a variety of adverse health effects for fish (for a review, see: Foley *et al.* 2018; Parker *et al.* 2021).

A major issue when evaluating potential adverse health effects of MP in laboratory experiments is that the concentrations of MPs applied tend to be much higher than

those occurring routinely in the environment. Often the discrepancy is greater than two orders of magnitude, sometimes as many as seven, making it difficult to extrapolate the results and inferences of such studies to natural conditions are potentially leading to biased conclusions (Lenz *et al.* 2016; de Sá *et al.* 2018). The size of the particles also plays a decisive role. Very small particles, for example can pass through the intestinal wall, migrate into organs like the liver (Collard *et al.* 2017; De Sales-Ribeiro *et al.* 2020) and therefore cause a different pathology to larger particles restricted to the intestinal passage. These and other factors make it difficult to assess the actual harmfulness of microplastics for fish (Cunningham *et al.* 2020).

A variety of different approaches have been applied to study the detrimental effects of pollutants on aquatic organisms. Generic baseline parameters such as growth and mortality (Austin 1998), are often considered alongside other established stress parameters such as levels of cortisol or heat shock proteins or evidence for lipid oxidation (Iwama 2004; Vinagre *et al.* 2012). However conflicting findings to date suggest that these parameters might not be sensitive enough to indicate the physiological effects of MP concentrations currently found in the environment. For this reason, recent studies have begun to adopt more sophisticated molecular biological approaches, including omics techniques (Petitjean *et al.* 2019; López-Pedrouso *et al.* 2020). A striking advantage of mass spectroscopy when searching for a needle in a haystack is that the technique permits simultaneous identification of thousands of proteins in a sample, up to the analysis of the entire proteome (Tomanek 2011). The identities and abundances of the various proteins may then suggest pathways by which, for example, particular concentration differences, have come about, and may thus provide important insights into the effects of short- and long-term MP exposure.

To date, proteomic approaches have been used only sporadically when investigating the effects of MP exposure on aquatic organisms. A recently conducted proteomic analysis of blue mussels (*Mytilus edulis*) exposed to MPs for 52 days identified 40 differentially regulated proteins in the haemolymph compared to a control group (Green *et al.* 2019). These were involved in immune regulation, detoxification and other vital biological processes. Another proteomic study on zebra mussels (*Dreissena polymorpha*) suggested a threshold exposure level beyond which stimulation with MPs began to modulate the proteome in the short-term and furthermore pointed to high sensitivity of proteomic analyses (Magni *et al.* 2019). So far, this approach has not been used to address potential MP impacts in fish. However several studies have evaluated transcriptomic

alterations in aquatic organisms exposed to MP and produced greatly varying lists of differentially regulated genes (Assas *et al.* 2020; LeMoine *et al.* 2018; Veneman *et al.* 2017).

In the present study, a proteomic approach was trialed alongside established methods in an investigation of fish experiencing long-term exposure to environmentally relevant concentrations of MPs. Two groups of rainbow trout (*Oncorhynchus mykiss*), a widely used model organism for ecotoxicological studies (Thorgaard *et al.* 2002), were exposed to MP concentrations currently encountered by wild fish and an increased concentration, expected to occur in the near future (Everaert *et al.* 2020; Geyer *et al.* 2017). These groups were compared to a control group maintained in MP free conditions. The plastic was administered in a controlled manner using a specially designed diet. In addition to a selection of typical performance and stress parameters, the liver proteome was examined using a label-free quantification (LFQ) approach (Tate *et al.* 2013). Proteins which exhibit obvious shifts in regulation were selected, and gene expression analysis was used to confirm and extend the results at transcript level. Based on the results, different hypotheses on the impact of microplastics on fish were developed and possible biomarkers for future studies were considered.

6.3. Methods/Experimental

6.3.1. Husbandry and experimental setup

Juvenile rainbow trout were acclimatized for three weeks in nine tanks (100 fish per tank, tank size: 50 × 55 × 55 cm) and fed twice a day with a commercial dry feed (Inicio, BioMar, Brande, Denmark) and an experimental diet (Gesellschaft für Marine Aquakultur mbH, Büsum, Germany; for more details, see: Roch *et al.* 2021). All fish were held in a flow-through system (8 L/min water exchange rate) with an average water temperature of 8 °C and oxygen levels between 8 and 12 mg/L. Light intensity was around 300 lx, with 12 h illumination and 30 min transition periods simulating dawn and dusk. Water parameters were monitored continuously, and tanks were cleaned daily.

In the MP exposure experiment the fish were divided into three groups, of which two received the experimental diet modified to contain different concentrations of polymethylmethacrylate particles of (PMMA) (Glow-Side, Kretz, Germany; details on the concentrations, see below). PMMA is a widely used plastic with a variety of applications and is routinely present in the environment (Ballent *et al.* 2016; Mani

et al. 2015; Scudo *et al.* 2017). A third group served as a control and fed the experimental diet without MPs. All experiments were performed in triplicate, resulting in nine groups. Generally, the quantity of food supplied was equivalent to 1.5 % of fish body weight and was dispensed within the same hour each day, six days a week. After 10 weeks, the pellet size of the diet was changed from two to four mm and the food ration was increased to 1.8 % of fish body weight. The MP concentration was 2729 ± 1639 particles per gram diet for the two mm pellets and 3526 ± 966 particles per gram diet for the four mm pellets. Particle sizes ranged from 27 to 778 μm (mean $\pm$ standard deviation (SD) = $149 \pm 128 \mu\text{m}$). The MP dose delivered to the low exposure group was 19 mg MP diet per fish, while the high exposure group received feed dosed with 85 mg MP per fish respectively. The portion of the experimental diet containing MP particles (hereafter: MP diet) was thus modified after each sampling event dependent in order to maintain the same dose. The MP diet was provided separately before the untreated diet every 48 h to ensure an even uptake of the MP and to allow the particles to pass the gastrointestinal (GI) tract prior to the next exposure (Roch *et al.* 2021).

6.3.2. Sampling and tissue preparation

The experimental fish were treated for 17 weeks and sampled on a regular basis. At the start and the end of the experiment, all individuals were weighed to the nearest 0.1 g and total length was measured to the nearest 0.1 cm. During the exposure experiment, sampling took place on three consecutive days, with three tanks being sampled per day. An overview over the sampling time points is given in [Figure 6.1](#). Five individuals per time point and treatment were anesthetized with clove oil (0.1 mL per L water, Euro OTC Pharma, Boenen, Germany), the wet weight and total length was measured and the caudal fin was ablated. A blood sample was taken for further analysis (see below). Subsequently, the fish were euthanized with a cut at the gills and stored on ice until further examination.

To prepare tissue samples for further analysis, for each individual the liver and intestine was dissected and cleaned with 1x PBS (phosphate-buffered saline). The liver was also weighed to calculate the hepatosomatic index (see below). The tissues were then dried with a cloth and stored in 1.5 mL Eppendorf tubes at - 80 °C.

Additional sampling events took place after two and seven weeks of exposure, in order to compare MP concentrations in experimental fish fed the 2 mm MP diet and 4 mm diet respectively ([Fig. 6.1](#)). After anaesthetisation and euthanization,

the whole gastrointestinal tract was removed and MP concentration was determined according to Roch et al., 2021.

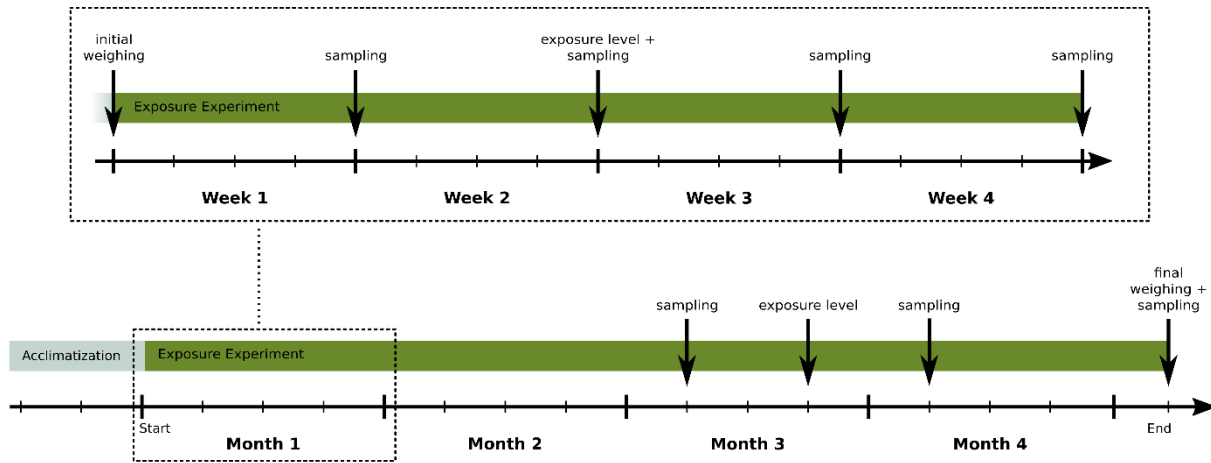


Figure 6.1. Overview of the sampling scheme in the exposure experiment (additional weighing of all experimental fish was conducted at the beginning and the end of the experiment).

All experiments were conducted according to the German Animal Welfare Act (TierSchG) and approved by Referat Tierschutz of Regierungspräsidium Tübingen (LAZ 2/16, AZ 35/9185.81-4).

6.3.3. Calculation of performance and health parameters

Specific growth rate (SGR, in % per day) for fish in each treatment was calculated using the mean weight of all fish at the start and end of the experiment according to the formula:

$$SGR = \frac{\ln(\text{mean initial weight [kg]}) - \ln(\text{mean final weight [kg]})}{\text{length of the experiment [days]}}. \quad (1)$$

The feed conversion rate (FCR) describes the amount of food needed in order to produce one kg of fish. It was calculated at the end of the experiment, using the formula:

$$FCR = \frac{\text{amount of feed [kg]}}{\text{weight gain [kg]}}. \quad (2)$$

The hepatosomatic index (HSI) was calculated for each sampling event, using the formula:

$$HSI = \frac{\text{liver weight [g]}}{\text{fresh weight [g]}}. \quad (3)$$

In order to determine haematocrit levels, two haematocrit capillaries (sodium heparinized; 75 mm; Hettich, Tuttlingen, Germany) were filled with blood, centrifuged for 10 min at 14 000 $\times g$ in a haematocrit centrifuge (HAEMATOCRIT 210, Hettich, Tuttlingen, Germany) and the mean haematocrit value of both capillaries was determined. Blood glucose concentrations were measured using a standard blood glucose meter (ACCU-Chek Aviva, Roche, Mannheim, Germany).

A TBARS (thiobarbituric acid reactive substances) assay kit (TCA method; Cayman Chemical, Ann Arbor, USA) was used to test for increases in lipid peroxidation (LPO) resulting from oxidative stress (Valavanidis *et al.* 2006). The assay detects malondialdehyde (MDA), which is generated by membrane oxidation and damage (Vinagre *et al.* 2012), and was performed according to the manufacturer instructions. Briefly, 100 mg of liver tissue was blended using a tissue homogenizer (Bead Ruptor 4, OMNI International, Kennesaw, USA). The homogenized sample was centrifuged at 1600 $\times g$ for 10 min and the supernatant mixed with thiobarbituric acid (TBA). After boiling for one hour, the TBARS formation was quantified using a plate reader (BioTek ELx 800, BioTek Instruments GmbH, Germany) at a wavelength of 540 nm.

6.3.4. Protein extraction and digestion using pressure cycling technology (PCT)

The samples were prepared by measuring 0.2 to 1.1 mg of tissue into a 150 μL FEP MicroTube (Pressure BioSciences Inc., South Easton, USA) with 30 μL of freshly prepared lysis buffer (8 M Urea (Sigma-Aldrich, St. Louis, USA). 0.1 M ammonium bicarbonate (Sigma-Aldrich, St. Louis, USA), 1x complete protease Inhibitor (Roche, Basel, Switzerland) were then added and the tubes sealed using a PTFE MicroCap (50 μL , PBI). Tubes were placed in the Barocycler (NEP2320; Pressure BioSciences Inc., South Easton, USA) and pressure alternated for 60 cycles according to the following plan: high pressure (45 000 psi) for 50 s followed by ambient pressure for 10 s. The temperature of the reaction chamber was maintained at a steady 33 °C using a circulating water bath. The MicroTubes were sonicated for 20 s in a water bath sonicator (VWR, Radnor, USA). The MicroCap was removed and Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma-Aldrich, St. Louis, USA) and iodoacetamide (IAA; Sigma-Aldrich, St. Louis, USA) were added to a final concentration of 10 mM and 40 mM, respectively. The solutions were mixed by pipetting up and down and the tubes were then sealed with the MicroCap and incubated for 30 min at 25 °C in a Thermo Shaker (Eppendorf, Hamburg, Germany) at 600 rpm. The samples were diluted to 6 M

Urea with 0.1 M ammonium bicarbonate and Lys-C (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany) was added in an enzyme to protein ratio of 1:40 w/w. The tubes were placed in the Barocycler and pressure in the tube was alternated for 45 cycles of high pressure (20 000 psi) for 50 s followed by ambient pressure for 10 s, again at 33 °C. The samples were diluted to 1.6 M Urea with 0.1 M ammonium bicarbonate and Trypsin (Promega) in an enzyme to protein ratio of 1:50 w/w. The tubes were sealed using a 150 µL MicroCap and placed in the Barocycler. Pressure in the tube was alternated for 90 cycles of high pressure (20,000 si) for 50 s followed by ambient pressure for 10 s, at 33 °C. The samples were transferred to a fresh 1.5 mL Eppendorf tube and acidified with trifluoroacetic acid (TFA; Honeywell, Charlotte, USA) to a final concentration of 0.5 %. Peptides were cleaned with C18 Stagetiip (selfmade). The samples were dried and re-solubilized in 3 % acetonitrile (ACN; Merck, Darmstadt, Germany) and 0.1 % formic acid (FA; Sigma-Aldrich, St. Louis, USA) for MS analysis and the peptide concentration was normalized by with Nanodrop (DS-F11 FX+; DeNovix, Wilmington, USA; Abs at 280 nm).

6.3.5. Liquid chromatography-mass spectrometry analysis

Mass spectrometry analysis was performed on a Q Exactive HF-X mass spectrometer (Thermo Scientific, Waltham, USA) equipped with a Digital PicoView source (New Objective, Littleton, USA) and coupled to a M-Class UPLC (Waters, Milford, USA). Solvent compositions in the two channels were 0.1 % formic acid for channel A and 0.1 % formic acid, 99.9 % acetonitrile for channel B. For each sample, 3 µL of peptides were loaded on a commercial MZ Symmetry C18 Trap Column (100 Å, 5 µm, 180 µm x 20 mm; Waters, Milford, USA) followed by nanoEase MZ C18 HSS T3 Column (100 Å, 1.8 µm, 75 µm x 250 mm, Waters, Milford, USA). The peptides were eluted at a flow rate of 300 nL/min by a gradient from 8 to 27 % B in 85 min, 35 % B in 5 min and 80 % B in 1 min. Samples were acquired in a randomized order. The mass spectrometer was operated in data-dependent mode (DDA), acquiring a full-scan MS spectrum (350–1,400 m/z) at a resolution of 120 000 at 200 m/z, after accumulation to a target value of 3 000 000, followed by HCD (higher-energy collision dissociation) fragmentation on the twenty most intense signals per cycle. HCD spectra were acquired at a resolution of 15 000 using a normalized collision energy of 25 and a maximum injection time of 22 ms. The automatic gain control (AGC) was set to 100 000 ions. Charge state screening was enabled. Single, unassigned, and charge states higher than seven were rejected. Only precursors with intensity above 250 000 were selected for

MS/MS. Precursor masses previously selected for MS/MS measurement were excluded from further selection for 30 s, and the exclusion window was set at 10 ppm. The samples were acquired using internal lock mass calibration on m/z 371.1012 and 445.1200.

The mass spectrometry proteomics data were handled using the local laboratory information management system (LIMS; Türker *et al.* 2010) and all relevant data will be deposited to the ProteomeXchange Consortium via the PRIDE (<http://www.ebi.ac.uk/pride>) partner repository.

6.3.6. Protein identification and label free protein quantification

The acquired raw MS data were processed using MaxQuant (version 1.6.2.3), followed by protein identification using the integrated Andromeda search engine (Cox & Mann 2008). Spectra were searched against a Uniprot *Oncorhynchus mykiss* reference proteome (taxonomy 8022, version from 2018-09-03), concatenated to its reversed decoyed fasta database and common protein contaminants. Carbamidomethylation of cysteine was set as a fixed modification, while methionine oxidation and N-terminal protein acetylation were set as variables. Enzyme specificity was set to trypsin/P allowing a minimal peptide length of 7 amino acids and a maximum of two missed-cleavages. MaxQuant Orbitrap default search settings were used. The maximum false discovery rate (FDR) was set to 0.01 for peptides and 0.05 for proteins. Label free quantification was enabled and a 2 min window for matches between runs was applied. In the MaxQuant experimental design template, each file is kept separate in the experimental design to obtain individual quantitative values.

6.3.7. Gene expression analysis

Based on the proteomics analysis, proteins exhibiting substantial up- or downregulation (> 10) were selected to validate their different expressions at the transcript level. Additionally, four genes involved in general immune and stress responses were analysed: complement component C3 (isoform 3; *c3-3*); immunoglobulin mu (heavy chain, membrane-bound form; *ighm*); interleukin-1 β (*il1b*); and hepcidin antimicrobial peptides (*hamp*). Finally, the gene 60S ribosomal protein L7 (*rpl7*) was added to the gene expression analysis as an additional reference gene, which was also upregulated in the proteomic analysis. The primer design was performed using the Pyrosequencing Assay Design software v.1.0.6 (Biotage, Uppsala, Sweden), subject to the condition that either the sense or

antisense primer was placed on an exon–exon boundary. The oligonucleotide primers listed in [Table S6.1](#) were tested via standard PCR and the resulting PCR products were sequenced. The quality of the PCR products was assessed by gel electrophoresis and melting-curve analysis.

RNA was isolated from the liver samples of treated and control rainbow trout ($n = 4$ per treatment and time point) in separate tubes using TRIzol (Thermo Fisher Scientific, Bremen, Germany) and subsequently purified with the ISOLATE II RNA Micro Kit (Bioline/Meridian Bioscience, Luckenwalde, Germany). RNA concentration and quality was assessed using the NanoDrop 1000 spectrophotometer (NanoDrop Technologies/Thermo Fisher Scientific, Wilmington, USA). RNA specimens from week 4 (low exposure) did not meet the criteria and were excluded from further investigations. For cDNA synthesis, 1 μg of RNA was reverse-transcribed in a total volume of 150 μL using the SensiFAS cDNA Synthesis Kit (Bioline/Meridian Bioscience, Memphis, USA).

Quantitative real-time expression (qPCR) analysis was performed using the LightCycler 96 System (Roche, Basel, Switzerland). The LightCycler 96 protocol was optimized for a 12- μL -reaction volume using 6 μL SensiFAST SYBR No-ROX Mix (Bioline/Meridian Bioscience, Memphis, USA), 1 μL primers and a total cDNA equivalent of 50 ng RNA. The qPCR program included an initial denaturation (95 °C, 5 min.), followed by 40 cycles of denaturation (95 °C, 5 min.), annealing (60 °C, 15 s) and elongation (72 °C, 15 s) steps and fluorescence measurement (72 °C, 10 s).

6.3.8. Statistical analysis

A series of linear models were utilized to analyse MP concentrations in fish stomachs, as well as performance and health parameters obtained throughout the exposure experiment. The models were generally constructed as follows:

$$y_i \sim \beta_0 + \beta_1 x_1 + \dots + \beta_i x_i + \varepsilon_i, \quad (4)$$

where y_i is the dependent variable, β_0 is the intercept, β_i is the regression coefficient, x_i is the predictor variables, and ε_i is the random residual error. Further details of the models used and associated model effects are summarized in [Table S6.2](#). Data for fish weights at the start and end of the exposure experiment, FCR, SGR and mortality for each tank were tested for differences between treatments using a contrast test (Abdi & Williams 2010).

In order to analyse the results of the LFQ, protein fold changes were computed based on the peptide intensity values reported by MaxQuant, using linear mixed-effects models (Bates *et al.* 2015). The reported peptide intensities were pre-processed as follows: all proteins with only one identified peptide and intensities equal to zero were removed. Non-zero intensities were $\log_2$ transformed and modified using robust z-score transformation (using the median and median average deviation). For each protein, a linear mixed-effects model was fitted to the peptide intensities:

$$medpolish \sim Sampling\ time * Exposure\ level \quad (5)$$

Fold changes and p-values for each contrast were computed using the R package lmerTest (Kuznetsova *et al.* 2017) and filtered for Benjamini-Hochberg false discovery rate (FDR; Benjamini & Hochberg 1995) to adjust p-values (hereafter referred to as q-value). An overview of all computed contrasts is provided in [Table S6.3](#).

To estimate fold-changes for proteins to which a mixed-effects model could not have been fitted because of an excess in missing measurements, the mean intensity of all peptides for each condition was computed first. For proteins with no measurements in that condition, peptide intensities were imputed using the mean of the 10 % smallest average peptide intensities calculated in step one. Afterward, the contrasts (differences between conditions) for each peptide were computed. Finally, the median of the peptide estimates was used to provide a per protein fold change estimate (pseudo estimate). Only proteins with fold-change values $> |2|$ were considered “differentially expressed”. These proteins (with unique UniprotIDs) were re-annotated using the Basic Local Alignment Search Tool for amino-acid sequences (BLASTP) tolerating only $> 80\%$ sequence coverage and identity.

QPCR data for the target genes was analysed using the LightCycler 96 analysis software v.1.1 (Roche, Basel, Switzerland) and normalized against the geometric mean of the *ef1a1* and *rps5* transcript values (Bowers *et al.* 2008; Köbis *et al.* 2017). One sample (week 1, low exposure) was excluded from any further statistical analysis, as the normalization factor (0.120) deviated extremely from the expected value of 1.0. A linear model was then utilized to investigate factors influencing RNA concentrations in experimental fish ([Tab. S6.2](#)). If statistically significant differences between treatments were detected, a Dunnett’s test (Dunnett 1955) was performed to compare the mean of the low and high exposure groups against the mean of the control group. Finally, a regression analysis (Sokal & Rohlf 2003)

was conducted in order to evaluate the correlation between differential protein concentrations and gene expression.

6.4. Results

6.4.1. Microplastic exposure level

The number of particles in the gastrointestinal tract of the experimental fish, and thus the average exposure level for each treatment, is summarized in [Table 6.1](#). The linear model (whole model: 90 observations, $df = 5, 84$, $r^2_{\text{adjusted}} = 0.5112$, $p < 0.0001$) revealed statistically significant differences between treatments ($F = 54.863$, $p < 0.0001$). However, differences between food pellet sizes were not apparent ($F = 0.0$, $p > 0.05$) and there was no significant influence of the interaction between exposure level and pellet size ($F = 2.0846$, $p > 0.05$).

Table 6.1. Mean microplastic concentration in the experimental fish for each treatment. n = number of examined fish. SD = Standard deviation.

Food pellet size	Treatment	n	Particle concentration $\pm$ SD
2 mm	Control	15	0
	Low exposure	15	15 $\pm$ 16
	High exposure	15	61 $\pm$ 47
4 mm	Control	15	0
	Low exposure	15	12 $\pm$ 16
	High exposure	15	86 $\pm$ 47

6.4.2. General performance and health parameters

An overview of the relationship of total length to wet weight of all sampled fish shown in [Figure 6.2 \(A\)](#). When considering all fish at the start and the end of the experiments, it is revealed that starting weight did not differ statistically between the treatments (whole model: 900 observations, $df = 2, 897$, $r^2_{\text{adjusted}} = -0.00199$, $p > 0.05$; [Fig. 2 \(B\)](#)). Overall mean wet weight ($\pm$ SD) and total length ($\pm$ SD) were 21.1 ± 3.6 g and 12.6 ± 0.7 cm respectively. In contrast, for end weight, SGR and FCR, contrast tests revealed statistically significant dose dependent differences between treatments (end weight: $df = 1, 371$, $F = 8.2067$, $p = 0.0044$; SGR: $df = 1, 6$, $F = 9.7445$, $p = 0.0205$; FCR: $df = 1, 6$, $F = 8.8148$, $p = 0.0250$; [Fig. 6.2 \(C-E\)](#)). Mortality ranged from two to five individuals per tank during the course of the experiment but did not differ between treatment groups (whole model: 9 observations, $df = 2, 6$, $r^2_{\text{adjusted}} = -0.03704$, $p > 0.05$).

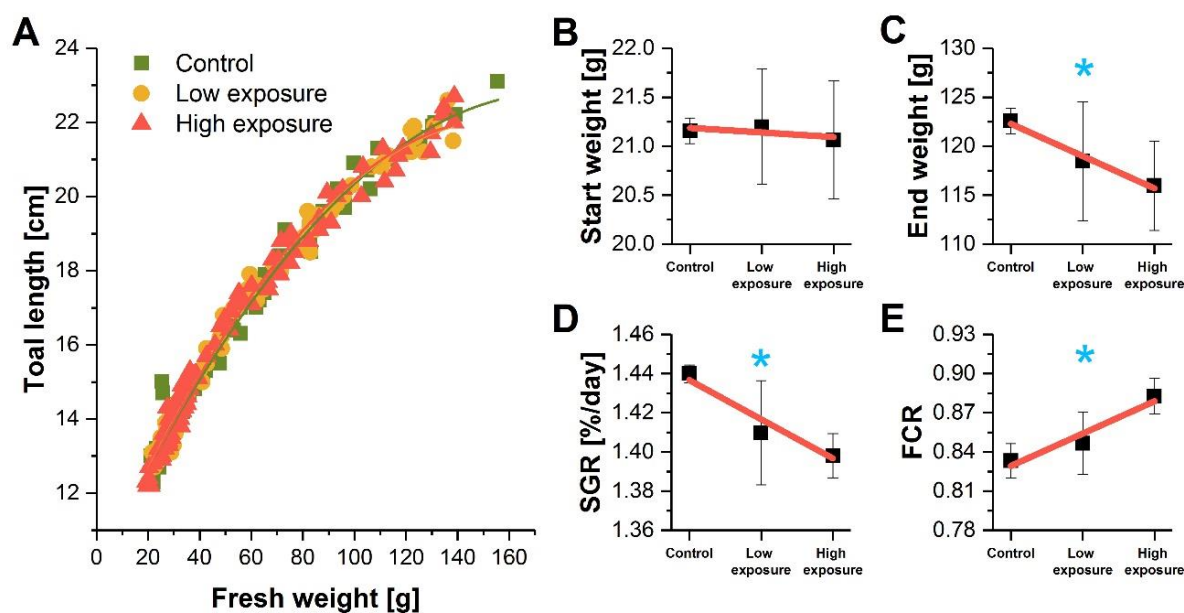


Figure 6.2. Growth and performance parameters of rainbow trout (*Oncorhynchus mykiss*) for each treatment in the exposure experiment. **(A)** Relationship between total length and wet weight. **(B)** Mean weight ($\pm$ standard deviation; SD) of fish at the beginning of the experiment. **(C)** Mean weight ($\pm$ SD) of fish at the end of the experiment. **(D)** Mean specific growth rate (SGR $\pm$ SD) of fish. **(E)** Mean feed conversion ratio (FCR $\pm$ SD) of fish. Asterisks indicate statistically significant dose dependent effect between treatments (contrast test, $p < 0.05$; for details please refer to the text).

For the blood glucose levels, the utilized linear model (whole model: 315 observations, $df = 6, 308$, $r^2_{\text{adjusted}} = 0.1033$, $p = 0.0201$; Fig. 6.3 (A)) revealed significant differences over the course of the experiment ($F = 4.2788$, $P = 0.0394$). However, there were no statistically significant differences between treatments ($F = 1.1565$, $P > 0.05$), for fish total length ($F = 0.6181$, $P > 0.05$) or the interaction of fish total length and treatment ($F = 2.1262$, $P > 0.05$). Haematocrit levels showed no statistically significant change over time, between treatments and for fish total length (whole model: 315 observations, $df = 6, 308$, $r^2_{\text{adjusted}} = -0.14043$, $p > 0.05$; Fig. 6.3 (B)). For the HSI (whole model: 315 observations, $df = 6, 308$, $r^2 = 0.3682$, $p < 0.0001$, statistically significant differences were revealed between fish of differing total length ($F = 8.3918$, $p = 0.0040$; Fig. 6.3 (C)), but not for any of the other variables (treatment: $F = 0.1808$, $p > 0.05$; sampling time: $F = 0.4921$, $p > 0.05$; fish total length x exposure level: $F = 0.9471$, $p > 0.05$). Analysis of liver MDA concentrations (Fig. 6.3 (D)) revealed statistically significant differences with sampling time ($F = 8.5565$, $p = 0.0041$) and total length of fish ($F = 4.5318$, $p = 0.0352$). However, model effects did not reveal a statistically significant influence on the overall response (whole model: 133 observations, $df = 4, 128$, $r^2_{\text{adjusted}} = 0.0945$, $p = 0.0914$). MDA concentrations in the intestine were strongly elevated during the first week compared to other sampling occasions (whole model: 133 observations, $df = 4, 128$, $r^2_{\text{adjusted}} = 0.2483$, $p = 0.0010$; Fig. 6.3 (E)). There

were statistically significant differences for the factors total length ($F = 4.5318$, $p = 0.0352$) and time ($F = 8.5565$, $p = 0.0041$), but not for treatments ($F = 0.2423$, $p > 0.05$).

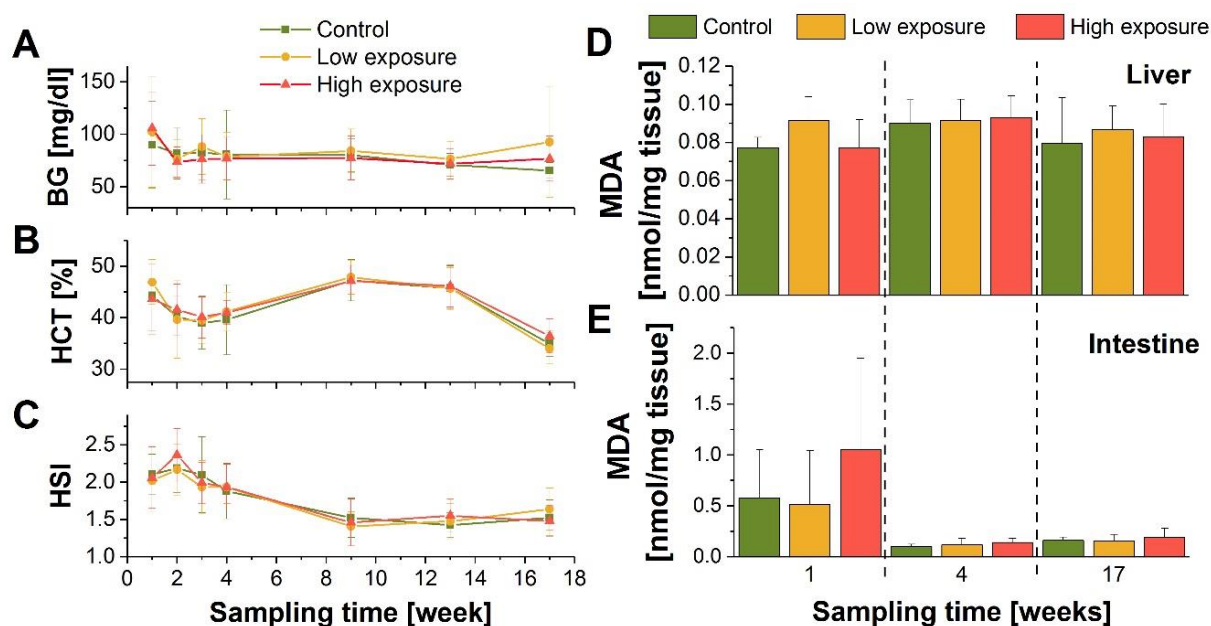


Figure 6.3. Health parameters of rainbow trout for each treatment in the exposure experiment. **(A)** Mean blood glucose concentration ($\pm$ SD) for each sampling. **(B)** Mean haematocrit levels ($\pm$ SD) for each sampling. **(C)** Mean hepatosomatic index ($\pm$ SD) for each time point. **(D)** and **(E)** Results of the TBARS Assay for three time points. The mean malondialdehyde (MDA) concentration ($\pm$ SD) in the liver and intestine is shown. For statistically significant differences, please refer to the text.

6.4.3. Proteomic analysis

In total, 6071 unique proteins were identified in the proteomic analysis. An overview of the LFQ results are presented as a heatmap based on protein intensity correlation among samples (Fig. 6.4 (A)). The number of statistically significant up- or downregulated proteins (q -value < 0.05) for each computed contrast is summarized in Figure 6.4 (B).

The pairwise comparison of the datasets from low or high MP exposure versus control groups revealed no proteins with a statistically significant regulation ($q < 0.05$) and a $\log_2$ fold change $> |2|$ respectively (Fig. 6.5(A)). In some cases, however, considerable differences in concentration were apparent (Fig. 6.5 (B)). For instance, in livers of rainbow trout exposed to the lower MP concentration for one week, the level of otopetrin-3 (*otop3*) was 44-fold higher ($\log_2$ fold change = 5.47, 95 % CI [3.06, 7.88]) than in control fish (Fig. 6.5 (B)). Concomitantly, the hepatic level of the bile salt export pump *abcb11* was 68-fold ($\log_2$ fold change = -6.09, 95 % CI [-7.19, -4.99]) reduced. After one week of exposure

to the higher MP concentration, levels of collagen (*colla1*) increased by 30-fold ($\log_2$ fold change = 4.90, 95 % CI [-3.08, 12.89]), while those of the *mhc2* antigen decreased by 13-fold ($\log_2$ fold change = -3.68, 95 % CI [-6.47, -0.89]) relative to the controls. Likewise, levels of other proteins were markedly altered after four and 17 weeks of exposure, when comparing both low and high MP treatments with the control. (Fig. 6.5 (B)).

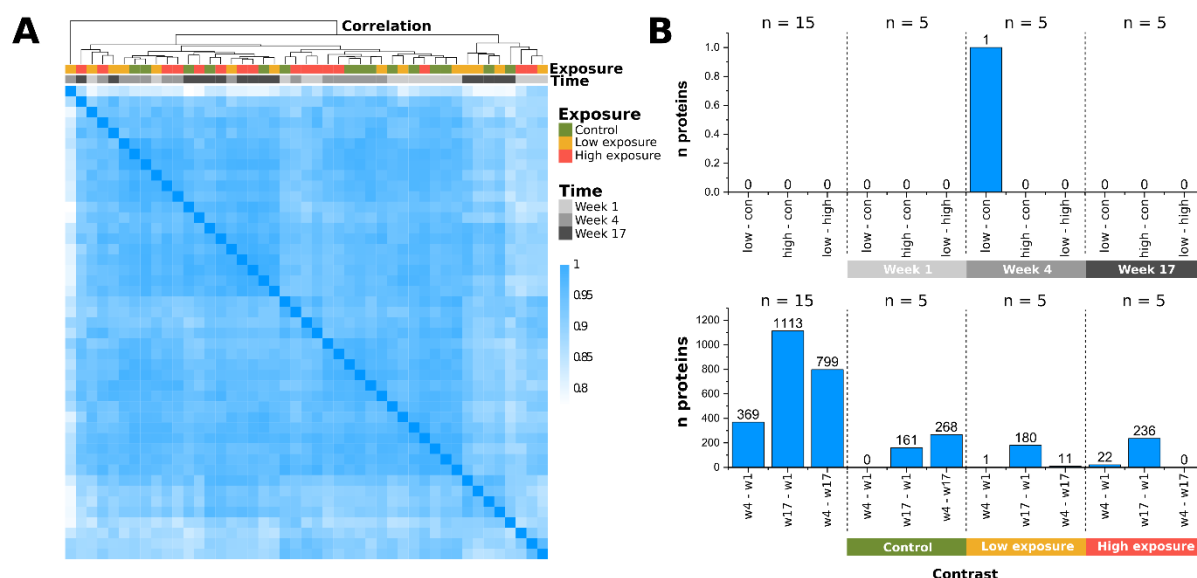


Figure 6.4. Overview of the results of the label-free proteomics quantification (LFQ). **(A)** Heatmap based on protein intensity correlation among samples. **(B)** Number of statistically significantly regulated proteins ($q < 0.05$) for each analysed contrast (w = week, con = control, low = low exposure, high = high exposure). n = number of samples in each condition.

In contrast to the pairwise comparisons between treatments, comparisons between sampling points identified a number of statistically significantly regulated proteins. Of particular note were the pairwise comparison of datasets from week 17 versus week one, which highlighted statistically significant ($q < 0.05$) differences in concentration for the low MP exposure (180 proteins, -7.0 to 3.9-fold) and high MP exposure (239 proteins, -12.5 to 4.9-fold) groups (Fig. 5 (B)). Comparisons between Week 17 and week 4 revealed significantly different concentrations for 11 proteins ($q < 0.04$, -3.5 to 1.7-fold) between the control and low-exposure group only. These three datasets share significantly modulated levels of the enzyme formimidoyltetrahydrofolate cyclodeaminase and the hormone insulin-like growth factor 2 (Fig. 6.6 (A)). However, if only variable proteins with a $\log_2$ fold change $> |2|$ are considered, the large majority of candidates are eliminated leaving just two in the low exposure group and three proteins in the high exposure group exhibiting significant differences in concentration over time, in both cases when comparing week 17 and week 1 (Fig. 6.6 (B)).

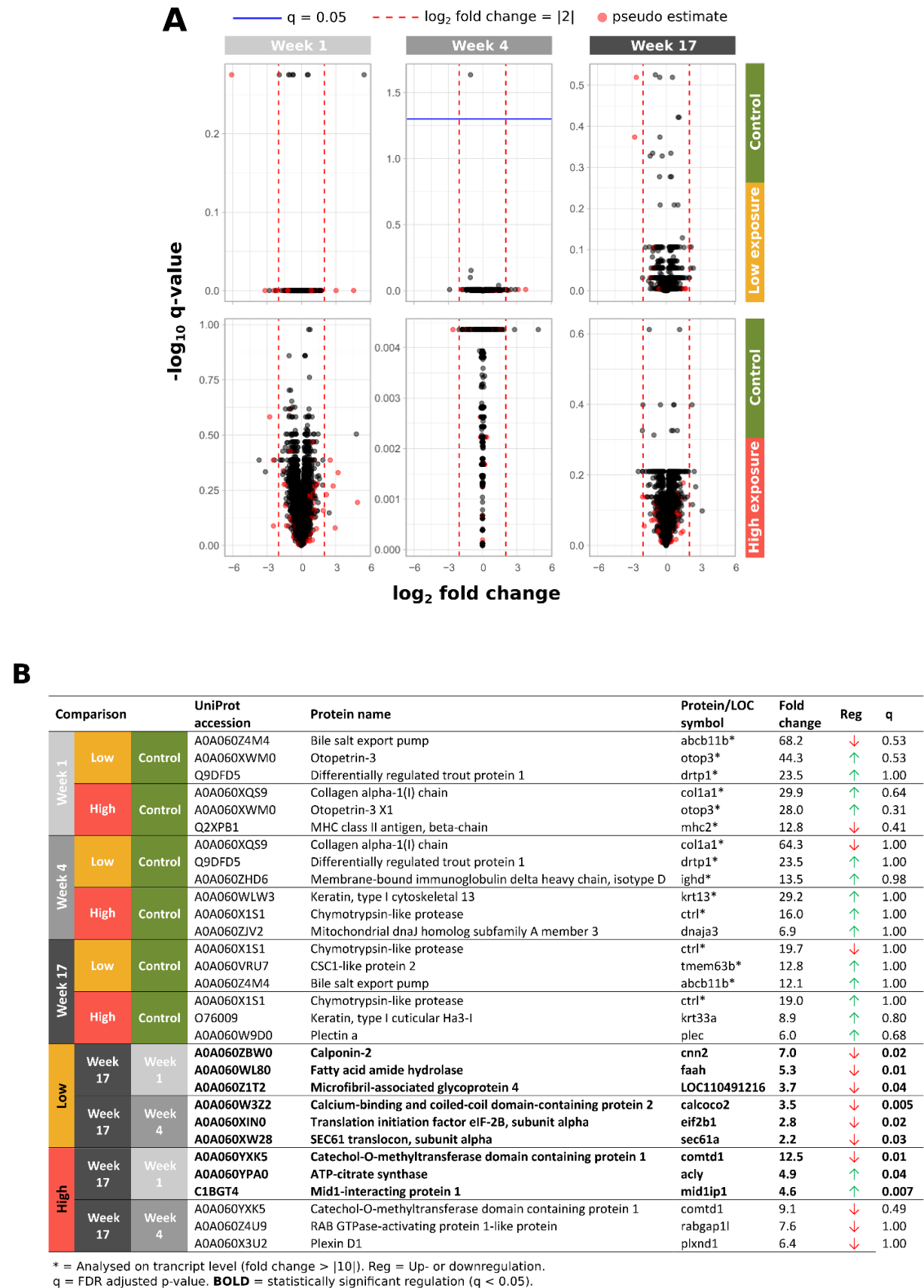


Figure 6.5. Results of the linear mixed-effects models of the label-free proteomics quantification (LFQ). **(A)** Volcano plot showing q-values plotted against $\log_2$ fold changes. **(B)** Table of the top three regulated proteins identified by contrast analysis for each contrast.

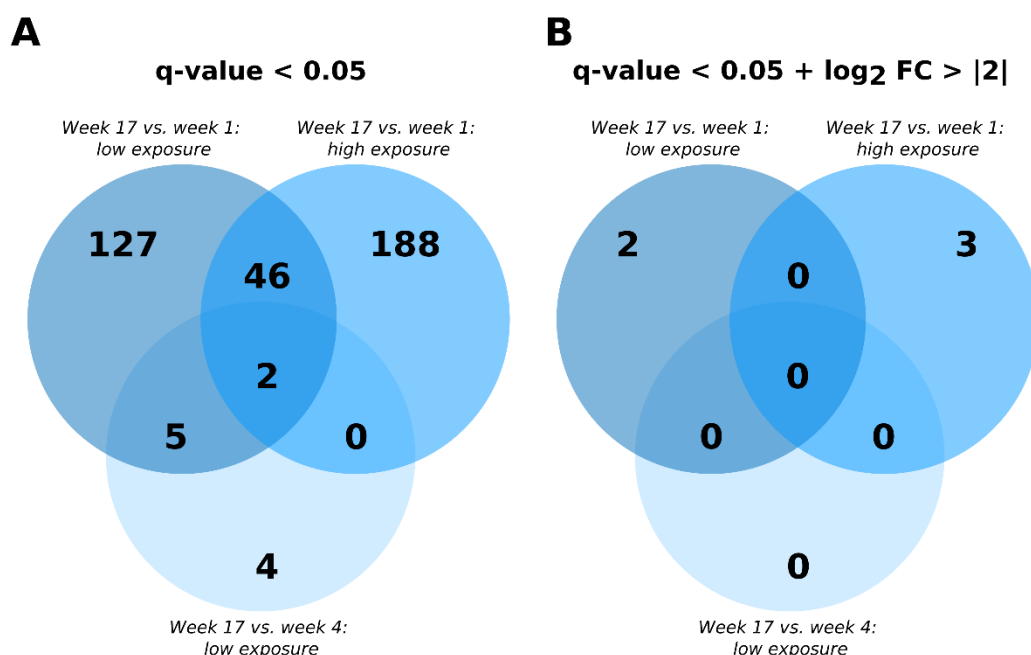


Figure 6.6. Venn diagram illustrating the number of individual and shared significantly different protein concentrations for three selected contrasts. **(A)** Number of proteins with a $q\text{-value} < 0.05$. **(B)** Number of proteins with a $q\text{-value} < 0.05$ and a $\log_2$ fold change (FC) $> |2|$.

6.4.4. Gene Expression analysis

Based on the list of proteins exhibiting differential regulation in response to low or high MP exposure (Fig. 6.5 (B)), the transcript levels of 11 genes were profiled in the liver samples of respective treatment groups. The gene *methfd1* was also analysed, as it was upregulated 12-fold in the proteomic analysis (week 1: low exposure *vs.* high exposure). However, it was not found among the top three regulated proteins and therefore does not appear in Figure 6.5 (B). This list was extended to four genes (*c3-3*, *ighm*, *il1b*, and *hamp*) with vital roles in stress responses and immunity. The concentrations of three transcripts coding for *krt13*, *methfd1* and *otop3* (derived from the list of differentially regulated proteins), as well as *il1b* and *hamp* (derived from literature research), were very low and were therefore excluded.

The results of the linear models used to analyse individual effects on gene expression are summarized in Figure 6.7 and Table S6.4. The model effect “exposure level” had a statistically significant effect on the expression of *colla1*, *ighd*, *rpl7* and *c3-3* (Fig. 6.7). Dunnett’s tests showed that for *colla1* ($p = 0.0227$), *ighd* ($p = 0.0159$) and *rpl7* ($p < 0.0001$), the low exposure group differed significantly from the control, whereas for *c3-3* ($p > 0.05$) no differences were apparent between the control and either of the exposure groups. The model effects “sampling time” and “total length” had a statistically significant effect on the

expression of *tmem63b* only (Fig. 6.7). Finally, the interaction of sampling time and exposure level had a statistically significant effect on *ctrl*, *tmem63b* and *c3-3* (Fig. 6.7).

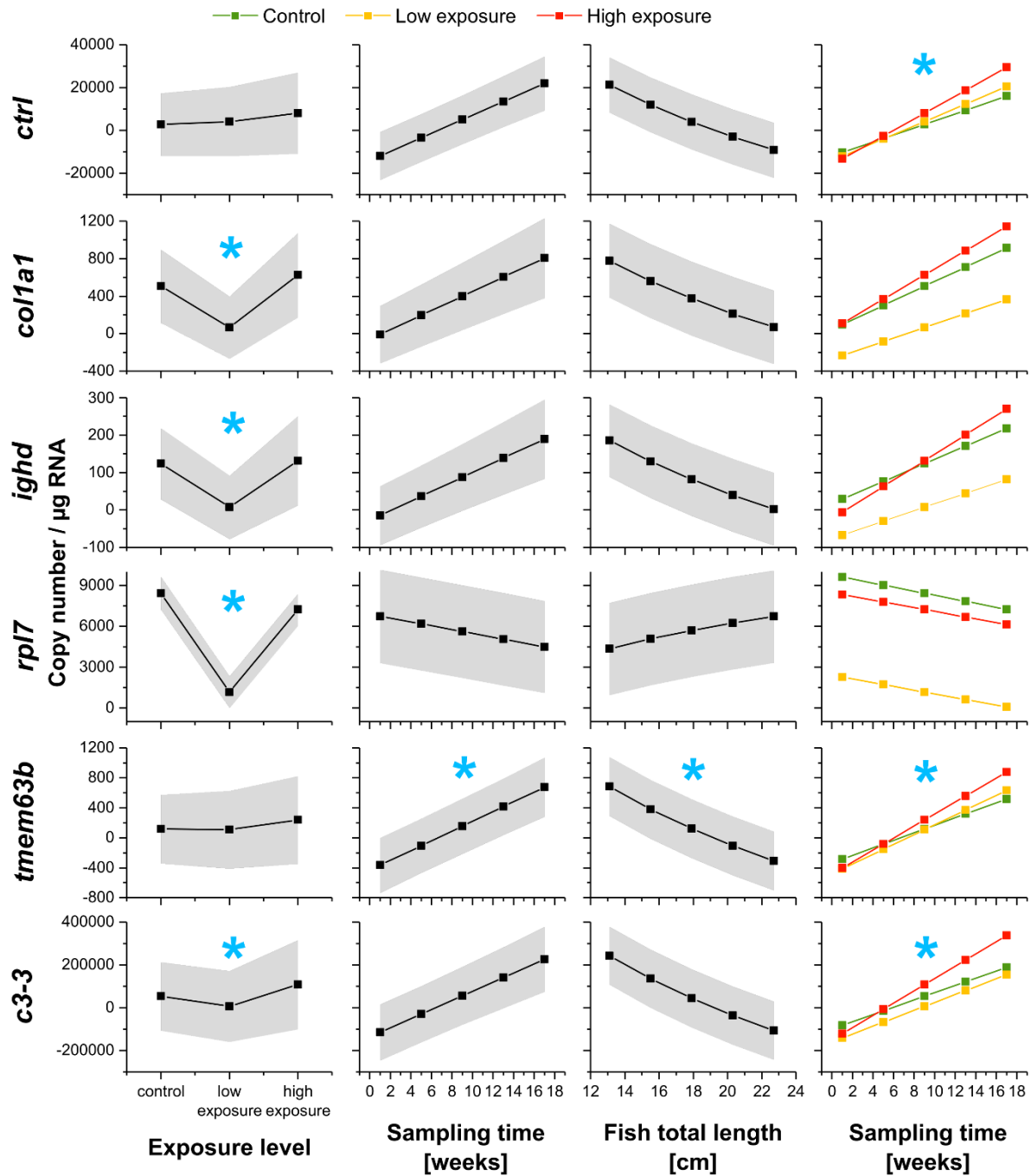


Figure 6.7. Grand marginal means of selected linear models, examining statistically significant model effects on transcript concentrations of selected features derived from the proteom analysis and established stress genes from the literature. Grey bands indicate standard deviation. Asterisks indicate statistically significant differences between model effects (for more details, please refer to Table S6.4).

The modulation of protein levels correlated only marginally with that of transcription ($df = 1, 12, F = 1,1339, p > 0.05$), with an r^2 of 0.0863 (Fig. 6.8). However, particular changes in transcription did reflect those observed at protein level. After one week of exposure to high MP concentrations, the levels of *drtp1* increased 8.6-fold ($\log_2$ fold change = 3.1; $p = 0.2$), corresponding with a 7.8-fold ($\log_2$ fold change = 2.9) higher concentration of *drtp1* proteins. After four weeks' exposure to high MP concentrations, the levels of *abcb11b* and *ctrl* decreased approximately 3-fold ($p < 0.05$) matching with the below 6-fold elevated levels of the respective proteins.

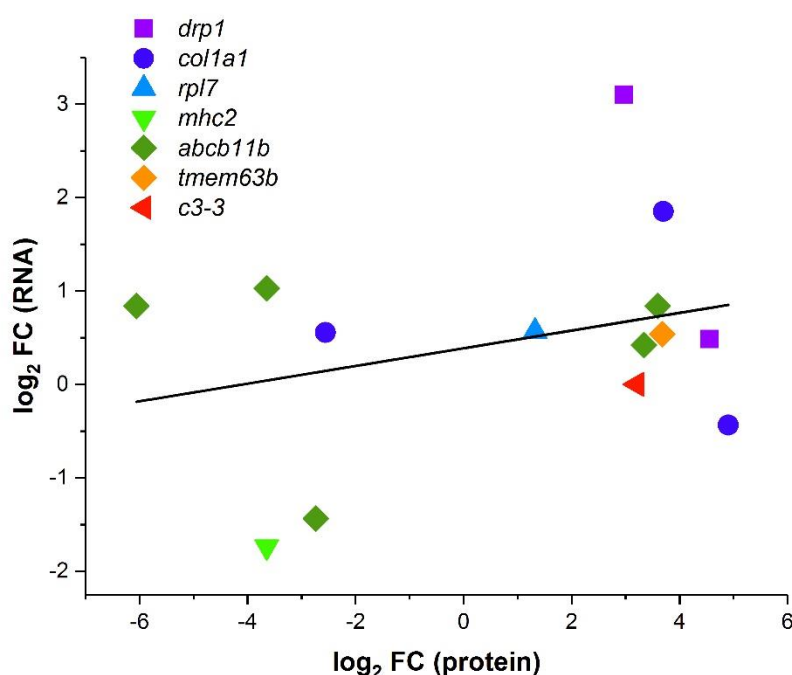


Figure 6.8. Regression analysis of the correlation between differential protein concentrations and differential gene expression ($F(1,12) = 1.1339, p > 0.05, r^2 = 0.0863$). Some proteins occur more frequently if they have been detected more than once in the contrast analysis of proteomic data. FC = fold changes.

6.5. Discussion

The negative effects of MPs on fish are part of a complex issue which, despite intensive research, remains far from fully understood (Cunningham *et al.* 2020). The many confounding factors, including a lack of standardization in experimental setups (fish species, age, length) and the sheer variety of different types, sizes, concentrations of plastic and secondary contaminants involved, have led to widely varying results (de Sá *et al.* 2018). A major shortcoming of several studies is the obvious discrepancy between concentrations of MP seen in the environmental and those applied in exposure experiments (de Sá *et al.* 2018; Lenz *et al.* 2016). In the present study, concentrations of around 14 particles in the gastrointestinal tract

per fish in the "low exposure" group are comparable to those frequently detected in environmental fish samples (Collard *et al.* 2019; Parker *et al.* 2021; Tanaka & Takada 2016). Furthermore, the use of consistent MP concentrations, and a reduced exposure schedule (every two days), ensured that the present experiments reflected natural conditions as closely as possible. In the "high exposure" group, MP values were increased to around 75 particles per fish in order to examine dose dependent effects and simulate increases in environmental MP concentrations expected in the near future. The global volumes of plastic waste is expected to triple by 2060, assuming no drastic measures are taken in waste reduction (Lebreton & Andrady 2019). Everaert *et al.* (2020) calculated that the risk to marine ecosystems of floating MPs at the ocean surface layer will increase almost ten-fold by the year 2100, assuming a worst-case scenario of plastic discharge. A study of preserved fish samples from the Chicago region (USA) between 1900 and 2017 revealed a correlation between plastic production and microplastic contamination of fish (Hou *et al.* 2021). It is therefore to be expected that the contamination of fish with microplastics will continue to increase in line with plastic production in the near future (Geyer *et al.* 2017).

There are many different approaches to studying the impact of pollutants on fish. Performance parameters, such as growth and mortality, are regularly considered in ecotoxicological studies, as they serve as an integrative proxy for the fitness or stress exposure of an individual (Crossin *et al.* 2014). While the present study agrees with other investigations in suggesting MP exposure for 120 days has no significant effect on mortality (Pannetier *et al.* 2020; Parker *et al.* 2021), it does reveal a significant negative effect on fish growth. There was a dose dependent effect between MP concentration and weight at the end of the experiment, with the "high exposure" group being 3.5 % underweight compared to the control group. These differences were mirrored in significantly reduced SGR values and an increased FCR for this group. Exposure experiments carried out in other studies have revealed significantly reduced growth rates but the scale of the effect varies, presumably as a result of differences in the duration of studies, in the age of fish and the type of MP particle used (Naidoo & Glassom 2019; Yang *et al.* 2020), meanwhile some failed to record any effect at all (Mazurais *et al.* 2015; Müller *et al.* 2020). This lack of consistency in the literature reinforces the urgency of establishing a sound experimental framework for such studies (Jacob *et al.* 2020).

In principle, there are several possible mechanisms of action by which increased MP concentrations can lead to the observed impairments in performance. Nutrient dilution, caused by increased amounts of non-digestible material in the diet, can

affect growth in fish (Reinitz 1984). However such an effect due to the presence of MP can be excluded in the present study, as MP particles constituted only a fraction of the weight of the diet (see calculations in Table S5). Even at the beginning of the experiment, when the proportion of microplastic in the diet was highest, it accounted for less than 0.15 % of the total diet weight. Previous studies on non-digestible feed supplements clearly show that those supplements, when applied below 0.3 % weight, have no influence on the growth of salmonids (Brinker & Reiter 2011). Similar results have been reported in experiments where markers such as chromic oxide, have been used in digestibility studies (Fernandez *et al.* 1999).

Growth effects due to differences in diet quality are also rather unlikely in the current context, as the diet used was produced specially for this study and subjected to strict quality controls (for more details about the diet composition, see Roch *et al.* 2021). Furthermore, the MP diet was provided alongside the untreated experimental diet supplied to all groups. At the beginning of the exposure experiments, when the proportion of the MP diet was highest, the proportion in the "high exposure" group comprised on average < 22 % of the total diet weight. As the experiment progressed, the proportional weight of the MP diet decreased continuously, as MP concentrations were kept steady throughout the experiment.

Another potential factor could be physical interference with the gastrointestinal tract by MPs. Available literature on the topic is ambivalent with regard to MPs. Lei *et al.* (2018) demonstrated significant histopathological damage in the gut of zebrafish (*Danio rerio*) and Ahrendt *et al.* (2020) found an impact of MPs on gut integrity in *Girella laevis*. However, comparable studies failed to confirm such physical damage and attributed the findings to incorrect sample preparation and post-mortem autolytic processes which can easily lead to misinterpretation of histopathologic results (Batel *et al.* 2020; Baumann *et al.* 2016; De Sales-Ribeiro *et al.* 2020). Generally, a direct physical effect seems implausible in this respect, as fish are exposed to a variety of different non-digestible inert materials in the wild, which they routinely ingest (Doyle *et al.* 2011; Johansson 1991). Similarly, wild fish experience regular exposure to large quantities of fine sediment, *e.g.* due to flooding (Kemp *et al.* 2011) which does not appear to cause physical damage, *e.g.* to the gills (Lake & Hinch 1999; Redding *et al.* 1987).

A more likely scenario seems to be an indirect detrimental effect of MP, either due to their hydrophobic surface properties or the presence of certain additives in the particular plastic used in the experiments. In humans, the addition of bran-like

plastic particles to the diet led to a laxative effect and a higher water content in the stool (Lewis & Heaton 1999; Tomlin & Read 1988). It was suggested that it was not the amount of particles that was responsible for the observed changes, but that certain sizes and shapes may cause a tactile stimulation of the enteric nervous system (Lewis & Heaton 1999). Similar effects are also observed with plant-based diets in salmonid fish and result in a reduced lipid digestibility (Refstie *et al.* 2005). Furthermore, the gastrointestinal tract of fish can react very sensitively to certain substances, potentially leading to an allergic reaction and subsequently reduction in lipid digestibility (Penn *et al.* 2011). Such reactions are regularly observed with the alternative plant-based protein sources increasingly used in diets for carnivorous fish. For example, saponins in soybean meal trigger inflammation processes in the intestine in several fish species (Hedrera *et al.* 2013; Kokou *et al.* 2015). In humans no allergic reaction or histamine-mediated inflammation was observed in different human cell types exposed to several polystyrene concentrations (0.5 - 1000 µg/mL) and sizes (0.46 - 100 µm); however, there was some evidence of local inflammation caused by small MPs in high concentrations (Hwang *et al.* 2020) and exposure to polypropylene particles (size: 20- 200 µm) may cause hypersensitivity at elevated concentrations (Hwang *et al.* 2019). Transposing mammalian results to fish is problematic, since there are fundamental differences in the nature of intestinal immune responses, especially regarding associated antibodies and cell types (Gomez *et al.* 2013; Jirillo *et al.* 2007). However the influence on the intestinal microbiota is of interest, as disturbances thereof are known to impact on the immunity and health of fish (Dawood 2021; Montalban-Arques *et al.* 2015). For example Jin *et al.* (2018) reported that in adult zebrafish when exposed to polystyrene particles (concentration: 1000 µg/L, size: 0.5 and 50 µm) for 14 days, the abundance of *Bacteroidetes* and *Proteobacteria* decreased significantly, while the abundance of *Firmicutes* increased. A similar microbiota dysbiosis was found in a further study where zebrafish were exposed to three different shapes (fibers, fragments, beads) of MP (Qiao *et al.* 2019). Lastly, a number of additives, including plasticizers, flame retardants and antioxidants routinely used in the production of plastics are known to be harmful to fish (Hahladakis *et al.* 2018; Jovanovic 2017). Although the leaching of such substances from MPs has so far been considered a rather minor issue for fish compared to other sources of environmental pollution (Koelmans *et al.* 2016; Koelmans *et al.* 2014), additives remain a potential hazard for aquatic organisms (Hermabessiere *et al.* 2017). Notably, the majority of studies conducted to date use virgin plastics, and rarely is a distinction made between the effects of MP particles alone versus potential associated substances (Jacob *et al.* 2020). In the present study, the

influence of specific additives on the performance of the fish cannot be excluded, as the plastic particles used were not tested for the presence of such compounds.

Finally, it may be that the presence of MPs in the diet could trigger a general stress response that influences the performance of exposed fish, for example by reducing feed conversion efficiency via increased energy consumption or the disruption of metabolic processes (Barton *et al.* 1986; Leal *et al.* 2011; Mommsen *et al.* 1999). Various stress markers are regularly used as indicators of fish health status, as they provide clues to the presence of a disturbance (Conte 2004; Iwama *et al.* 1995), which may be chemical, physical and perceptual (Barton 2002). So far, stress markers have been used only sporadically in studies on the impact of MPs on aquatic organisms. In adult water fleas (*Daphnia magna*), expression levels for two stress response genes (heat shock protein (HSP) 60 and 70) were significantly altered after exposure to a mixture of different MP types (around 30 particles per individual, size: 40 μm ; Imhof *et al.* 2017). Cortisol, another frequently used biomarker for stress in fish, was detectable in early life stages of sea trout (*Salmo trutta*) exposed to polystyrene particles but not in the control group (Jakubowska *et al.* 2020), although the number of examined samples was low, so results must be treated with caution.

Beyond these theoretical considerations, investigation of established health parameters in the present study does not offer direct indication of the causes of the observed differences in growth. The parameters blood glucose, hematocrit and HSI varied significantly during the course of the experiment, but differences between the treatments were not apparent. A significant decrease in HSI, with increasing fish length was also observed, in line with previous studies (Torrissen *et al.* 1984). No differences in LPO in the liver and intestine were found between treatments, although MDA levels were significantly elevated in the intestine after one week. Oxidative stress is often reported as a response to MP exposure in fish, however it seems to be associated with high concentrations of MP: significant increases in reactive oxygen species (ROS) levels in male and female zebrafish gonads and livers were observed after a 21-day exposure to polystyrene particle concentrations $> 100 \mu\text{g/L}$ (Qiang & Cheng 2021). Iheanacho & Odo (2020) found significantly increased LPO levels in the liver of juvenile *Clarias gariepinus* after exposing them to polyvinyl chloride particles for 45 days (concentration: 0.5 – 3 % of total diet weight). Since the strong increase in the present study was observed in both treatment groups and the control, a connection with MP exposure seems unlikely. Instead, it may be a general reaction to the provided experimental diet (Birnie-Gauvin *et al.* 2017). In summary, the present results strengthen the argument that

more sensitive approaches are needed to study the negative effects of MP exposure at environmentally relevant concentrations (Petitjean *et al.* 2019).

Omics approaches are becoming a favored method by which to understand the complex interactions of MPs and aquatic organisms (Liu *et al.* 2020; López-Pedrouso *et al.* 2020), but to our knowledge have not yet been applied to salmonid fish in this context. The rainbow trout data presented here indicates that long-term exposure to both environmentally realistic and increased MP concentrations did not induce any significant change in hepatic protein concentrations compared to their matching controls, although certain parameters were regulated many times up or down. As comparable experiments do not yet exist, transcriptomic studies may indicate the proteins and associated pathways affected. It is worth noting that a transcriptomic study on the liver of adult zebrafish reported differential expression in 147 and 201 genes, after 20-day exposure to 100 µg/l or 1000 µg/l polystyrene and high-density polyethylene respectively (Limonta *et al.* 2019). Regulated features in common with the present list of differentially concentrated proteins include members of the abc transporter family (*abca1b/abcb11b*), transmembrane channel proteins (*tmem106bb/tmem63b*) and keratins (*krt96/krt13, krt33a*). Of these, the first two groups are associated with transport processes and their altered regulation might indicate a pollution response (Ferreira *et al.* 2014). Keratins provide barrier functions (Roth *et al.* 2012) similar to the complement components shown to exhibit differential expression in other fishes exposed to MPs (Banaee *et al.* 2019; Veneman *et al.* 2017; Wen *et al.* 2018). When the change in protein regulation over time were analyzed, a large number of statistically significant changes were found. However, most of these exhibited a $\log_2$ fold change $< |2|$ and therefore their biological relevance is questionable (McCarthy & Smyth 2009). In the present study, liver tissue was chosen for proteomic and subsequent transcriptional analysis, since MP exposure has previously been demonstrated to induce toxicity (Lu *et al.* 2016), metabolic disorders (Zhao *et al.* 2020) and lesions (Abarghouei *et al.* 2021; Hamed *et al.* 2022) in the livers of several fish species. Furthermore, it has been shown that fish gut and liver act in collaboration during intestinal inflammation (Wu *et al.* 2016). As there was no clear evidence of inflammation or stress in the liver tissue and considering the above mentioned hypotheses on the impact of microplastics on fish, an investigation of the gut proteome would be a logical next step.

The small number of highly up- or down-regulated proteins identified in the current study were selected and examined at the transcript level. Generally, the correlation between protein and RNA regulation was very low, but a clear

correlation was found for the differentially regulated trout protein 1 encoded by *drtp1* when comparing the high exposure group and control group at week one of the experiment. This protein has been previously identified as a suitable biomarker for acute stress in fish (Eissa & Wang 2016; Talbot *et al.* 2009). Further studies should clarify whether this marker is a suitable candidate for studying the effects of MPs on trout. One reason for this otherwise low correlation between protein and RNA regulation seems to be the small sample size, together with strong individual differences between sampled fish (Levin 2011; Todd *et al.* 2016). Furthermore, the variations in levels of transcripts and their associated proteins do not necessarily coincide in time and also can vary dynamically in their association with other factors (Vogel & Marcotte 2012). No evidence of a negative influence of MPs on fish were seen when analyzing several of the “established” genes regularly used to investigate fish health (Najafian & Babji 2012; Tort 2011). For example, *Il1b*, an indicator of inflammation (Mantovani *et al.* 2019; Reis *et al.* 2012), were not detected in relevant concentrations. However, the gene expression analysis did suggest that the duration of exposure may be a key factor governing the effects of MPs on fish. For example, the data indicates that for *ctrl*, *tmem63b*, and *c3-3*, gene expression increased significantly more over time in the “high exposure” group than in the other two groups. This should be a consideration in future studies, as previous investigations have rarely used exposure times longer than two months (Bucci *et al.* 2020; de Sá *et al.* 2018). Interestingly, the gene expression results for *coll1a1*, *ighd* and *rpl7* showed significantly lower expression in the “low exposure” group, an observation for which there appears to be no obvious biological explanation.

6.6. Conclusion

The results presented here provide clear evidence that the long-term exposure with environmentally relevant MP concentrations has a dose-dependent negative effect on the performance of rainbow trout, and highlight the importance of measures to prevent further increases of MPs in the environment. With increasing concentrations, growth was clearly inhibited, indicating a reduced feeding efficiency. Several hypotheses were put forward that could explain the observed differences, of which nutrient dilution, quality differences in the provided diet and physical interference MP particles in the intestine were considered improbable. More likely explanations include an allergic reaction due to the hydrophobic properties of MP or the presence of additives. Furthermore, it is suggested that MP exposure could cause a general stress reaction and thus lead, for example, to

disruption of metabolic processes. Unfortunately, neither established approaches nor the combined proteomic and gene expression analysis provide support for these presented theories. One reason for this could be the use of liver tissue in the analysis. An analysis of the intestine proteome may provide further insights in the observed effects. Despite these limited results, the study clearly identifies *drtp1* as a potential biomarker, demonstrating the potential value of omics approaches in this complex topic. Future studies should combine histological examinations and proteomic and transcriptomic analyses of intestinal tissue in order to better understand the detrimental effects of MPs in fish. Furthermore, targeted markers for hypersensitivity reactions and changes in energy uptake and lipid digestibility should be considered. Finally, for environmentally relevant MP concentrations, the duration of exposure must be taken into account.

6.7. Acknowledgements

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6.8. Supplemental information

Table S6.1. List of oligonucleotide primers used in the gene expression analysis. Genes were derived from the previous proteomic analysis or from literature.

Gene/ LOC symbol	Gene product	NCBI accession code	Sense primer (5' -3')	Antisense primer (5' -3')	Fragment length [bp]
<i>abcb11b</i>	ATP-binding cassette, sub-family B (MDR/TAP), member 11b	NM_001124656	CACGAAACAGGACACAAAAGTCTC	CGTACTCAATGAAGGTGTCTGTG	197
<i>c3-3^a</i>	Complement component C3, isoform 3	XM_021568201	CGACCAGGGAAGATGTTTGGGA	AGTACAGCGCTAATTTTGGCTAC	168
<i>col1a1</i>	Collagen, type I, alpha-1 chain	NM_001124177	CCCCCTCGCCTCAAAAGAGT	GTGCGATCGTCTCGCCTTG	127
<i>ctrl</i>	Chymotrypsin-like protein	XM_021625136	CACGAAACAGGACACAAAAGTCTC	CGTACTCAATGAAGGTGTCTGTG	127
<i>drp1</i>	Differentially regulated trout protein 1	NM_001124601	GATCGAAAGACTCTCTGAAGAAAG	TTTCAGGCCCTCTCCAAAAGCTA	94
<i>hamp^a</i>	Hepcidin antimicrobial peptide	XM_021595153	AGTGTTCAGTTGCAGTGGTACTC	AAATGCTCCGGCAGACGC	164
<i>ighd</i> (<i>LOC110485215</i>)	Immunoglobulin delta, heavy chain (secreted form)	JQ034605	ACTTCTGCCTCATCAAACTGCTC	CCCGCCTTCGTCAATCCATTTG	141
<i>ighm^a</i> (<i>LOC1100136770</i>)	Immunoglobulin mu, heavy chain (membrane-bound form)	U04616	AAGCCTACAAGAGGGAGAGACCGA	GGCAGTTGCTCCAACGCCATA	151
<i>il1b^a</i>	Interleukin-1 beta	XM_036979104	GAGAGTGCTGTGGAAGAACAATAT	GCAGCTCCATAGCCTCATTCAT	156
<i>krt13</i>	Keratin, type I cytoskeletal 13	XM_021624710	CCAGAGCCTCCAGCTTCAGTAT	TGTGAACCTTTCCGCCAGGTAGGT	120
<i>mhc2</i> (<i>LOC110494409</i>)	MHC class II antigen, alpha-1 chain	FR688130	CAGTGATTCAGATGGAGTGAATAT	CATAATATCCAGGGAAGAAATCT	131
<i>mthfd1</i>	Methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1	XM_021600758	CTATCAGTTTCTCTTGCAGGGAG	GGTCATCCTCATCTGCCTGTAT	120
<i>otop3</i> (<i>LOC110499463</i>)	Otopetrin-3	XM_021576601	TGGCCTACAGACCTATTGCTC	ATACGTGGCGCGGCCATCCT	184
<i>rpl7^b</i>	60S ribosomal protein L7	XM_021562213	TCAAACCTGAACAAGGCTTCCATC	TATTTACCCAGGGCCTTCTCGA	178
<i>tmem63b</i> (<i>LOC110493827</i>)	CSC1-like protein 2, Transmembrane protein 63b	XM_021568328	ATAACGGCTTCTGTTCTCTGTTA	CGCTGGAAGGAAGGTAGTGC	103

^a Genes derived from a literature survey. ^b Reference gene, which also upregulated 2.5-fold in the proteomic analysis

Table S6.2. Overview of utilized linear models to analyse the microplastic concentration in fish, as well as the performance parameters, health parameters and gene expression, obtained from the exposure experiment.

Dataset	Method	dependent variable	time points	Model effects
microplastic exposure	standard least-square (method: REML ^a)	MP concentration	n.a.	exposure level, pellet size, exposure level x Pellet size
start weight, end weight, food conversion ratio (FCR), specific growth rate (SGR), mortality	standard least-square	weight, FCR, SGR, mortality	n.a.	exposure level
blood glucose, hematocrit, hepatosomatic index	standard least-square (method: REML ^a)	blood glucose, hematocrit, hepatosomatic index	all	exposure level, sampling time, fish total length, exposure level x fish total length
TBARS Assay	standard least-square (method: REML ^a)	MDA concentration	week 1, week 4, week 17	exposure level, sampling time, fish total length, exposure level x fish total length
gene expression	standard least-square	RNA concentration	week 1, week 4, week 17	exposure level, sampling time, log(fish total length), exposure level x sampling time

^a restricted maximum likelihood (Tank[Fish ID] was added as a random effect).

Table S6.3. Overview of all computed contrasts for the analysis of the label-free proteomics quantification (LFQ).

Contrast	Given
low exposure	- control
high exposure	- control
low exposure	- high exposure
low exposure	- control
high exposure	- control
low exposure	- high exposure
low exposure	- control
high exposure	- control
low exposure	- high exposure
week 1	- week 4
week 1	- week 7
week 4	- week 7
week 1	- week 4
week 1	- week 7
week 4	- week 7
week 1	- week 4
week 1	- week 7
week 4	- week 7
week 1	- week 4
week 1	- week 7
week 4	- week 7

Table S6.4. Results of the linear models utilized to analyse individual effects on gene expression. n = number of observations. df = degrees of freedom. Asterisks indicate statistically significant differences ($p < 0.05$).

Gene	n	df	model effect	F-value	p-value
<i>abcb11b</i>	31	6, 24		3.2753	0.0169*
			exposure level	1.2696	> 0.05
			sampling time	3.0424	> 0.05
			log(fish total length)	2.8391	> 0.05
			sampling time x exposure level	2.9555	> 0.05
<i>col1a1</i>	31	6, 24		3.0798	0.0222*
			exposure level	6.0119	0.0076*
			sampling time	1.3454	> 0.05
			log(fish total length)	0.6650	> 0.05
			sampling time x exposure level	0.8984	> 0.05
<i>ctrl</i>	31	6, 24		4.5394	0.0033*
			exposure level	1.4621	> 0.05
			sampling time	3.7395	> 0.05
			log(fish total length)	3.3125	> 0.05
			sampling time x exposure level	3.9598	0.0326*
<i>drtp1</i>	31	6, 24		1.6120	> 0.05
<i>ighd</i>	31	6, 24		2.8762	0.0295*
			exposure level	5.1342	0.0139*
			sampling time	1.1121	> 0.05
			log(fish total length)	0.6952	> 0.05
			sampling time x exposure level	1.3161	> 0.05
<i>mhc2</i>	26	5, 20		1.1139	> 0.05
<i>rpl7</i>	31	6, 24		10.1064	< 0.0001*
			exposure level	28.046	< 0.0001*
			sampling time	0.2714	> 0.05
			log(fish total length)	0.1834	> 0.05
			sampling time x exposure level	0.0046	> 0.05
<i>tmem63b</i>	31	6, 24		4.4931	0.0035*
			exposure level	1.2617	> 0.05
			sampling time	4.6046	0.0422*
			log(fish total length)	4.6796	0.0407*
			sampling time x exposure level	4.4805	0.0222*
<i>c3-3</i>	31	6, 24		4.1745	0.0052*
			exposure level	3.9413	0.0331*
			sampling time	3.3181	> 0.05
			log(fish total length)	3.6714	> 0.05
			sampling time x exposure level	4.9804	0.0155*
<i>ighm</i>	31	6, 24		2.6638	0.0400*
			exposure level	2.8778	> 0.05
			sampling time	0.9781	> 0.05
			log(fish total length)	1.0300	> 0.05
			sampling time x exposure level	0.0572	> 0.05

Table S6.5. Example calculation of the percentage by weight of microplastics (MP) in the utilized fish feed. For a pellet size of 2 mm, a fish weight of 20 g and an amount of feed of 1.5 % of the body weight is assumed. For the pellet size of 4 mm, the fish weight is assumed to be 65 g and the feed amount is 1.8 % of the body weight.

Pellet size	Exposure level	MP particle weight per gram MP feed [g]	Weight MP feed per fish [g]	Total feed weight [g]	% MP by weight
2 mm	Low exposure	5.0×10^{-4}	0.019	0.3	0.032
	High exposure		0.085	0.3	0.141
4 mm	Low exposure	1.0×10^{-2}	0.019	1.17	0.017
	High exposure		0.085	1.17	0.076

7. General discussion

For several decades we have known that the environment is polluted with plastic debris (Carpenter & Smith 1972). However, the extent and consequences of this pollution have only been researched intensively in the last 20 years. As expected, the growing production and use of plastic has led to an increase in plastic waste in the environment. For a long time, the world's oceans were seen as the main ecosystem affected by plastic debris (Derraik 2002; Thompson *et al.* 2004). However, as research has progressed and the impact of plastic on the environment has become more apparent, it is clear that freshwater and terrestrial ecosystems are similarly exposed (Wagner *et al.* 2014; Dris *et al.* 2015; Horton *et al.* 2017). In particular, as most of the plastic waste entering the environment is land based, the role of rivers as pathways for the spread of plastic waste solidified (Lebreton *et al.* 2017; Jambeck *et al.* 2015). Today, virtually every body of water on earth is affected, no matter how remote or protected. An extremely high abundance of plastic debris can be found in the ocean's large subtropical gyres, where it accumulates over time (Moore 2008; Brach *et al.* 2018). The seafloor also acts as an important sink for plastic waste, as even buoyant debris can eventually subside (Kooi *et al.* 2017; Woodall *et al.* 2014).

7.1. Key challenges in evaluating microplastic pollution

Regardless of where plastic debris is detected, it can be assumed that as particle size decreases, the number of particles increases exponentially (Lindeque *et al.* 2020; Conkle *et al.* 2018; Imhof *et al.* 2016). This is logical, since larger waste breaks down into smaller fragments over time, and the degradation rate of plastic is very low (Barnes *et al.* 2009; Andrady 2011). Unfortunately, the smaller the fragments, the greater the risk that they will be ingested by aquatic organisms (Wright *et al.* 2013). Thus, in addition to potential entanglement with and colonization of plastic debris, new dangers are becoming apparent. Furthermore, with smaller particle size comes a number of challenges which complicate the detection of microplastics in the environment, and impede inter-study comparability (Hidalgo-Ruz *et al.* 2012).

7.1.1. Lack of standardised sampling protocols

Regardless of the type of sample, standardised methods for the detection of microplastics are still lacking (Koelmans *et al.* 2020; Hermesen *et al.* 2018). This is mainly due to the diversity of microplastics, which comprise a range of different

shapes, polymer types and particle sizes (Hüffer *et al.* 2017). For water and sediment samples, much is determined by the mesh size of the chosen sampling net or sieve (Van Cauwenberghe *et al.* 2015; Conkle *et al.* 2018). While the upper limit of microplastic particles is established and thus comparable between studies, the lower limit is heavily dependent on the sampling equipment utilised (Lindeque *et al.* 2020). This means that only a certain size range is quantified, which can differ from study to study. This already complicates comparison, especially if the unit of measurement differs as well (Koelmans *et al.* 2020; Connors *et al.* 2017). In addition, recovered particles that are smaller than the utilised mesh size are regularly included when evaluating the particle concentration of a sample; at best, this allows a semi-quantitative estimation of the microplastic burden (Eerkes-Medrano *et al.* 2015; Lindeque *et al.* 2020). Related to this, there is the question of temporal and spatial variability. Particle abundances in the environment can vary greatly, and are dependent on the sampling date, water level and/or location in the water column (Mani *et al.* 2015; van Emmerik *et al.* 2019; Schmidt *et al.* 2018). As such, most available studies only provide a spatially and temporally localised “snapshot”, and do not allow any general statements to be made about the long-term exposure levels or distribution of microplastics throughout the body of water (Beer *et al.* 2018; Mani *et al.* 2015).

Similar limitations exist for the detection of microplastics in aquatic organisms. In fish, for example, either the entire gastrointestinal tract can be examined, or the stomach/intestinal contents alone (Lusher *et al.* 2017). When examining singular parts of the digestive tract or its content, there is a risk that only a fraction of particles is isolated. Moreover, available studies show that the prevalence of affected individuals varies widely (Collard *et al.* 2019; Barboza *et al.* 2018). It is therefore important to ensure that the number of samples taken is sufficiently high (Hermsen *et al.* 2018). Since most microplastics ingested by fish are excreted again relatively quickly, one must assume that a measured microplastic concentration only reflects the current burden; no statements can be made about the general exposure level without repeated testing.

The aforementioned constraints must also be taken into account when evaluating the results presented here. Although a relatively large number of bodies of water were sampled in Baden-Württemberg, sampling took only place once per sampling site and distances between the sites were large. As such, these investigations should be repeated to gain a more complete picture of the burden on the local fish fauna. As they stand, these results only facilitate substantiated statements about the examined sites, because the burden in adjacent water sections could differ

significantly (Mani *et al.* 2015). Furthermore, additional fish species in the selected rivers and lakes should be examined; for example, the presented here from Lake Constance showed that piscivorous fishes significantly less burdened.

These current field investigations show that we still know very little about the complex pollution situation in our local waters. This lack of understanding is linked to one fundamental drawback: even now, there are no adequate methods for detecting microplastics, especially very small particles.

7.1.2. Isolation of microplastics from samples

A number of approaches allow the isolation of microplastics from an environmental sample. In principle, organic material must be removed from water samples, otherwise the identification of plastic particles is challenging (Cole *et al.* 2014; Hidalgo-Ruz *et al.* 2012). For sediment samples, mineral materials, such as stones and sand, must be separated from particles of potentially human origin (Van Cauwenberghe *et al.* 2015; Nuelle *et al.* 2014). For fish samples, organic components of the stomach or intestinal contents, or the entire gastrointestinal tract, must be dissolved first (Lusher *et al.* 2017; Collard *et al.* 2019). Depending on the method chosen, certain plastics or particle sizes may not be detected, which can lead to a further invalidation of the results.

Corrosive chemicals were established early on as a methodological compromise between efficacy and efficiency. Although the use of enzymes might seem an ideal method for complete digestion of all organic matter without harming the plastic particles, it is extremely time-consuming and cost-intensive for higher organisms (Lusher *et al.* 2017; Cole *et al.* 2014). In addition, the high number of processing steps means that there is a significant risk of particle loss and contamination. One regularly used and recommended method for the decomposition of organic matter is the use of potassium hydroxide (KOH, 10 %; Hermesen *et al.* 2018); compared to other approaches, this method has proven to be the most effective, while also not causing too much damage to the plastic particles (Dehaut *et al.* 2016; Lusher *et al.* 2017). However, the time required to completely digest organic material using potassium hydroxide is temperature dependent, ranging from 24 h at higher temperatures, to several weeks at lower temperatures. Unfortunately, higher temperature protocols can result in deformation and clumping of the very microplastics that they are designed to isolate (Hermesen *et al.* 2018).

In the present study, digestion time was substantially reduced by using the combination of a base (NaOH) and an acid (HNO₃). This made it possible to

increase the number of samples while both ensuring that no plastic particles were missed and minimising the number of processing steps. Nevertheless, all available methods have drawbacks, and are a factor that continues to hamper the comparability of studies (Lusher *et al.* 2017). For now, harmonisation of methods is still a long way off (Koelmans *et al.* 2020). However, it is crucial that new ways are found to effectively isolate microplastics from organic samples, and facilitate the identification of microplastics in different tissues. Investigating new approaches is becoming especially important now it is known that very small particles can translocate into fish tissues and organs (Collard *et al.* 2019); these particles are challenging to isolate as they are often fragile and easily missed. Currently available methods are either too aggressive (damaging the particles) or simply cannot digest the organic material effectively enough to isolate these small particles (Lusher *et al.* 2017).

7.1.3. Identification of microplastics

Even when conducting appropriate and thorough pre-processing of a sample, the human origin of the isolated particles must be verified (Hermesen *et al.* 2018). A purely visual inspection and identification of microplastics based on shape or colour has been shown to be insufficient, as inconspicuous particles are overlooked or particles of natural origin are not discarded (Wesch *et al.* 2016). Today, spectroscopic methods are favoured for the identification of microplastics. For particles $\geq 20\text{ }\mu\text{m}$, FTIR (often coupled with a microscope) is routinely used because it can reliably determine the polymer type and size of plastic debris (Löder *et al.* 2015). For smaller particles Raman spectroscopy can theoretically be utilised as it allows the identification of particles $<1\text{ }\mu\text{m}$. However, it is a prerequisite of Raman spectroscopy that the sample does not contain any impurities (*e.g.* biological material), as this would lead to interferences which impede further analysis (Löder & Gerdt 2015). Spectrograms obtained from FTIR or Raman are usually compared against a database of known polymer types, and can even be used to identify cellulose-based materials and dyes (Remy *et al.* 2015).

That said, these spectroscopic approaches have some major disadvantages: firstly, they require thoroughly trained personnel. Secondly, the required equipment is expensive to purchase and maintain. Lastly, the identification of all isolated particles is extremely time-consuming, which often means that only sub-samples are analysed (Koelmans *et al.* 2019). To overcome this, some efforts have been made to automate the analysis in order to reduce the time required and improve the overall quality and reliability of results (Primpke *et al.* 2017).

As the available spectroscopic methods are not yet able to provide the required sample throughput and accuracy, it was decided pragmatically that in the present studies isolated particles would be identified using the “hot needle” method. This method takes advantage of the fact that plastics are deformed or begin to melt when exposed to high temperatures, while mineral or plant materials do not (Devriese *et al.* 2015; Vandermeersch *et al.* 2015). Unfortunately, this does not allow the type of polymer to be determined. However, it is not known whether identification of the plastic type provides useful information, as strong fragmentation makes an assignment to a source extremely difficult. Furthermore, available spectroscopic methods do not provide any information on additives or adsorbed pollutants (Löder & Gerdt 2015).

Intensive work is still being done on alternative ways to identify microplastics. A practical and inexpensive approach is staining plastic particles with fluorescent dyes. Nile red is one promising stain because it binds to hydrophobic substances and thus stains plastic (Shim *et al.* 2016; Maes *et al.* 2017). So far, however, this method has only been tested on environmental samples. Whether it also works on biological samples such as fish has not been clarified. Regardless, here too contamination can lead to false-positive results, and less hydrophobic polymers might be overlooked (Maes *et al.* 2017; Erni-Cassola *et al.* 2017).

One of the most promising approaches is the identification of plastics through thermal degradation. Pyrolysis, coupled with a gas chromatography-mass spectrometry system (PY-GC-MS), allows the identification of very low concentrations of micro- and even nanoplastics in environmental and biological samples (Dümichen *et al.* 2017; Ter Halle *et al.* 2017; Fischer & Scholz-Böttcher 2017). However, no information is obtained on the number, colour, shape or size of the particles. It is therefore important that additional methods, such as asymmetrical flow field-flow fractionation (AF4), are used before conducting PY-GC-MS to obtain at minimum information on the number and size of recovered microplastics (Mintenig *et al.* 2018). How successful such complex systems are in praxis when using field samples remains to be seen.

7.1.4. The prevention of contamination

Contamination of environmental fish samples is a concern in all laboratories where microplastic samples are processed. As plastic has become indispensable and is used in all aspects of our lives, contamination of samples is practically unavoidable (Woodall *et al.* 2015). Although measures have been established to reduce contamination, airborne fibres in particular are regularly detected in procedural

blanks (Wright *et al.* 2021; Hermesen *et al.* 2018). It is therefore essential that negative controls are carried out in order to correct for any contamination that has occurred when evaluating the results.

For samples in which microplastic abundances are generally low, contamination can lead to erroneous results (Vandermeersch *et al.* 2015; Wesch *et al.* 2016). This is particularly relevant when examining the microplastic burden in the tissues or organs of fish. Some studies found larger microplastics of up to 600 μm in size in the liver of fish (Avio *et al.* 2015; Collard *et al.* 2017). As there is no extant explanation of how such large particles could translocate from the gastrointestinal tract, contamination is the most likely explanation (De Sales-Ribeiro *et al.* 2020; Jovanović *et al.* 2018). Contamination is also a major challenge when using highly sensitive identification methods for microplastics (such as PY-GC-MS) as exclusion based on shape, size and colour is not possible (Mintenig *et al.* 2018).

When examining the microplastic exposure levels of wild fish in the present study, several measures were taken to reduce any form of contamination. All digestion steps were carried out under a fume hood, synthetic-free clothing was worn at all times and all labware was rinsed with filtered ultrapure water. In addition, procedural blanks were used, which were processed similarly to the samples. The blanks were checked for plastic particles and their shape and colour was noted. Particles that had a similar appearance in the fish samples were excluded from any further analysis as a precaution. Together with the reduction of the digestion steps established in the newly developed method, this ensured that contamination had a negligible influence on the present results.

7.2. Future trends in the level of microplastic exposure

The present results in conjunction with other available studies on the burden of fish with microplastics suggest that current abundances are low, and therefore may not pose an imminent threat (Parker *et al.* 2021; Adam *et al.* 2019). However, as our utilisation of plastic continues to trend upward, the question arises: will microplastic burden increase in the near future (*i.e.* the next 30 years)? Currently, there are very few studies on the subject available. Although the production of plastic is expected to increase (Geyer *et al.* 2017; Jambeck *et al.* 2015), awareness of the problem and efforts to reduce the amount of plastic waste are also growing (Borrelle *et al.* 2020; Lau *et al.* 2020).

7.2.1. Long-term progression of the plastic pollution in the environment

The difficulty in predicting the long-term trajectory of plastic pollution on the environment lies in the large number of confounding factors, many of which are still only partly understood. To date, it has been difficult to assess all possible input pathways and quantify their emissions into the environment (Jambeck *et al.* 2015; Burns & Boxall 2018). Further to this, losses of microplastics into the environment are not yet well studied; the seafloor, as well as lake and river sediments, are considered important sinks (Woodall *et al.* 2014; Vaughan *et al.* 2017; Tibbetts *et al.* 2018), but how much of plastic waste is residing there is almost impossible to estimate. Finally, the vertical movement of plastic particles within a water body is ambiguous, as biofouling can change the density of individual polymer types (Kooi *et al.* 2017). As described previously, due to inconsistent use of available methods for detecting microplastics, we have an incomplete picture of the current microplastic burden that impedes estimation of global trends (Galgani *et al.* 2021).

That the amount of plastic waste found in the environment has increased in the second half of the last century is evident. The number of plastic fragments and fibres detected in zooplankton samples from the Atlantic Ocean increased significantly between 1960 and 1990 (Thompson *et al.* 2004). Brandon *et al.* (2019) found an exponential increase in microplastics in sediment cores between 1945 and 2009, with a doubling rate of 15 years. A significant increase in plastic particles was also detected in the western North Atlantic between 1986 and 2015 (Wilcox *et al.* 2020). Additive statistical models using this dataset showed that the input of plastic waste is higher than all losses combined (*e.g.* sinking, washing up on beaches, etc.), which suggests that the release of plastic waste into the ocean is not declining (Wilcox *et al.* 2020). A comparable assessment was also made by Borrelle *et al.* (2020), who concluded from their calculations predicting the growth of global plastic waste up until 2030 that efforts to reduce emissions will not be sufficient to reduce pollution of the environment in the near future. For remote regions in particular, the burden of plastic waste will continue to increase sharply, and will likely have a long-term effect on the ecosystems (Barnes *et al.* 2009; Horton & Barnes 2020). By contrast, many short-term datasets from the beginning of the 21st century no longer show a clear increase in plastic pollution during the last 10–20 years. No temporal trends in plastic pollution were found between 2001 and 2011 in the OSPAR beach litter monitoring dataset in the North-East Atlantic (Schulz *et al.* 2013), nor in beach sediments and water samples in Chinese seas

between 2007 and 2014 (Zhou *et al.* 2016) or in floating marine debris at the coast of the Balearic Islands between 2005 and 2015 (Compa *et al.* 2019).

Unfortunately, there is very limited data available examining the long-term exposure levels of aquatic organisms. So far, there are only three studies, all of which examined samples from long-term surveys or collections from museums. A case study from the Baltic Sea showed no increase in the microplastic burden in either plankton samples or two fish species between 1987 and 2015 (Beer *et al.* 2018). Similarly, a study examining two deep-sea benthic invertebrates found no increase in microplastics between 1967 and 2015 (Courtene-Jones *et al.* 2019). In contrast, Hou *et al.* (2021) found a clear increase in microplastic abundance in several freshwater fish species between 1950 and 2018 from the urban area of Chicago. The concentration of microplastics increased in parallel with global plastic production and changes of plastic pollution found in other ecosystems (Hou *et al.* 2021). Unfortunately, comparison between studies is difficult, as the study designs were vastly different. It therefore remains unclear how the pollution of the environment with plastic, and therefore microplastics, will progress. Given the many limitations in the detection of microplastics in environmental samples and water organisms, whether currently available data allows us to make any statement at all about the present microplastic burden is fundamentally questionable.

7.2.2. The particle size factor

The results of the present thesis highlight the outstanding importance of particle size as a factor in the research of microplastics, while also delineating the clear limitations of current methods for the detecting small particles. However, information about particle size is essential for a comprehensive assessment of the detrimental effects of microplastics on fish and other water organisms. Although there is much discussion about future developments in microplastic pollution of the environment, the fraction of very small microplastics – which is currently very difficult to measure – is rarely considered. Only recently have a growing number of studies recognised this problem and, furthermore, provided evidence that environmental abundance increases dramatically with smaller particle size.

A recent literature review revealed that ~80 % of surveys use a mesh size >300 µm when examining the microplastic burden in water samples (Conkle *et al.* 2018). Lindeque *et al.* (2020) have demonstrated that using nets with this mesh size results in significant underestimation of microplastic abundance; a reduction in the mesh size from 333 µm to 100 µm resulted in a 2.5-fold higher particle

concentration. In addition to this, larger mesh sizes increase the risk that particles with a small width, such as fibres, will not be collected (Covernton *et al.* 2019). Many primary plastics, *e.g.* beads in personal care products, are much smaller than 300 μm and so will be overlooked in most studies (Conkle *et al.* 2018).

Accordingly, smaller particles are expected to be more prevalent in aquatic organisms. In the present study it was demonstrated that in the local fish fauna, particles were more abundant with smaller size, following a power law. These data facilitated extrapolation of theoretical particle abundances for size classes below the study detection limit. Merga *et al.* (2020) followed a similar approach and showed a comparable increase of particle abundance with smaller size in four fish species and sediment samples from an African lake. A meta-analysis of available datasets showed that a power law often accurately predicts the size distribution of microplastics in the environment (Kooi & Koelmans 2019). This follows other findings: similar particle frequency distributions are regularly found for different particulate matters in the environment (Kavanaugh *et al.* 1980). Such insights should be accounted for when estimating the abundance of microplastics in the environment or calculating future developments. What is certain is that with the use of more sophisticated methods, it will be possible to lower detection limits and thus detect higher microplastic abundances in the future. At the same time, the input of plastic waste will continue to increase or at least stagnate at the current level (Borrelle *et al.* 2020). Together with the ongoing fragmentation of already discharged plastic waste, there will almost certainly be an increase in plastic pollution in the near future, especially when considering small particle sizes. One size-related topic beyond the scope of this thesis is the occurrence of nanoplastics in the environment. Nanoplastics are formed by the further fragmentation of microplastics, but can also enter aquatic systems directly, *e.g.* via medical products (Koelmans *et al.* 2015). Laboratory studies show that nanoparticles can have far-reaching effects on organisms (*e.g.* Chae *et al.* 2018; Mattsson *et al.* 2017). However, as yet there are virtually no methods for detecting them in the environment (Koelmans *et al.* 2015).

7.2.3. Ways to reduce microplastic pollution

As interest increases about the topic of microplastics in research, the media and society, the discussion has ensued on how to reduce the microplastic burden in the environment (Moore 2008). The most obvious approach is to reduce emissions of plastic waste into the environment. There are countless national and international efforts to facilitate this; for example, bans on single use plastics, incentives to reuse

and recycle, or support for a more circular economy for plastic materials (Eriksen *et al.* 2018). Concurrently, there are efforts to reduce the release of microplastics from certain point sources into the environment. For example, wastewater treatment plants (WWTPs) are quite effective at reducing microplastic burden of wastewater, though also are still considered an important contributor (Murphy *et al.* 2016). A recent study calculated that in Germany, the median microplastic burden from WWTPs was 7×10^{12} items a year (Schmidt *et al.* 2020). Additional cleaning steps, such as membrane filtration, can reduce the release of microplastics, but necessitate high investment costs and are sometimes technically difficult to implement (Conley *et al.* 2019; Liu *et al.* 2021).

Another approach is to remove plastic waste directly from the environment. When the so-called "garbage patches" in subtropical gyres became known to the general public, a number of projects were initiated aiming to recover the waste directly from the ocean (Eriksen *et al.* 2018). However, it is questionable whether such efforts are feasible or beneficial; they can have a strong impact on the ecosystem, often have not been tested under real conditions, and are economically challenging (Cordier & Uehara 2019; Sherman & van Sebille 2016; Rochman 2016). Nevertheless, programmes to actively remove plastic waste from surface waters and on beaches, especially for larger items (*e.g.* fishing nets), are seen as a necessary measure that should be used until the release of plastic waste is significantly reduced (UN Environment 2017; Wurpel *et al.* 2011).

That said, these efforts are very unlikely to lead to a significant reduction in microplastic pollution of the environment (Borrelle *et al.* 2020). As such, this problem will continue to grow and, critically, have an increasing impact on aquatic fauna. In addition to improving detection methods to more accurately estimate and predict exposure levels, it is essential that their impact is studied under controlled conditions in order to conduct any valid assessment of risk.

7.3. Experimental challenges when studying negative effects of microplastics

Once the extent of environmental pollution with microplastics became undeniable, research began to address the question of whether an inert material like plastic could have detrimental effects on aquatic organisms (Thompson *et al.* 2004). Given the diversity of the plastic pollutants, there are numerous different challenges facing the conduct of controlled experiments in the laboratory. Firstly, there are a wide variety of available microplastic polymer types, shapes and sizes, which may

also contain a number of different chemical additives (Andrady & Neal 2009). Then, there is the question of what microplastic concentrations should be used, and how they should be administered to the organism (de Sá *et al.* 2018). Finally, appropriate experimental methods and endpoints must be determined (Connors *et al.* 2017; Phuong *et al.* 2016). All of these challenges, and the very different approaches to solving them, mean that data on the impact of microplastics on aquatic organisms has so far been quite inconsistent, leading to an increased risk of drawing incorrect or biased conclusions.

7.3.1. The gap between real-world conditions and laboratory experiments

The discrepancy between microplastic composition and exposure levels in laboratory experiments versus in the environment was quickly recognised as a concern. In many studies, virgin spherical plastic particles of only one type and size are used (de Sá *et al.* 2018). Additionally, concentrations are often two to nine orders of magnitude higher than those encountered in the environment (Lenz *et al.* 2016; Connors *et al.* 2017). Furthermore, according to a meta-analysis, 80 % of included studies used particle sizes smaller than those currently observed in environmental samples (Bucci *et al.* 2020). It is imperative that these limitations are taken into account and critically discussed when evaluating and interpreting study results, as they can easily lead to misunderstandings, especially among the public (Lenz *et al.* 2016). At the same time, it must also be clearly stated that due to the limitations of the currently available detection methods and lack of standardisation, exposure levels in environmental samples cannot reliably be determined (Hermsen *et al.* 2018). Therefore, future studies must consider new approaches within standard regulatory frameworks in order to facilitate an airtight assessment of risk, and with it the identification of the most harmful polymers, shapes, sizes, and so on (Connors *et al.* 2017; Burns & Boxall 2018).

In the present exposure experiments, efforts were made to ensure that the microplastic exposure of the experimental fish was as realistic as possible. The artificially produced particles used in the exposure experiments were of the same size range (around 20 to 1000 µm) as those detected in the examined field samples. In addition, small particles were more abundant than larger ones. The number of ingested particles per fish was approximately 14 particles, which is much lower than in many other ecotoxicological studies (Anbumani & Kakkar 2018), and reflects relatively well the reported exposure of fish in the environment (Collard *et al.* 2019; Parker *et al.* 2021). The experimental fish were also only exposed to

microplastics every two days, to allow excretion of the particles before re-exposure. Finally, unlike many other studies, the duration of the experiment was relatively long at 17 weeks (Anbumani & Kakkar 2018), which allowed a true long-term effect analysis in a realistic exposure scenario. As a result, the present study is one of the first of its kind to find a negative dose-dependent effect on growth and other performance parameters using realistic particle concentrations. Such effects have previously only been observed at substantially higher exposure levels (Naidoo & Glassom 2019; Yang *et al.* 2020).

Another issue that is rarely considered in laboratory experiments at present is the weathering or biofouling of plastic particles. Biofouling occurs when plastic debris remains in the water for a certain time, microorganisms and invertebrates are able to settle on its surface (Reisser *et al.* 2014). Weathering alters the odour of plastic particles, which may lead to increased ingestion by water birds and fish (Savoca *et al.* 2016; Savoca *et al.* 2017). Mediterranean mussels (*Mytilus galloprovincialis*) have exhibited similar behaviour, taking up significantly more weathered particles by weight than virgin ones (Brate *et al.* 2016). These findings are especially relevant to the results of the present study, which examined the uptake pathways of microplastics in fish and revealed that sensory-oriented benthic fish species (such as carp) are able to detect and actively avoid non-digestible items. Future experiments need to clarify whether this is due to a more pronounced sense of taste or because of other factors, such as particle hardness and size. Whether the weathering of plastic particles hampers the ability of fish to distinguish between edible and non-edible items should also be assessed, as it could lead to an increased uptake of microplastics. It is apparent that there is a general need for studies that align with real-life conditions, such as mesocosms, in order to better assess the impact of microplastics on fish (Connors *et al.* 2017).

7.3.2. New insights from next-generation approaches

A number of established methods are currently available to study the adverse effects of pollutants; experimental endpoints, such as mortality, growth or fecundity, are typically investigated (Austin 1998; Crossin *et al.* 2014; Connors *et al.* 2017). However, to better understand the mechanisms behind certain effects, new approaches are being developed and increasingly applied. In particular, "omics" approaches (*e.g.* genomics, transcriptomics, metabolomics, proteomics) are able to identify complex mechanisms of action, detect alterations between samples and generally improve understanding of how certain toxicants affect an organism and a variety of physiological layers (Van Aggelen *et al.* 2010; Tomanek 2011). This

is made possible by the technical advances in the field of mass spectroscopy over the last decades (Chalmers & Gaskell 2000). In the field of proteomics, for example, thousands of proteins can be identified and quantified simultaneously in one sample (commonly referred to as “shotgun proteomics”; Zhang *et al.* 2013). Nowadays, there are label-free techniques, which reduce processing time and costs but still allow quantification of individual proteins (Tate *et al.* 2013). However, these approaches generate a large amount of data, which must be analysed using advanced statistical algorithms (Tomanek 2011) and suitable follow-up experiments. This is often seen as the main limitation of these approaches, as the interpretation of the results strongly depends on the bioinformatics tools used (Yu *et al.* 2004).

Proteomics is a tool used more and more in ecotoxicological studies, including in research on the detrimental effects of microplastics on aquatic organisms (Liu *et al.* 2020). Most available studies to date are confined to experiments with mussels. These showed that microplastics influence molecular processes including energy metabolism, RNA/DNA binding and immune regulation (Green *et al.* 2019; Sussarellu *et al.* 2016; Magni *et al.* 2019). Before the present study, the proteome of fish exposed to microplastics had not been investigated. Although this meant that there was no comparator for the results, they provide a valuable basis for future investigations. For example, there are signs that even low concentrations of microplastics affect transport processes or barrier functions in liver cells after a long-term exposure. Furthermore, individual proteins, such as *drtp1*, were identified as potential biomarkers and require more study. However, it is important that these next-generation approaches are used in conjunction with “traditional” experimental endpoints in order to assess the risk within standard regulatory frameworks (Connors *et al.* 2017; Miracle & Ankley 2005).

7.3.3. Is the popularity of the subject affecting the quality of research?

With the increasing interest of the media, politicians and the general public in the topic of microplastics, the pressure on the scientific community to provide reliable data and assessments of their harmfulness has also increased. This has led to the implementation of numerous projects, resulting in a substantial increase in publications in recent years (see Fig. 1.2(B) in ‘General introduction’). However, this has also led to a number of critical voices raising concerns about the lack of standardisation, and questioning the quality standards of certain studies (Provencher *et al.* 2020). As previously discussed, numerous challenges complicate

the detection of microplastics in the environment and increase the likelihood of misinterpretation of the data obtained. One example is the detection of microplastics in drinking water and related freshwater sources. A review by Koelmans *et al.* (2019) found that only four of the 50 examined studies met all of their proposed quality criteria, which included an assessment of sampling methods, sample processing, contamination prevention and the use of controls. The authors concluded that the lack of high quality data to date necessitates a comprehensive and standardised estimation of exposure levels, and that improved quality assurance is urgently needed in future studies (Koelmans *et al.* 2019).

Similar concerns have been raised about studies of the physical effects of microplastic particles in fish. Several studies have claimed that exposure to microplastics results in significant histopathological damage in the gut, or an overall reduction in gut integrity (Lu *et al.* 2016; Lei *et al.* 2018; Ahrendt *et al.* 2020). However, in the case of Lu *et al.* (2016), inconsistencies and fundamental errors in data interpretation were found by a group of pathologists (Baumann *et al.* 2016). Other studies have failed to prove physical harm, and attributed previous results to incorrect sample preparation and *post-mortem* autolytic processes (Batel *et al.* 2020; De Sales-Ribeiro *et al.* 2020).

One case that caught the attention of the scientific community was a study published in the renowned journal “Science” on the impact of microplastics on larval fish ecology (a citation is not provided for the reasons stated below). The results clearly showed, among other things, how microplastics influence predator-prey interactions in perch, and the study was used to demonstrate the significant ecological effects of microplastic pollution. However, an investigation later revealed that the experiments had no ethical approval and that parts of the experimental data had been fabricated (Berg 2017a). Ultimately, this led to a retraction of the article (Berg 2017b), but it continues to be cited today (Carney Almroth *et al.* 2020). This is dangerous because nowadays scientific results are directly interpreted by the media and used by policy-makers, and furthermore, such cases of unethical behaviour cast science and research in a bad light (da Costa 2018; Kramm & Völker 2018; Katavić 2014). Although the explanation for this incident is not known, it demonstrates how the popularity of a topic, combined with the opportunity or pressure to publish in high impact journals, might increase the risk of fraudulent research (Franzen *et al.* 2007). Quality assurance and scientific integrity are essential when researching and publishing data on microplastic exposure, as these data can have a direct impact on environmental policies and assessments of risk to human health.

7.4. The impact of microplastics on human health

Several studies have detected microplastics in commercially important aquatic species used for human consumption (Barboza *et al.* 2018; Santillo *et al.* 2017; Van Cauwenberghe & Janssen 2014). Concerns quickly emerged that this might pose a pathway for plastic particles to enter humans, and therefore, increase the risk of humans being exposed to adsorbed organic pollutants or chemical additives (Thompson *et al.* 2009). Furthermore, physical effects, such as inflammation, immune responses and oxidative stress are seen as possible responses to plastic particle exposure, which might lead to tissue damage or carcinogenesis (Wright & Kelly 2017).

The transmission of toxic substances to humans has been a big concern to both the public and researchers in recent years. This could be due to the fact that there is already a lot of information about the toxicity of these substances available (Thompson *et al.* 2009; Bouwmeester *et al.* 2015). However, whether the quantity of microplastics ingested via food is high enough to transmit and release relevant concentrations of adsorbed pollutants into the human body is questionable. Van Cauwenberghe & Janssen (2014) calculated that European consumers, who regularly eat mussels, ingest between 1,800 and 11,000 microplastic particles per year, dependent on the weight of mussels consumed (11.8 g up to 72.1 g per day, respectively). The theoretical annual intake of bisphenol A (BPA; one of the most widespread additives in plastics) for a person who consumes 72.1 g of mussels a day has been estimated based on the highest concentration of BPA found on microplastics in the environment. Per this estimate, the amount of BPA transmitted via eating seafood was 40 million time lower than that from exposure to other sources (*e.g.* through food packaging; Rist *et al.* 2018).

When considering fish as food, the risk of ingesting microplastics appears much lower, as particles are mainly detected in the fish gastrointestinal tract (Lusher *et al.* 2017). Since in most cases only the fillet of fish is consumed, where particle numbers are generally low, the risk of transfer were thought to be negligible (Cunningham *et al.* 2020). However, an increasing number of studies have shown particle translocation from the gastrointestinal tract, fuelling concern that edible parts of fish could also be contaminated with plastic particles. Indeed, some studies have detected microplastics in muscle tissue (Abbasi *et al.* 2018; Jovanović *et al.* 2018), although others found no evidence (De Sales-Ribeiro *et al.* 2020). As discussed, the currently available data from studies do not yet allow a substantiated statement on how severely certain organs and tissues of fish are affected. Nevertheless, this form of exposure must be taken into account in future

assessments of the effects of microplastics on humans, as current detection limits might underestimate microplastic burden.

In summary, based on the available data, seafood seems an unlikely pathway for microplastics and related toxic chemicals to reach humans. It is also becoming apparent that much discussion around this topic does not account for the degree of exposure to plastic and microplastics in our daily lives. For example, humans inhale airborne plastic fibres (which are ubiquitously present indoors and outdoors) to a much higher extent than they are likely to be ingested directly via food (Vianello *et al.* 2019; Wright *et al.* 2020; Dris *et al.* 2017). Unfortunately, a lack of quality standards and limitations in particle size detection have thus far prevented a robust estimation of the abundance and toxicity of airborne microplastics (Wright *et al.* 2021). Another source of microplastics is the packaging for food or drinks, which can contain and release harmful chemicals in relevant quantities (Muncke *et al.* 2020). For this reason, it seems logical to bring these direct contacts with plastics to the forefront of future research when addressing human health impacts.

8. Conclusions and future directions

The present thesis provides novel insights into the current burden of microplastics on local freshwater fish, and contributes to the understanding of microplastic uptake pathways and behaviour in the gastrointestinal tract. Critically, it also shows that realistic concentrations of microplastics have a relevant impact on fish performance. Another key result is the fundamental significance of particle size. It is imperative that this mostly overlooked factor should be given greater consideration in future studies. Particle size has an important influence on all research areas, be it in determining exposure levels, interactions with fish or evaluating negative effects on health. So long as the entire size spectrum is not fully considered, and in particular if small particles are completely excluded, current exposure and its potential threat is almost certainly considerably underestimated.

Generally, the present results have demonstrated that microplastic research is still in its infancy. Many important challenges are still unaddressed: lack of standardisation, imprecise and inaccurate methods for microplastic detection and insufficient quality/sampling standards. Fortunately, these issues are increasingly recognised in the scientific community and solutions are being developed. Hopefully, this will make it possible to conduct consistent and well-founded

assessments of risk and, based on these, develop appropriate measures that reduce the impact of microplastics on the environment. It is vital that the limitations of current methodologies are clearly communicated by the scientific community, so that the society and decision-makers are able to understand the research and its implications. Microplastics show the lasting and profound impact humans have on the environment. At present, there are no indications that the threat is receding. Therefore, it will continue to occupy the scientific community and also the public for a long time to come. As with other complex issues that have global implications, science has a special responsibility to fulfil.

9. Publication and presentation list

List of peer-reviewed publications related to the present thesis:

Roch S., Brinker A. (2017). Rapid and Efficient Method for the Detection of Microplastic in the Gastrointestinal Tract of Fishes. *Environmental Science & Technology* **51**, 4522-4530. DOI: 10.1021/acs.est.7b00364

Roch S., Walter T., Ittner L. D., Friedrich C., Brinker A. (2019). A systematic study of the microplastic burden in freshwater fishes of south-western Germany - Are we searching at the right scale? *Science of the Total Environment* **689**, 1001-1011. DOI: 10.1016/j.scitotenv.2019.06.404

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Roch S., Rebl A., Wolski W., Brinker A. (In preparation). Combined proteomic and gene expression analysis to investigate reduced performance in rainbow trout (*Oncorhynchus mykiss*) caused by environmentally relevant microplastic exposure.

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10. Author contributions

Manuscript I: Rapid and Efficient Method for the Detection of Microplastic in the Gastrointestinal Tract of Fishes

Authors: Samuel Roch^{1,2} (SR), Alexander Brinker^{1,2} (AB)

SR and **AB** designed the study; **SR** conducted the investigation; **SR** and **AB** conducted the statistical analysis; **SR** drafted large parts of the manuscript; **AB** contributed to writing and editing the manuscript; **AB** supervised the study.

Manuscript II: A systematic study of the microplastic burden in freshwater fishes of south-western Germany - Are we searching at the right scale?

Authors: Samuel Roch^{1,2} (SR), Thomas Walter¹ (TW), Lukas D. Ittner¹ (LI), Christian Friedrich³ (CF), Alexander Brinker^{1,2} (AB)

SR and **AB** designed the study; **SR**, **TW** and **LI** conducted the investigation; **All authors** conducted the statistical analysis; **SR** drafted large parts of the manuscript; **CF** and **AB** contributed to writing and editing the manuscript; **AB** supervised the study.

Manuscript III: Uptake routes of microplastics in fishes: practical and theoretical approaches to test existing theories

Authors: Samuel Roch^{1,2} (SR), Christian Friedrich³ (CF), Alexander Brinker^{1,2} (AB)

SR and **AB** designed the study; **SR** conducted the investigation; **All authors** contributed to the statistical analysis; **SR** drafted large parts of the manuscript; **CF** and **AB** contributed to writing and editing the manuscript; **AB** supervised the study.

Manuscript IV: Microplastic evacuation in fish is particle size-dependent

Authors: Samuel Roch^{1,2} (SR), Albert F. H. Ros¹ (AR), Christian Friedrich³ (CF), Alexander Brinker^{1,2} (AB)

SR and **AB** designed the study; **SR** conducted the investigation; **All authors** contributed to the statistical analysis; **SR** drafted large parts of the manuscript; **AR**, **CF** and **AB** contributed to writing and editing the manuscript; **AB** supervised the study.

Manuscript V:

Authors: Samuel Roch^{1,2} (SR), Alexander Rebl⁴ (AR), Witold Wolski⁵ (WW), Alexander Brinker^{1,2} (AB)

SR and **AB** designed the study; **SR, AR and WW** conducted the investigation; **All authors** contributed to the statistical analysis; **SR** drafted large parts of the manuscript; **All authors** contributed to writing and editing the manuscript; **AB** supervised the study.

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