

Microplastics in the Aquatic Environment

Insights into Biological Fate and Effects in Fish

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MICROPLASTICS IN THE AQUATIC ENVIRONMENT: INSIGHTS INTO BIOLOGICAL FATE AND EFFECTS

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In the memory of my Mother

Dissertation abstract

According to the United Nations, plastic pollution in the natural environment has been identified as one of the biggest environmental challenges of this century and has become a cause for an emerging international concern. It has been predicted that up to 12 million tons of plastic waste reach the aquatic environment annually. Therein, UV-radiation induced photo-oxidation, mechanical weathering and biological degradation contribute to the fragmentation of plastic litter to the micro- or even nanoscale. Microplastics (MPs) thus have become prominent pollutants in the aquatic environment, and their prevalence has been documented in every aquatic ecosystem studied. MPs enter aquatic food webs, also reaching humans, the top consumers in the food chain.

The omnipresence of small microscopic plastic particles in the aquatic environment presents several ecotoxicological concerns. Firstly, MP fragments can interact with aquatic organisms and act as physical or mechanical stressors. Secondly, MPs can be toxic, as some polymers consist of potentially hazardous monomers. Synthetic, petroleum-derived polymers can also contain functional additives, impurities or chemical residuals, which are not chemically bound to the polymeric material and thus have the potential to leach out and cause diverse toxicological effects. Lastly, plastic polymers are known to absorb persistent hydrophobic organic pollutants from the environment. MPs have been suggested to act as vectors of environmental contaminants into organisms, promote bioaccumulation of toxic compounds, and cause biological effects in aquatic biota. It remains widely debated whether MPs are important vectors of chemicals for aquatic animals, including fish, and whether MP ingestion by edible fish species can impact human food quality and safety. This PhD project addressed some of these prevailing concerns, and investigated biological fate and impacts of MPs and associated chemicals in fish.

It has been shown that exposure route can play an important role in particle-organism interactions and can determine the organismal uptake and localization of plastic particles in fish [**Paper I**]. Plastic nanoparticles interact with aquatic organisms: they can enter fish via contaminated prey (trophic transfer) and they can be directly ingested and/or adhere to organismal surfaces. Ingested nanoplastics can accumulate in the gastrointestinal tract and can then be internalized by the intestinal cells. Plastic ingestion is regarded as an environmentally relevant particle pathway in fish, and it facilitates their entrance into aquatic food chains.

Studies included in this thesis also explored biological effects derived from the ingestion of larger, micro-sized plastic particles, at sizes commonly extracted from biological and environmental matrices, and which entail environmentally relevant chemical exposures [**Papers II-III**]. Direct impacts resulting from MP ingestion were found to be negligible, as no adverse effects were observed on fish intestinal physiology. Indirect, chemical exposure related effects resulting from ingestion of contaminated MPs were also minor. No indications of hepatic stress (oxidative stress, detoxification, endocrine disruption) were observed. It was concluded that MPs did not act as mechanical and chemical hazards upon ingestion, and are unlikely to cause adverse effects on organismal health. Although MPs showed capacity to associate with environmental contaminants [**Papers II-IV**], the transfer of pollutants from particles into fish via ingestion, as well as accumulation and biological impacts were suspected to be low [**Papers II-IV**]. The early findings presented in this thesis suggest that ingestion of MPs by commercial fish species does not significantly diminish the oxidative stability of commercial fish products, and MP-mediated chemical exposure does not pose an evident concern for human food quality and product shelf-life.

Keywords: Microplastics, nanoplastics, plastic pollution, effects, environmental chemicals, chemical mixtures, ingestion, ecotoxicology, fish, vector effects

Sammanfattning

Förenta nationerna har identifierat plastförorening i den akvatiska miljön som en av världens största miljöutmaningar vilket har lett till en växande internationell oro. Det har förutsetts att upp till 12 miljoner ton plastavfall når den akvatiska miljön varje år. Därefter bidrar UV-strålningsinducerad foto-oxidation, mekanisk förväxling och biologisk nedbrytning till fragmentering av plastmaterial till fragment på mikro- eller nanoskala. Mikroplaster har således blivit vanligt förekommande föroreningar i den akvatiska miljön och deras förekomst har dokumenterats i akvatiska ekosystem över hela världen. Mikroplaster finns idag i livsmedel vi får från havet, inklusive fisk, vilket har lett till en oro för att plasten även ska tas upp av människor som konsumerar dessa livsmedel.

Närvaron av mikroskopiska plastpartiklar i vattenmiljön leder till flera ekotoxikologiska problem. För det första kan plastbitar interagera med vattenorganismer och fungera som fysiska eller mekaniska stressorer. För det andra kan små plastpartiklar vara giftiga. Syntetiska polymerer kan innehålla föroreningar, produktionsrester, eller funktionella tillsatsmedel, vilka inte är kemiskt bundna till det polymera materialet och därmed har en potential för att läcka ut och leda till toxikologiska effekter. Slutligen har man funnit att plastpolymerer kan absorbera persistenta hydrofoba organiska föroreningar. Små plastfragment kan verka som vektorer och föra in miljöföroreningar i organismer, främja bioackumulering av giftiga ämnen och orsaka negativa effekter i vattenlevande biota, inklusive fisk. Trots den senaste tidens framsteg på fältet är möjliga interaktioner, de biologiska effekterna och de ekologiska konsekvenserna av mikroplaster i vattenmiljön fortfarande i stort sett okända. Det är inte heller klarlagt om mikroplaster kan påverka livsmedelskvalitet och säkerhet för människor. Detta doktorandprojekt fokuserade på några av dessa rådande ekotoxikologiska problem kring mikroplast och undersökte det biologiska ödet och effekterna av mikroplaster samt tillhörande kemikalier i fisk.

Det har visats att exponeringsvägen kan spela en viktig roll för interaktioner mellan partiklar och organismer vilket kan bestämma upptag och lokalisering av partiklarna i organismen. Plastpartiklar på nano-skala interagerar med vattenlevande organismer och kan tas upp via förorenad föda, direktupptag eller genom vidhäftning på organismerna. Plast på nano-skala som tas upp via födan ackumuleras i mag-tarmkanalen, och kan därefter tas upp av tarmceller. Plastintag i fisk anses vara en miljömässigt relevant väg för partikelupptag till akvatiska näringskedjor.

Studierna som ingår i denna avhandling undersökte även biologiska effekter som orsakats av intag av större partiklar i mikroskala, som vanligen extraheras från biologiska prover och fältprover, och som kan föra med miljöföroreningar. Den direkta biologiska effekten hos mikroplaster som tagits upp via födan visade sig vara försumbar, eftersom inga negativa effekter observerades hos fiskens tarmfysiologi. Slutsatsen drogs att mikroplaster i den storleksklassen inte fungerade som mekaniska och kemiska risker vid intag, och kan inte orsaka negativa effekter på organismens hälsa. Indirekta kemiska exponeringsrelaterade effekter som härrör från förtäring av förorenade mikroplaster var också mindre. Inga indikationer på leverstress (oxidativ stress, avgiftning, endokrina störningar) observerades. Även om mikroplaster visade förmåga att associera med miljöföroreningar, så var misstänks överföringen av föroreningar från partiklar till fisk via föda, samt ackumulering och biologiska effekter vara låg, vilket leder till slutsatsen att vektor-effekter på biota har liten betydelse. Vad gäller livsmedelskvalitet tyder resultaten på att intag av mikroplaster inte signifikant påverkar kvaliteten av kommersiella fiskprodukter, och att den kemiska exponeringen inte utgör en uppenbar risk för kvalitet och hållbarhet hos livsmedelsprodukterna.

Nyckelord: Mikroplastik, nanoplastik, plastförorening, effekter, miljökemikalier, kemiska blandningar, intag, ekotoxikologi, fisk, vektoreffekter

List of publications

This doctoral thesis is based on the following publications and a manuscript.

The articles are referred to their Roman numerals in the text.

- I. Skjolding, L. M.*, **Ašmonaitė, G.***, Jølck, R. I., Andresen, T. L., Selck, H., Baun, A., Sturve, J. An assessment of the importance of exposure routes to the uptake and internal localisation of fluorescent nanoparticles in zebrafish (*Danio rerio*), using light sheet microscopy, *Nanotoxicology*, 11:3, 351-359, **2017**

*authors contributed equally

- II. **Ašmonaitė, G.**, Sundh, H., Asker, N., Carney Almroth, B. Rainbow trout maintain intestinal transport and barrier functions following exposure to polystyrene microplastics. *Environmental Science and Technology*, 52:24, 14392-14401, **2018**
- III. **Ašmonaitė, G.**, Larsson, K., Undeland, I., Sturve, J., Carney Almroth, B. Size matters: ingestion of relatively large microplastics contaminated with environmental pollutants posed little risk for fish health and fillet quality. *Environmental Science and Technology*, 52:24, 14381-14391, **2018**
- IV. **Ašmonaitė, G.**, Tivefålh, M., Westberg, E., Magnér, J., Backhaus, T., Carney Almroth B. Microplastics as a vector for exposure to hydrophobic organic chemicals in fish: a comparison of two polymers and silica particles spiked with three different model compounds, *Manuscript*, **2019**

Publications and a report not appended in this thesis:

Ašmonaitė, G., Carney Almroth, B. Effects of microplastics in organisms and impacts in the environment: balancing between the known and unknown. Swedish Environmental Protection Agency (Naturvårdsverket), Report, **2019**

Asnicar D., **Ašmonaitė, G.**, Birgersson L., Kvarnemo C., Svensson O., Sturve J. Sand goby - an ecologically relevant species for behavioural ecotoxicology, *Fishes*, 3, 1-18, **2018**

Ašmonaitė, G., Boyer, S., de Souza, K. B., Wassmur, B., Sturve, J. Behavioural toxicity assessment of silver ions and nanoparticles on zebrafish using a locomotion profiling approach. *Aquatic Toxicology*, 173, 143–153, **2016**

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List of abbreviations

AChE	Acetylcholine esterase
AOP	Adverse outcome pathway
B α P	Benzo(α)pyrene
CPS	Chlorpyrifos
CYP	Cytochrome P450
DLS	Dynamic light scattering
EE2	17 α -ethynylestradiol
ER	Estrogen receptor
FTIR	Fourier transform infrared spectroscopy
HDPE	High-density polyethylene
HOC	Hydrophobic organic chemical
HPLC	High-performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
LC/MS-MS	Liquid chromatography–mass spectrometry
LDPE	Low-density polyethylene
Log K _{ow}	Octanol-water partitioning coefficient
LSM	Light sheet microscopy
MP	Microplastic
NNP	Nanoplastic
NP	Nanoparticle
PA	Polyamide
PAH	Polycyclic aromatic hydrocarbon
P _{app}	Apparent permeability coefficient
PBDE	Polybrominated biphenyl ether
PBTs	Persistent bioaccumulative toxic substances
PCB	Polychlorinated biphenyl
PE	Polyethylene
PET	Polyethylene terephthalate
POP	Persistent organic pollutant
PP	Polypropylene
PS	Polystyrene
PVC	Polyvinylchloride
qPCR	Quantitative polymerase chain reaction
SCC	Short circuit current
SEM	Scanning electron microscopy
TEP	Transepithelial potential
TER	Transepithelial resistance
T _g	Glass transition temperature
TJ	Tight junctions
UPLC	Ultra Performance Liquid Chromatography
VTG	Vitellogenin
ZF	Zebrafish

1 Introduction

1.1 Plastics in modern society

Plastics entail an extremely large group of very different materials with distinctive properties and applications¹. Plastics are made of polymers, which are long, repeated chains of low molar mass units (monomers), and chemical additives. Plastics are easily molded and shaped, especially under heat and pressure². The existing diversity of synthetic polymers has led to a broad spectra of applications ranging from packaging, fabrics and coatings to medical, automotive, construction and space applications. Plastics are lightweight, versatile, durable and low-cost materials. In contemporary society, plastics have become an indispensable part of everyday life. They provide numerous societal benefits ranging from health and safety and infrastructure elements to energy and material preservation².

Since the 1930s and 1940s, when the mass production of plastics began, global plastic production has been steadily increasing³ (**Figure 1**). The estimated annual global production volume in 2010 exceeded 200 million tons and is expected to proceed with continuous rapid growth in the foreseeable future (**Figure 1**). To a large extent, plastics have replaced many conventional materials, such as wood, glass and ceramics, and dominate the market⁴. While there are 50 or more chemically distinct classes of plastics, only a few plastic polymer types are commonly used as commodity polymers and are produced in high volumes⁵. Such plastic polymers include polyethylene (PE), polypropylene (PP), polystyrene (PS), polyethylene terephthalate (PET), and polyvinyl chloride (PVC). The majority of currently produced plastics are fossil-fuel based materials⁶, and up to 4% of the globally available fossil-fuel is used for raw plastic production⁷.

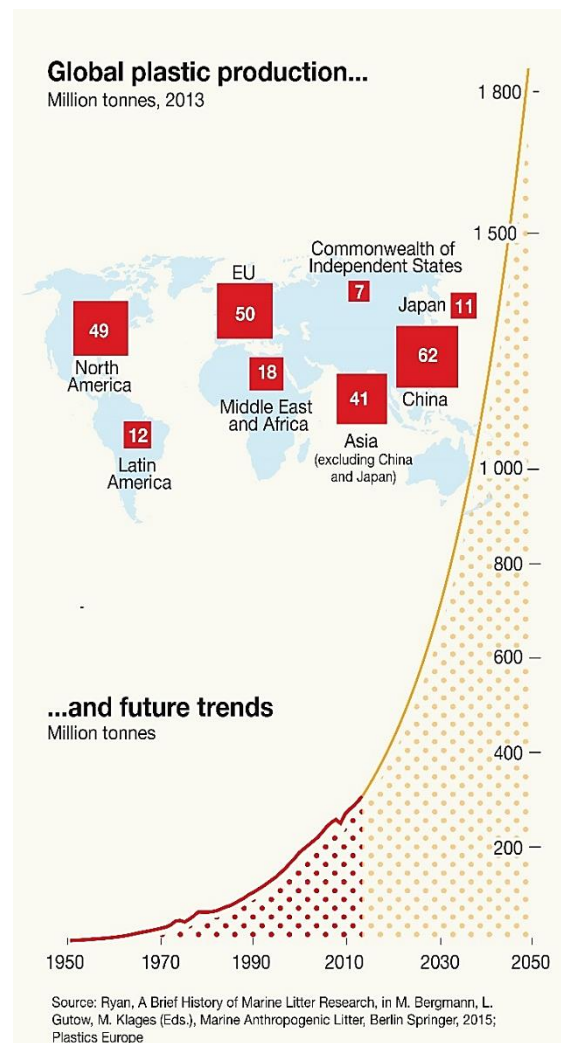


Figure 1 Global plastic production volumes (Source: Grid Arendal, Riccardo Pravattoni)

1.2 Plastic pollution - an emerging environmental problem

Over the years, intense use and disposal, inefficient material recycling and littering on land and sea have resulted in the accumulation of mismanaged plastic waste in the environment^{8,9}. The longevity, durability and resistance to degradation of plastic materials grant their persistence in the natural environment. Plastic deposition and accumulation in the natural environment became an emerging matter of environmental urgency.

It is not known how much plastic is in the aquatic environment, but it has been predicted that 4.8-12.7 million tons of plastic waste reach the marine environment from the anthropogenic land-based sources every year³. As a result of the exponential growth of the plastic industry, emissions to the environment are predicted to increase⁷. The estimated cumulative marine plastic debris is expected to reach 250 million tons by 2025, given the worst-case estimates³. Plastic waste has become abundant and widespread in the natural environment and the majority of marine litter (60-80%) is regarded as plastic debris¹⁰. In natural environment, plastics are subjected to physical and chemical degradation^{11,12}, which leads to formation of small particulate fragments on the micro- and nanoscale. These small plastic particles or fragments, currently commonly referred to as microplastics (MPs) size < 5 mm, have become recognized as an emerging global contaminant¹³. MPs are estimated to constitute be 15% of current disposed oceanic plastics by mass, yet they make up more than 90% of all plastics by particle count¹⁴. With some extrapolations, there are at least 5.25 trillion plastic particles floating on the surface of the ocean, weighing more than 200 000 tons¹⁴. More recent predictions suggest that up to 4.9×10^5 tons of buoyant plastic particles are present in the aquatic environment, with an estimated from 50 to 265-fold increase in 2100¹⁵. Due to its global ubiquity, plastic pollution has been referred to as a potential global boundary threat^{12,16}, contributing to the emissions of toxic and long-lived substances (or novel entities), which can cause irreversible impacts on the biotic and abiotic components of the natural ecosystems.

Early evidence of this emerging environmental problem was already observed in the late 1960s, when floating plastic pellets in the North Atlantic Ocean were documented¹⁷ and plastic ingestion by marine birds and fish was described^{17,18}. Retrospectively, these observations were early warning signs about the forthcoming plastic pollution problem. Although the scientific interest concerning plastic debris, was already evident as early as the beginning of the 1970s, it is only the last decade that has marked a substantial interest in research involving plastic litter. Concerns about the vast extent of the plastic pollution problem and its potential to cause negative consequences accelerated the development

of a new interdisciplinary research field that seeks to unravel the exposure (occurrence, typology and abundance), potential ecotoxicological consequences and risks associated with the presence of (micro)plastics in the natural environment.

1.3 Defining microplastics

Microplastics (MPs) are routinely defined as polymeric particles below 5 mm in size¹³. This pioneering definition acknowledges the synthetic chemical nature and generally small size but provides no lower size boundary specification. The lower size limit conventionally coincides with environmental sampling size limitations and analytical limits of detection¹⁹. To define ultrafine polymeric particles, the complementary term **nanoplastics (NNPs)** has been introduced and encompasses particles with sizes below 100 or 1000 nm (**Figure 2**). The current size-based nomenclature, however, remains ambiguous and non-standardized, as a consensus definition has not been established^{20,21}. Microplastic definition continues to span across a large magnitude of biological and physical scales, and in the scientific literature, small plastic particles can indistinguishably be referred to as MPs²⁰ (**Figure 2**). Considering the absence of established particle size cut-offs, the term MPs will be used throughout this thesis to inseparably address small-sized particles along the broad size span of < 5 mm. When specifically referring to NNPs, a respective arbitrary size margin of < 1 μm, was set.

Definitions

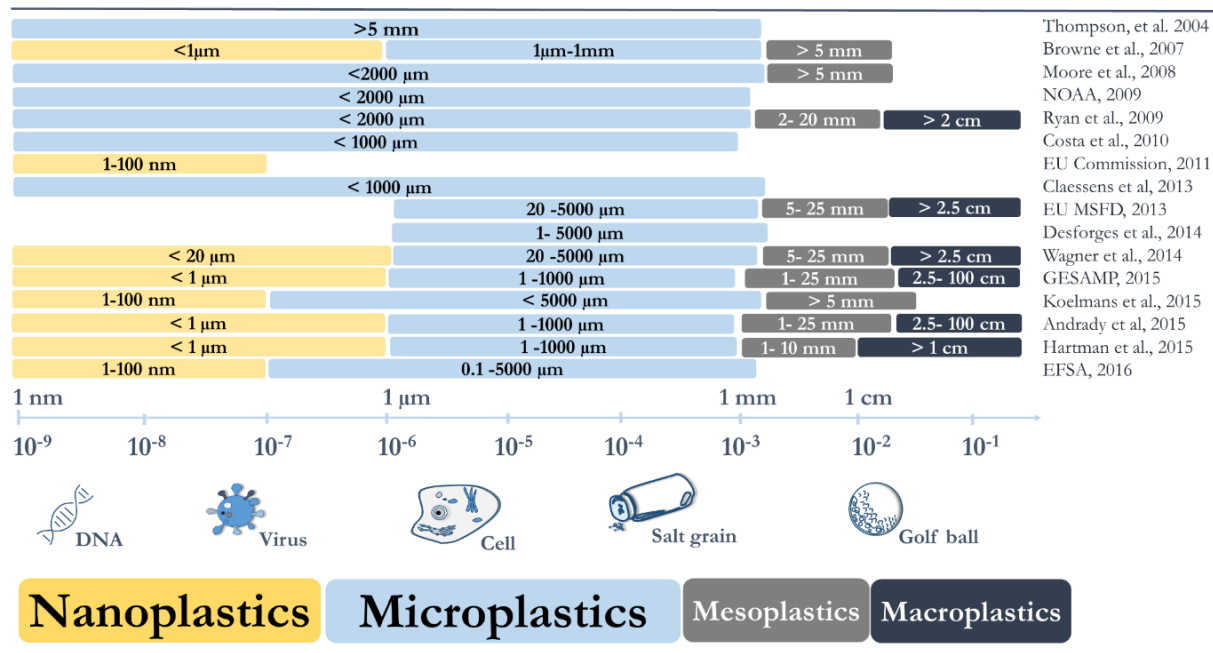


Figure 2 Prevalent size-based nomenclature for MPs and dimensional comparisons with biological references and physical objects^{21,22}.

The term MPs covers not only different dimensions but also implies high heterogeneity in regard to chemical composition, physical appearance and origin. MPs are generally referred to as polymeric materials, not restricted to a single type of monomer or a defined chemical profile of plastic additives²¹. While the composition of plastic formulation is generally unknown due to trade secrets, the structural and functional diversity of chemical substances associated with plastic materials is ought to be immense^{23,24}, making MPs a highly chemically diverse group of materials.

By origin, MPs are classified as either **primary** or **secondary**. Primary MPs are designed and manufactured to be “micro-sized”, whereas secondary MPs are created through degradation of larger plastic items²⁵. MPs can vary greatly in shape: from uniform microspheres to irregularly shaped plastic fragments, microscopic fibers, films and filaments^{21,26}. Primary MPs are often regularly shaped and have relatively consistent morphology, whereas secondary MPs are generally uneven and diverse in shape. Primary MPs constitute a small share of all MPs in the natural environment and are expected to have low global importance in the plastic pollution context, overshadowed by the presence of secondary MPs^{27,28}.

In recent years, the concept of MPs has evolved and currently encompasses even greater complexity than just size, shape and chemical composition. An updated definition for environmental MPs has been proposed and describes MPs as “a complex, dynamic mixture of polymers and additives, to which organic material and contaminants can successively bind to form an “eco-corona, increasing the density and surface charge of particles and changing their bioavailability and toxicity”¹¹. By addressing their dynamic nature, MPs can be described as constantly changing physical and chemical entities that have the potential to interact with the surrounding environment. Given the all-encompassing scope of its definition, the term of MPs continues to be ambiguous. In accordance, the terminology suited to describe MPs’ fate in the environment, and ways in which they interact with biological and ecological systems, remains equivocal and noticeably context-dependent.

1.4 Microplastics in the aquatic environment

Great scientific and technological progress has been achieved through sampling, characterizing and identifying MPs in various aquatic matrices, constructing global distribution patterns and elucidating their fate in the natural environment^{3,14,29}. From environmental sampling and monitoring programs worldwide, the research community is rapidly gaining understanding about the prevalence and spatial and temporal distributions of MPs in various compartments of aquatic ecosystems and is obtaining preliminary estimates about MP exposure to biota.

1.4.1 Degradation: from macro to micro- and nanoplastics

Microplastics are produced as a result of physical, chemical and biological degradation, or weathering, of larger plastics items that undergo changes in their physical integrity and chemical constitution¹². As plastics degrade, they not only facilitate the formation of small particulate fragments on the micro- and nanoscale but also release various chemical additives and low-molecular weight fragments (monomers, oligomers) into the ambient environment^{11,12} (**Figure 3**). While the detection of MPs and plastic degradation products has been documented³⁰⁻³², it remains technologically challenging to isolate (and concentrate) polymer-based nanoparticles from complex environmental matrices and quantify their presence in the aquatic environment^{22,33,34}. Experimental weathering and modeling studies demonstrated plastic degradation and formation of NNPs under environmentally representative conditions^{35,36}, and pioneering field-based studies have provided the first indications for their presence in the natural environment³⁷. NNPs (100-1000 nm) have been identified in colloidal fractions of seawater collected from plastic pollution-subjected locations, such as the North Atlantic subtropical gyre³⁷, demonstrating the prevalence of plastic-derived nanoscale pollutants in the world's oceans. However, it remains largely unclear to what extent and constitution such particles are available in the natural environment and what exposure levels are anticipated for aquatic biota. Current knowledge on MP occurrence and abundance remains biased towards larger-sized microparticles. An increasing number of studies suggest that smaller-sized particles are more abundant in the aquatic environment than those of a larger size^{15,38}. However, with current sampling, isolation, and analytical techniques, the detection of environmental MPs from complex environmental matrices remains size-limited ($> 1-100 \mu\text{m}$)¹⁹.

Plastic weathering in the aquatic environment

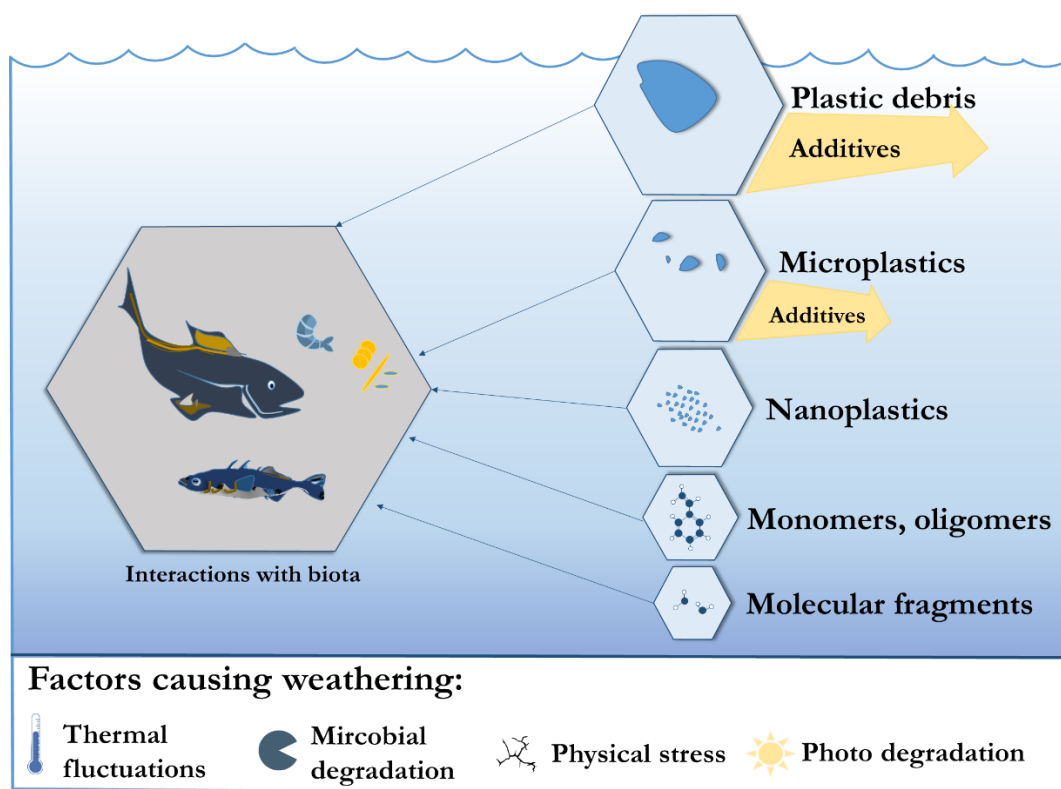


Figure 3 Plastic degradation in the aquatic environment

1.4.2 Environmental occurrence

MPs pollution crosses broad geographical scales: from tropic and temperate to polar regions^{39,40}. MPs are found in virtually every marine habitat: from shallow coastal waters to open seas^{9,41}. MPs are detected in the surface waters, water column, on the ocean seabed and in sediments⁹. In addition, MPs are also found in freshwater and brackish waterways: in lakes, rivers and streams⁴²⁻⁴⁴. Although the greatest global abundance of plastics is thought to concentrate in large-scale convergence zones (gyres), enclosed seas or in areas in close proximity to urbanized regions⁹, MPs are found everywhere, even in very remote and isolated locations around the globe⁴⁵⁻⁴⁷.

1.4.3 Interactions with environmental pollutants

Plastic debris has a high potential to accumulate persistent organic pollutants (POPs) from the marine environment^{48–50}. Notably, plastic fragments have been shown to concentrate hydrophobic organic chemicals (HOCs) up to six orders of magnitude greater than the surrounding seawater⁵⁰. A wide range of persistent organic pollutants, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polybrominated biphenyl ethers (PBDEs), organochlorine pesticides^{49,51} and metals⁵² were shown to associate with MPs present in the aquatic environment. The low polarity (hydrophobicity) of synthetic plastic polymer surfaces facilitates the sorption of HOCs from the surrounding medium, making plastic polymers good sorbents and promoting their use as passive sampling devices in environmental chemical monitoring⁵³.

In solution, with the presence of plastics and HOCs, the HOCs will migrate from the aqueous phase into the polymer phase until sorption equilibrium is reached (**Figure 4**). Depending on the governing mechanism, the chemical sorption can be differentiated into absorption and adsorption (**Figure 4**). **Absorption** generally refers to the partitioning (diffusion) of HOCs into the polymer matrix and infers that sorbent molecules penetrate into the solid phase and tightly associate with the polymer matrix⁵⁴. Such a molecular dissolution process is governed by weak van der Waals forces⁵⁵. **Adsorption** is described as a superficial process in which molecules adhere to surfaces or sorbent molecules are confined at the interface between the fluid and solid phase⁵⁴, and it is governed by a variety of intermolecular interactions, such as Van-der-Waals, ionic, steric, π - π interactions and covalent bonds⁵⁵.

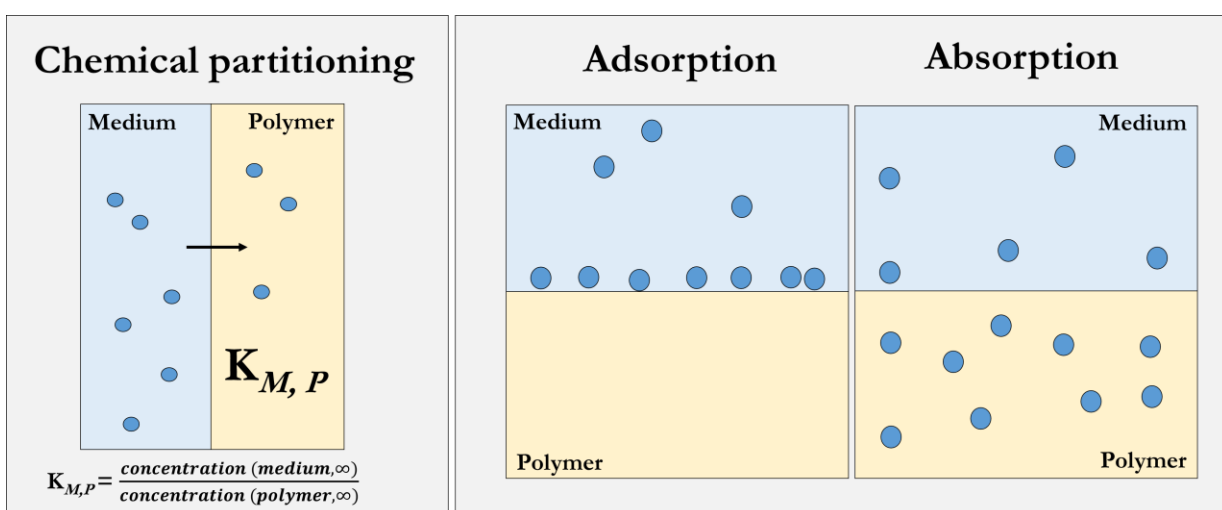


Figure 4 Chemical partitioning between the polymer and medium (left) and different molecular sorption mechanisms (right)

Due to polymer chemistry, different plastic polymers have differential affinity and capacity to sorb HOCs. Chemical sorption can be influenced by the nature of the polymer (i.e., type, density, crystallinity), which, in part, determines particle-chemical interactions. Sorption takes place in the amorphous regions of the plastic polymer⁵⁶, which, depending on its glass transition temperature, can be **glassy** or **rubbery**⁵⁵. Rubbery polymers have high mobility of polymer chains and faster successive diffusion of molecules compared to glassy polymers. Additionally, the properties of HOCs (i.e., molecular weight, volume, planarity and hydrophobicity) are important determinants of chemical sorption^{49,57}. For example, the hydrophobicity (octanol-water partitioning coefficient, log K_{OW}) of a chemical substance can be an important driver for the partitioning of chemicals onto plastics⁵⁸. Knowledge about the sorption behavior of pollutants and their mechanisms onto MPs is important for understanding their fate and transport in ecological and biological systems⁵⁹.

1.5 Plastic ingestion

An increasing number of scientific publications and biomonitoring studies report the ingestion of MPs by aquatic animals in the natural environment. The ingestion of MPs has been identified in more than 690 aquatic species⁶⁰, including many fish species from different habitats and geographical areas^{61–64}. As spatial and temporal differences in plastic occurrence and abundance in aquatic habitats exist, the likelihood and extent of ingesting (or encountering) MPs by aquatic organisms may depend on the geographical location and species ecology (habitat, diet, foraging behavior, trophic level)^{64–66}.

In aquatic ecosystems, MPs are expected to physically associate with naturally occurring sediment particulates or aggregates²⁶ or fecal pellets of zooplankton⁶⁷. Aquatic animals, including fish can accidentally ingest such particulates via unselective passive ingestion^{65,68} or by confusing MPs with prey items^{65,69,70}. Fish bite marks on plastics indicate selective feeding on plastic particulates⁷¹. In accordance with the existing hypothesis, plastics can associate with chemical substances that chemosensorically stimulate foraging behavior, leading to ingestion of plastic particles^{11,72}. The size of MPs coincides with the sizes of planktonic organisms²⁶; thus, a wide spectrum of aquatic organisms are expected to ingest MPs⁷³, thus facilitating secondary ingestion (via contaminated prey) in fish⁷⁴. Particulate accumulation in organisms from low trophic levels can serve as a pathway for particle entry in aquatic food webs^{26,75–77}.

Various plastic fragments, including microbeads, filaments from discarded fishing gear and textile fibers have been found in digestive systems of fish^{65,68,78,79}. The most common types of ingested plastic polymers include PE and PP⁸⁰. Other types of synthetic polymer

materials that occasionally prevail in fish include polyamide (PA), nylon, rayon, acrylic, PS, PET, polyester, and alkyd resin^{61,62,65,68,80,81}. The internal number of ingested MPs by fish vary from none to a few (1-2) particles per individual with the highest numbers reported up to 10 particles per animal^{64,82-84}. The analysis of plastic ingestion by biota remains predominantly biased towards detecting larger-sized particles (1-5 mm⁶⁴), potentially underestimating the smallest fraction of MPs. To date, the incidence of ingestion and abundance of MPs in the natural environment is generally expected to be low^{65,83} and it is unlikely that ingested MPs accumulate in fish⁸⁵. It is generally assumed that the accumulation of microscopic particles is restricted to the gastrointestinal tract, but limited knowledge exists about the uptake and translocation of particles in aquatic vertebrates, such as fish⁸⁶.

An increasing body of evidence suggests that ingestion is the most prominent and ecologically relevant route for the uptake of MPs in fish. However, the potential impacts associated with MP exposure cannot be predicted on the basis of ingestion monitoring alone¹⁵. Thus, experimental studies are becoming essential for exploring the biological fate and plethora of potential ecotoxicological impacts associated with MP ingestion.

1.6 Understanding the hazard

From an ecotoxicological point of view, MPs represent a rather unique and unconventional group of environmental contaminants and pose a novel challenge for ecotoxicological research. MPs represent a stressor consisting of inert and insoluble particulate matter and associated chemical components (**Figure 5**), which subsequently influence its hazard potential.

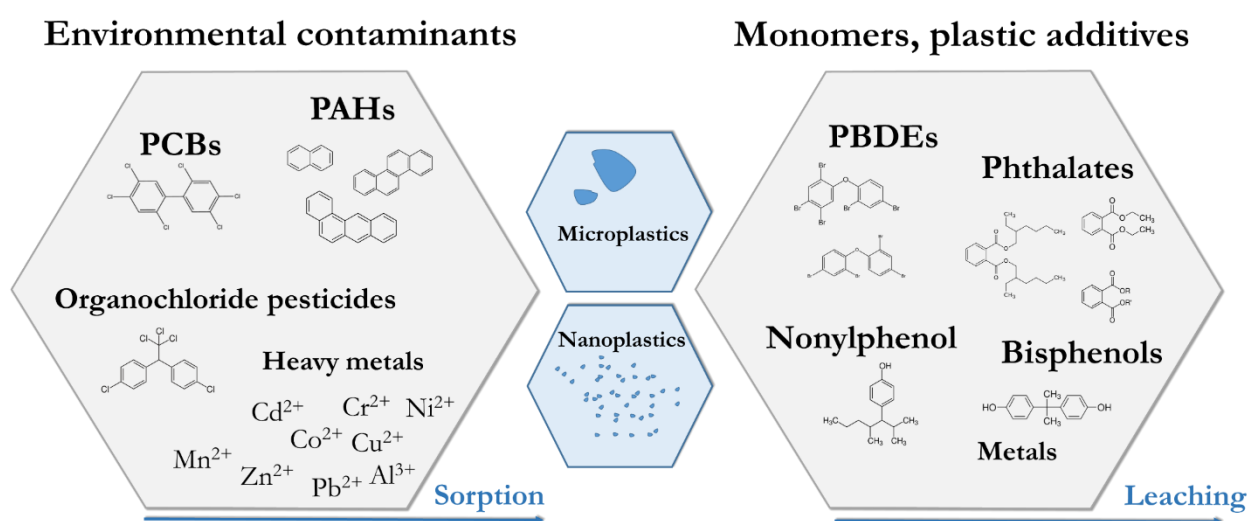


Figure 5 The interplay between the physical and chemical properties of MPs

1.6.1 Physical stressor

First, MPs are recognized as solid-phase substances rather than a molecular pollutants¹⁶. Given their physical nature, MPs can physically interact with certain biological receptors, such as organisms, tissues, membranes, and exert mechanical stress, such as clogging and abrasion²⁶. Physical attributes of the particles, such as size and shape, can influence the physical impacts²⁶. MPs can become kneaded or embedded into animal tissues and can be translocated and internalized by the cells⁶⁹. Additionally, due to their small size, MPs (especially NNPs) possess high surface reactivity and a high surface-to-volume ratio, which are toxicologically important aspects of particle toxicity.

1.6.2 Chemical toxicity

In regard to chemical toxicity, toxicological concerns arise from numerous additives (i.e., flame retardants, plasticizers, colorants, fillers, etc.) and chemical residuals which are trapped in the polymer matrix. Plastics can contain residuals from manufacturing, such as solvents, catalysts, processing aids, or other non-intentionally added substances (NIAS), such as oligomers, impurities, reaction byproducts, and breakdown products²⁴. A non-degraded polymer is biologically and chemically inert, and thus not able to cross membranes and interact with cellular organelles, molecules or receptors. While plastic polymers have high molecular mass ($> 10\,000\text{ g mol}^{-1}$)²¹, plastic resin can release chemicals with smaller molecular weight ($<1000\text{ g mol}^{-1}$) which then are capable of escaping polymer matrix and reaching molecular targets. Chemical additives in plastic are not chemically bound to the matrix, but rather are physically dispersed within the three-dimensional porous structure of polymer⁵⁶, and thus have the capacity to leach out⁸⁷ and induce toxicological effects^{23,87-89}. The large diversity of chemicals found in plastics raises concerns not only for the toxicity of individual components but also chemical mixtures associated with plastic materials²⁴.

1.6.3 Vector hypothesis

Apart from the intrinsic chemical nature of plastic materials, other chemical risks relate to exposure from persistent, bioaccumulative and toxic (PBT) substances adsorbed onto MPs. The documented association of environmental pollutants with MPs poses a concern that adsorbed chemical contaminants can be liberated from plastic and act as vectors, or carriers, for chemical contaminants into biota⁴⁹. The desorption of PBT chemicals following ingestion of MPs, has been proposed as a potential threat to wildlife^{56,90,91} and become a central theme in the discussions addressing the ecotoxicological risks of MP exposure. The abovementioned narrative has boosted the development of a wide range of hypotheses addressing this phenomenon⁹². A broad spectrum of investigations, including field observations, modeling and experimental studies, has been conducted to increase scientific knowledge about the propensity, pathways, mechanisms and impacts of MP-mediated vector effects. However, the role of MPs as potential carriers of environmental pollutants remains a subject of debate.

In recent years, the development of theoretical frameworks and formulation of different conceptual hypotheses have proliferated, enabling research on this subject. Syberg et al. (2015) developed a three-level framework addressing vector effects and described the main domains where interactions between MPs and biological systems can occur⁹³ (**Figure 6**). Firstly, MPs were described as **environmental vectors**, acting as vehicles for hydrophobic organic contaminant transfer, and altering their distribution and bioavailability in the natural environment. Secondly, MPs were denoted as **organismal vectors** that interact with biota via ingestion and release the adhered contaminants into organisms. Thirdly, MPs (more specifically, NNPs) are referred to as **cellular vectors** that are capable of interacting with cells and facilitating the cellular uptake of sorbed contaminants.

Conceptually, MP-mediated chemical transfer and bioaccumulation in an organism can either be **direct**, taking place via ingestion of contaminated MPs, or **indirect**, mediated via desorption of chemicals into environmental media prior to exposure⁹⁴. In an attempt to elucidate the underlying mechanisms for the transfer of HOCs between MPs and biota, four key diffusive mass transfer processes for HOCs have been identified and include exposure via water, organismal fluids, direct contact exposure externally or internally⁵⁵ (**Figure 6**). Direct contact of MPs (and associated HOCs) occurring through internal organismal surfaces, such as intestinal and gill epithelia, could be important for MP-mediated chemical uptake and can be viewed as an overlooked exposure pathway for HOCs into organisms⁵⁵.

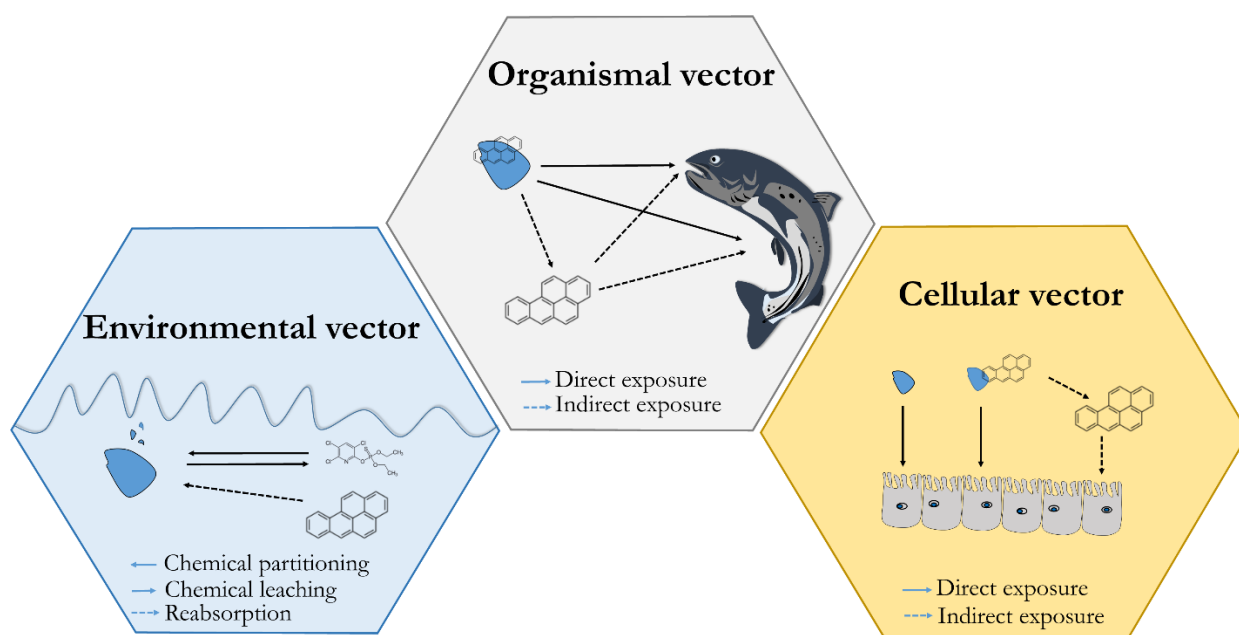


Figure 6 Conceptual representation of MP vector effects, summarizing the prevalent exposure pathways and mechanisms and pathways of vector effects

Over the years, it has been widely accepted that MPs are potential environmental vectors. However, the ability of MPs to act as carriers or organismal vectors by absorbing HOCs, transporting them from the environment to biota and causing adverse biological effects, was questioned and has led to a diverging scientific debate⁵⁵. It has been discussed whether MPs are important pathways for contaminant transfer compared to other exposure pathways^{92,95}. In particular, as MPs were suspected to have limited importance on the bioaccumulation of persistent chemicals, compared to other naturally occurring particulates, such as mineral particles or suspended organic matter⁹⁵. Additionally, some skepticism has been directed towards the potential of MPs to cause adverse effects, as well as negative ecological consequences^{96,97}. It is still debated whether MP-mediated transfer of chemicals can reach levels that can cause adverse effects in the natural environment^{92,97-99}. In this regard, the disputed role of MPs in facilitating “cleaning” of the chemical burden via reabsorption of contaminants and excretion has also been addressed^{99,100}. Thus, the notion that MPs act as significant vectors for environmental pollutants into biota remains hypothetical. The processes and mechanisms underlying the transfer of HOCs from MPs to biota are under investigation, and it remains controversial whether MP-mediated chemical transfer leads to bioaccumulation or can induce adverse biological effects in aquatic organisms, including fish^{90,101}.

1.7 Ecotoxicological impacts

In recent years, research efforts seeking to address interactions and impacts of MPs on biota have rapidly intensified¹⁰². The ecotoxicological studies have begun unravelling the potential of MPs to cause biological effects on organisms across different levels of biological organization (**Figure 7**).

The majority of ecotoxicological studies have investigated the impacts at an organismal level and documented adverse effects on animal ecophysiology, such as reduced growth and survival, fecundity, developmental impairments and changes in energy metabolism^{90,101,103,104}. Regarding the specific effects exerted by MPs via ingestion, several studies have addressed the physiological effects in the digestive systems of animals. The accumulation of large plastics (macro- or meso-plastics) in the digestive tract has been shown to cause physical harm through mechanical damage, such as injuries of the internal epithelium or ulcers²⁶. Plastic items have been shown to physically block the intestinal passage, impair food intake and/or cause false satiation^{70,74}. Such implications are thought to impose negative consequences on an organisms' body condition, health and fitness⁷⁰. The ingestion of MPs, in addition to having the potential to cause conspicuous physical damage, can have more subtle effects on aquatic animals at the molecular, tissue and organismal levels. Earlier studies have demonstrated that the exposure to MPs can induce inflammation, cause mechanical and chemical stress, or can lead to gut microbiota dysbiosis^{83,105–108}. While the ingestion-related impacts of large MPs are unequivocally perceived, studies on the biological fate (uptake, translocation) and implications of smaller MPs ingested by fish remain scarce and require further investigation.

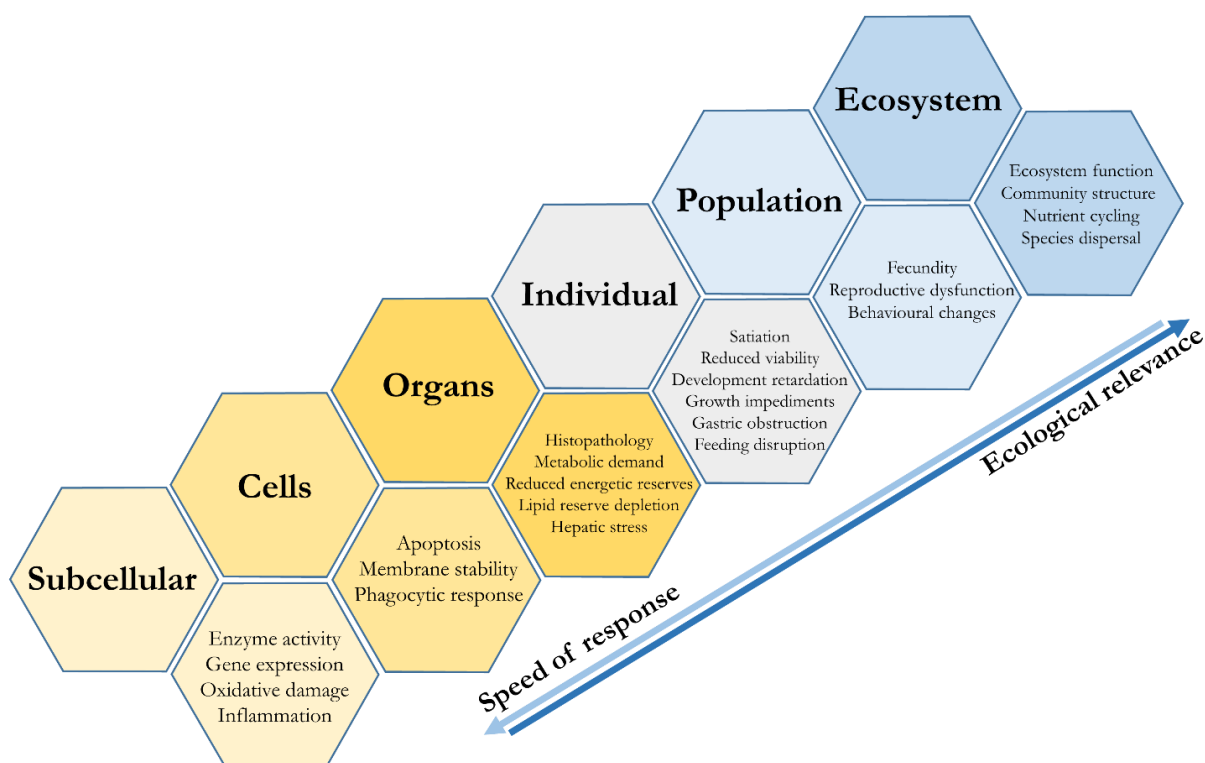


Figure 7 Graphical scheme depicting potential impacts of MPs across different levels of biological complexity^{11,109}.

An expansive growth in MP ecotoxicological research reflects not only an increasing interest in this research topic but also infers to rapid dynamics in knowledge generation. Knowledge gaps and uncertainties persist, especially relating to environmental and human risks¹⁵. Comprehensive toxicological knowledge about the interactions, impacts and mechanisms of particle uptake, physical and chemical toxicity, the vector effects of MPs is still largely lacking.

1.8 Potential impacts on human food quality and safety

MPs are omnipresent in the aquatic environment, entering aquatic food webs, and reaching humans, the top consumers. Microscopic plastic particles have been detected in seafood, such as mollusks (mussels, oysters)¹¹⁰, crustaceans¹¹¹ and fish^{62,63,112}. According to certain estimates, plastic particles were found in more than 25% of seafood commodities present in the market⁷⁹. As seafood products constitute an important dietary component in human diets, this information has triggered concerns regarding the potential impacts of MPs on human health, food safety and food security^{113–115}. Concerns have been primarily addressed towards the presence of anthropogenic particles in the seafood, leading to subsequent human exposure when these commodities are consumed. This aspect has been mainly discussed in regard to mussel and oyster consumption, which, via filtration of water, have the potential to accumulate

MPs in edible parts of the animal⁷⁹. For fish, on the other hand, this appears to be as a negligible issue, not only because the reported MP abundance in wild-caught fish is comparatively small but also because the majority of fish species are gutted prior to consumption^{79,114,115}. Other public health and food safety concerns for fish consumption have emerged during discussions on the ambiguous role of MPs in transferring and accumulating contaminants into the edible parts of the tissues of commercial animals⁷⁹ and/or MPs being carriers for toxicogenic and pathogenic microorganisms¹¹⁴.

Apart from the forecasted implications on human health, derived from direct particulate and indirect chemical exposure, collateral concerns relating to food quality have emerged. It has been hypothesized that plastic-associated chemical exposure can affect the edibility or quality of fish products. Chemical exposure can promote oxidation of lipids, which is a major factor affecting the quality of commercial fatty fish¹¹⁶. Lipid oxidization alters the color and texture of the fillet and/or induces the formation of aldehydes, which advance the development of rancid odor and taste¹¹⁷. Additionally, in some instances, the formation of genotoxic and cytotoxic metabolites can be enhanced¹¹⁸. Due to reduced oxidative stability, fish fillets lose commercial appeal and value¹¹⁹, and this may also have implications on food suitability and safety for human consumption.

The unequivocal presence of MPs in various other food products (salt, sugar, honey)^{120,121} and beverages (tap and bottled water, beer)¹²² intended for human consumption have exacerbated media interest and sparked public concerns on this issue¹²³. This has caused concern in governmental and industrial sectors and promoted assessments and evaluations of potential health risks associated with the prevalence of MPs in aquatic food chains^{79,114}. Potential implications of plastic-ingestion-mediated chemical influx into consumer products and the associated implications remain speculative and require further investigation.

2 Research scope and objectives

Plastic pollution is recognized as a global problem, and the occurrence of MPs in aquatic environments has been regarded as an environmental issue with potential ecotoxicological consequences on wildlife. As the interactions and impacts associated with MP exposure in aquatic organisms are not well understood, the overarching scope of the present thesis was to increase the understanding of the biological fate and impacts of MPs and associated chemicals in fish (**Figure 8**).

The specific aims of the thesis were as follows:

- To investigate the relative importance of exposure route for organismal uptake and internal localization [**Paper I**]
- To assess the prevalence of direct (particle) and indirect (chemical) effects associated with ingestion of MPs [**Papers II-III**]
- To assess the biological fate (desorption, transfer and accumulation) of MP-associated contaminants [**Paper IV**]
- To explore the interplay between MP exposure and potential hazard [**Papers II-IV**]
- To provide insights into ongoing discussions regarding the environmental relevance of MP-mediated vector effects [**Papers II-IV**]
- To assess chemical exposure-related consequences on commercial fish fillet quality [**Paper III**]

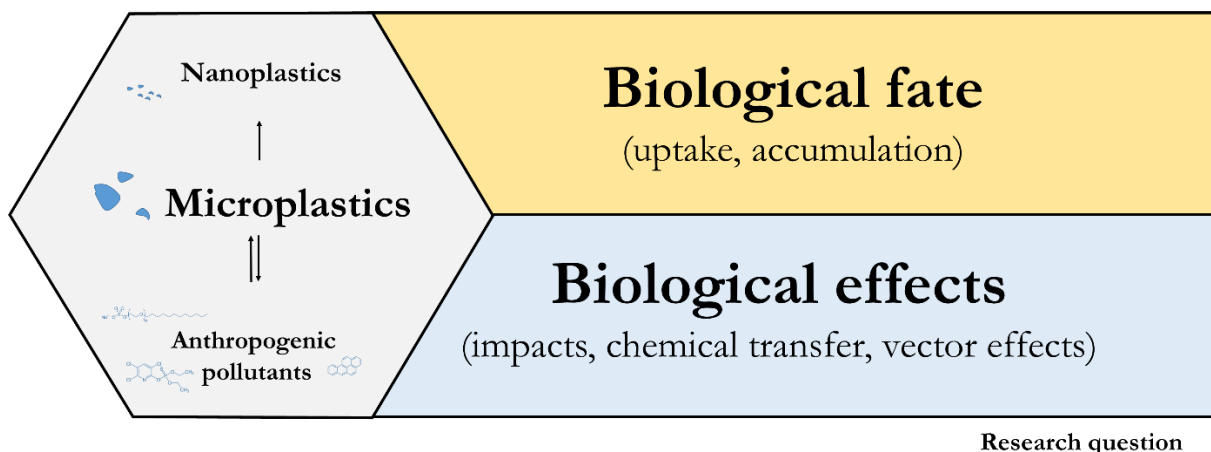


Figure 8 Graphical summary of materials and research questions addressed in this doctoral thesis

3 Materials and methods

3.1 Selection of experimental material

3.1.1 Polymers

Two different polymers were selected for uptake and ecotoxicological studies of MPs: polystyrene (PS) [Papers I-IV] and polyethylene (PE) [Paper IV]. These polymers are among the six most widely used synthetic plastic polymers and also commonly detected in the environment^{42,124,125}.

Polystyrene is a synthetic aromatic hydrocarbon polymer consisting of styrene monomers (Figure 9). PS is made of an aliphatic hydrocarbon backbone, substituted with aromatic rings¹²⁶. The presence of phenyl groups (C₆H₅) in PS hampers the rotation of the polymer chains around C-C bonds and restricts them from forming tight crystalline arrangements, making it a relatively rigid plastic¹²⁷.

Polyethylene is made by polymerizing ethylene monomers (Figure 9). The chain-like polymeric unit of PE where hydrogen connects onto the carbon backbone enables differential branching, leading to different types of PE (i.e., LDPE, HDPE, etc.).

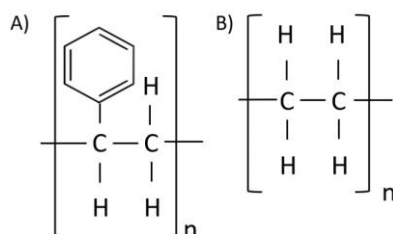


Figure 9 The building blocks (monomers) of synthetic polymers: polystyrene (A) and polyethylene (B)

Considering the glassiness state of the polymer (glass transition temperature; T_g), which influences the contaminant sorption mechanism, PS and PE polymer particles were selected for sorption experiment in **Paper IV**. PS is an amorphous glassy polymer ($T_g=100$ °C)^{55,126}. Being readily condensed and cross-linked, PS possesses lower diffusivity, favoring chemical adsorption of HOCs onto plastic¹²⁸. PE is considered an amorphous rubbery polymer ($T_g= -120$ °C) with a predominance of chemical absorption into the polymer⁵⁵.

3.1.2 Particle morphology: size and shape

To examine particle interactions with an organism and their biological fate, NNPs were used because they entailed sizes relevant for biological uptake. PS nanoplastics are commonly used model particles for biological uptake studies^{129–131}; thus, they were selected for investigation in **Paper I**.

Larger micro-sized plastic particles ($> 100 \mu\text{m}$) were selected for studies, investigating biological effects and plastic-mediated chemical transfer [**Papers II-IV**]. Hazard data that examine the effects of particles in that size range are currently lacking¹³² and represent a knowledge gap in the research field. Particles at this size range are detected in environmental matrices and are reportedly ingested by various fish species in the wild¹¹⁵, thus representing environmentally relevant MPs. For experiments, a combination of commercial industrial polymeric powder (PS) and microparticles produced for the research applications (PE) were used [**Papers II-IV**]. PS was obtained from bulk powder containing irregularly shaped particles, whereas PE particles were uniformly spherical.

The necessity of using naturally-occurring particles has been identified in the MP research field, not only for evaluating the relative importance of synthetic and natural particles for mediating contaminant transfer but also for understanding non-plastic particle effects^{92,133}. Inorganic silica glass particles were, therefore, selected as reference particles in **Paper IV**, due to their high abundance in the natural environment and their commercial accessibility in the desired particle size ranges.

3.2 Particle characterization techniques

The characterization of particles in experimental studies involving MPs is becoming indispensable not only for describing physicochemical properties but also for understanding their behavior under experimental conditions¹³⁴. Depending on the size and material preparation (suspension, powder), different analytical methods were applied to characterize MPs and are briefly overviewed in the following section.

Dynamic light scattering (DLS) is an analytical technique used for determining the size distribution of nanoparticles in a solution. When suspended in liquids, particles move in Brownian motions (erratic random movements). By measuring the intensity of light scattering (fluctuations in light intensity) of particles in motion, the hydrodynamic size of particles is estimated. DLS technique also enables measurement of the electrokinetic potential of the particles – zeta potential (ζ -potential), an indicator of the

stability of particles in the suspension. In **Paper I**, DLS was used to assess the stability of NNPs and to monitor particle behavior in the exposure medium.

The particle analyzer system CAMSIZER™ (Retch Technology) was used for dynamic image analysis. This technique is suited for automatic detailed morphological characterization of particles in micro-size range (5 μm - 3 mm). By employing dual camera technology, projection areas of free-flowing individual particles are collected, allowing determination of particle morphological parameters (diameter, symmetry, sphericity), size distribution and facilitating estimation of particle numbers in bulk polymeric powder. Data obtained from this analysis were used in **Papers III-IV** to characterize MP morphology, obtain particle size distributions and estimate alternative exposure metrics (i.e., particle number, surface area or volume).

Fourier transform infrared spectroscopy (FTIR) is a vibrational spectrometry technique used for synthetic polymer identification. With FTIR technology, the sample of interest is subjected to infrared radiation, and the oscillation (vibration) of chemical bonds in the molecules is investigated, allowing the collection of qualitative information about polymer composition. This technique was used to confirm the polymer identity of MPs used in **Papers II-III**.

Scanning electron microscopy (SEM) is a type of electron microscopy in which focused low-energy electron beams (few kV acceleration) are used to raster along the sample to obtain topographic information. SEM was used to obtain high-resolution images for qualitative assessment of the particle surface topology of microparticles in **Papers II and IV**. Gold-sputtering was applied on particles prior imaging to allow imaging of non-conductive particles and achieve better resolution.

Optical light microscopy was complementarily used to determine the MP morphology and assess the color and translucency of particles from bulk powder in **Paper III**.

3.3 Fish models

Zebrafish (ZF) (*Danio rerio*, Hamilton, 1822) is a widely used vertebrate model in biomedical, toxicological and environmental research. The species is an attractive model species in experimental research due to its relatively inexpensive maintenance, small housing space, high fecundity, fully sequenced genome and transgenic versatility¹³⁵. To study particle uptake and localization *in vivo*, the pigment-less ZF strain, named *casper*, was used in **Paper I**. This transgenic strain has an almost transparent body due to lack of melanocytes and iridophores, which persists during embryogenesis and adulthood¹³⁶. Translucency of the ZF body ensures greater light penetration into tissues during fluorescence imaging.

Rainbow trout (*Oncorhynchus mykiss*, Walbaum, 1792) is a common fish species used in aquatic ecotoxicology research. This salmonid species is also a well-recognized model in fish physiology research, including research into the intestinal barrier function^{137–140}. It was therefore selected for investigation of intestinal permeability and transport functions [**Paper II**]. The species are known to be readily adaptive to conditioned feeding¹⁴¹, and therefore were chosen for the feeding experiments in **Papers II-III**. Both farmed and wild fish of the species are suited for human consumption, and are popular in the global cuisine, making it a good choice for fish fillet quality assessment in **Paper III**. Fish were obtained from a local aquaculture farm (Vänneåns AB, Laholm, Sweden).

Three-spine stickleback (*Gasterosteus aculeatus*, Linnaeus, 1758) is an emerging fish species in ecotoxicological studies and is widely found across the Northern hemisphere. The species were selected as an environmentally and ecologically relevant test species in **Paper IV**, as the species inhabits marine coastal areas in close proximity to hotspots for plastic pollution. Fish were captured and collected from a stream at the reference site in Skaftö, Sweden.

3.4 Approaches to study biological fate

Fluorescence microscopy has been widely used as a detection tool in studies examining the uptake and localization of fluorescently-labeled plastic particles in various organisms^{129,142}. Herein, the use of fluorescently-labeled particles enabled qualitative assessment of uptake and localization of particles in fish at the organismal [**Paper I**], tissue and/or cellular level (**unpublished data**), as well as allowed documentation of dietary passage via the food chain [**Paper I**]. The biological fate (organismal uptake and localization) of particles *in vivo* was investigated using fluorescence light sheet microscopy (LSM) in **Paper I**. LSM is a confocal fluorescence imaging system that is suited for live 3D *in vivo* imaging. In contrast to conventional confocal microscopy, with LSM, an imaged sample is optically sectioned with sheets of focused light, reducing the potential for photo-toxicity and photo-bleaching allowing imaging of living specimens (**Figure 10**). As a non-invasive technique, LSM allowed investigation of the uptake of fluorescent particles without affecting the integrity of experimental animals. Additionally, confocal laser scanning microscopy was used to visualize interactions with fish intestinal epithelia and to demonstrate nanoplastic internalization by fish intestinal cells.

Quantification of polymer particles in biological tissues has been proven to be analytically challenging¹⁴³, and many uptake studies are limited to qualitative (descriptive) examinations. Therefore, a consideration was given to using metallic particles for mass based particle uptake quantification [**Paper I**]. Gold nanoparticles (Au NPs) were that were selected as reference particles for insoluble particle uptake quantification and were used as a proxy for polymer particle uptake *in vivo*. Experimental Au NPs possessed dual advantages: they were fluorescent, and could be directly employed in the elemental analysis with inductively coupled plasma mass spectrometry (ICP-MS).

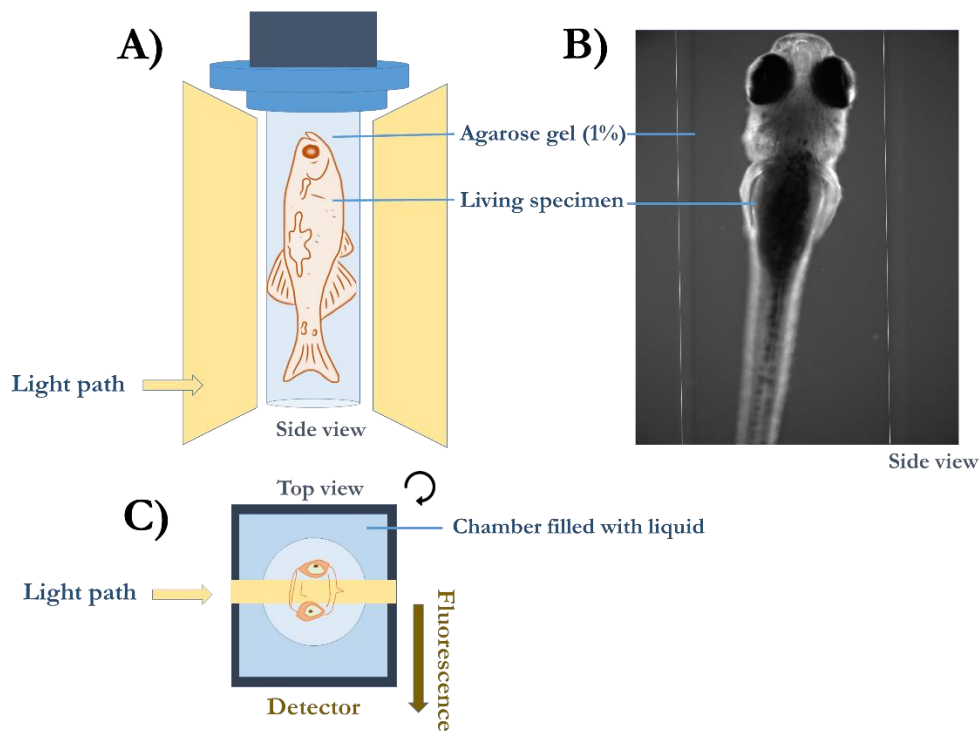


Figure 10 The principle of embedding and imaging setup of light sheet fluorescence microscopy from the side (A-B) and top view (C). The living specimen is mounted vertically in the gelled cylinder ejected from the capillary holder via plunger (A-B). The embedded sample is then subjected to light sheet illumination, and multi-view imaging is achieved by axial rotation (A-C).

Inductively coupled plasma mass spectrometry (ICP-MS) is an analytical technique that is primarily suited for detection and quantification of metals. In this method, the sample is subjected to inductively coupled plasma, causing ionization of the sample. Liberated ions are then separated and quantified with a mass spectrometer coupled to the system. ICP-MS allows elemental analysis with great sensitivity, making the technique very suitable for metallic nanoparticle detection [**Paper I**].

3.5 Methodologies for studying impacts of microplastics

The propensity of MPs to induce adverse biological effects was investigated across multiple levels of biological organization: molecular, gene, enzyme, tissue level and organismal levels [**Papers II-V**]. Different methods and techniques were employed to determine the bioavailability of chemicals and associated biological effects and are reviewed below (**Figure 11**). Detailed methodological descriptions are provided in the amended publications.

The **direct impacts**, associated with particle exposure on fish intestinal physiology were primarily investigated with the Ussing chamber technique and were complemented with histological assessment, hematological and gene expression analysis [**Paper II**]. The **indirect effects** associated with chemical exposure were investigated using established ecotoxicological biomarkers at the gene and enzyme levels [**Papers III-V**]. By using particles, exceeding thresholds typical for biological uptake, indirect effects associated with chemical release from particles were investigated. In **Paper III**, the focus was on determining the presence of chemical exposure in hepatic tissue associated with oxidative stress, detoxification and endocrine regulation. In **Paper IV**, biomarkers specific to organochloride pesticide, synthetic estrogen and PAH exposure were used to assess the bioavailability of the model compounds.

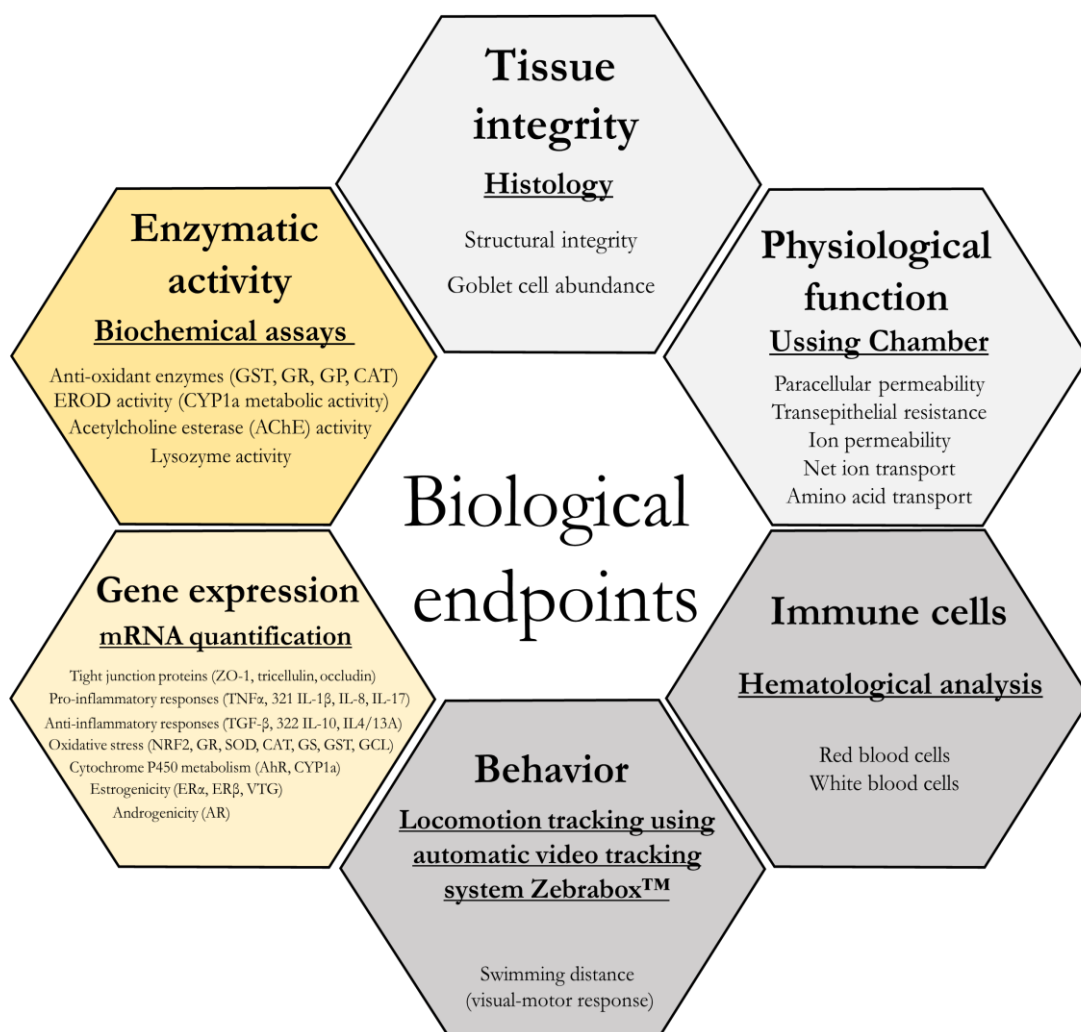


Figure 11 Summary of biological endpoints and methodologies used in **Papers I-IV**.

Main methodologies in focus

Gene expression analyses were performed using quantitative real-time polymerase chain reaction (qPCR). This method is routinely used for measuring mRNA transcript levels of genes of interest (**Figure 11**) [**Papers II-IV**]. In the qPCR reaction, gene-specific primers are amplified with DNA polymerase and were quantified using a specific fluorophore bound to double-stranded DNA.

Enzymatic measurements were conducted to assess enzymatic activity (not their abundance) in fractions of dedicated tissues. Kinetic enzymatic analyses rely on spectrophotometric or spectrofluorometric quantification of reaction product accumulation or reagent consumption over time¹⁴⁴. Established biochemical assays were deployed in the analyses [**Papers II-IV**].

Histological assessment was performed using histological staining (hematoxylin/eosin (H&E) and Alcian blue), which was used to quantify mucus-producing goblet cells, as a proxy for the disturbed extrinsic intestinal barrier in **Paper II**.

The Ussing chamber technique was used to study the electrophysiological properties of tissue, paracellular permeability and active nutrient transport across viable fish intestinal epithelia *ex vivo* [**Paper II**]. In vertical Ussing chambers, the intestinal epithelia was embedded in a two-compartment system, representing the gut lumen (mucosal side) and lamina propria (serosal side) of the intestine, supplied by aerated physiological solution (**Figure 12**). With the addition of radioactively labeled tracer molecules in the mucosal chamber, the time-dependent transport across the epithelia (to serosal chamber) was investigated. Additionally, the built-in setup allowed measurement of electrochemical parameters across the mounted epithelium. By applying alternating DC voltages that generate current, measurement of the transepithelial potential difference (TEP) across the epithelium was performed. Current-voltage pairs were then plotted, and a linear, least square analysis was applied in the regression analysis, where the slope of the line represents the transepithelial resistance (TER). Using Ohms law, the short circuit current (SCC) was calculated as $SCC = -TEP/TER$.

The integrity of the intrinsic barrier was investigated as the selective permeability of molecules across the epithelia (apparent permeability coefficient (P_{app})) and the paracellular transport of ions (transepithelial resistance (TER)). The apparent permeability coefficient (P_{app}) (cm/s) reflected the accumulation of the intestinal permeability marker ^{14}C -mannitol across the epithelia.

Investigation of active transport was achieved by measuring the time-dependent Na^+ -mediated transport of the essential amino acid 3H -lysine, TEP and SCC. Electrophysiological measurements provided information regarding ion transport capacity (TEP) and net flow of ions (SCC) across the viable epithelia.

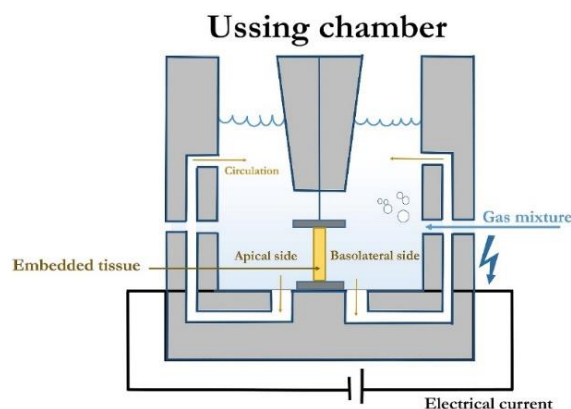


Figure 12 Vertical Ussing chamber setup used in **Paper II**.

3.6 Approaches to studying vector effects

3.6.1 Particle preparation for vector studies

The propensity of MPs to act as a vector for environmental contaminants into fish was investigated using two approaches, deploying different particle preparation procedures (**Table 1**):

***In situ* particle deployment** was conducted to facilitate the sorption of hydrophobic chemicals onto MPs under environmentally relevant conditions [**Paper II-III**]. While most of experimental sorption studies consider the affinity of chemicals in isolation¹⁴⁵ rather than in complex mixtures, in which MPs are exposed to during their presence in aquatic environments, in the present studies, it was important to incorporate the MP role as a carrier for mixtures of environmental contaminants. To achieve this, we adapted some previously used approaches for particle *in situ* deployment^{101,108}. Instead of using plastic pellets, plastic micropowder was selected and enclosed in a penetrable stainless steel net (> 100 μm) and placed into environmental compartments (**Table 1**).

Chemical spiking was exclusively used to acquire sorption of the selected model HOCs [**Paper IV**]. Selected model compounds had different hydrophobicity and toxicological modes of action, and included the following chemicals: **17 α -ethinylestradiol (EE2)**, **chlorpyrifos (CPS)** and **benzo(α)pyrene (B α P)** (**Table 1**). Chemical doping experiments were performed using established methodology¹⁴⁶.

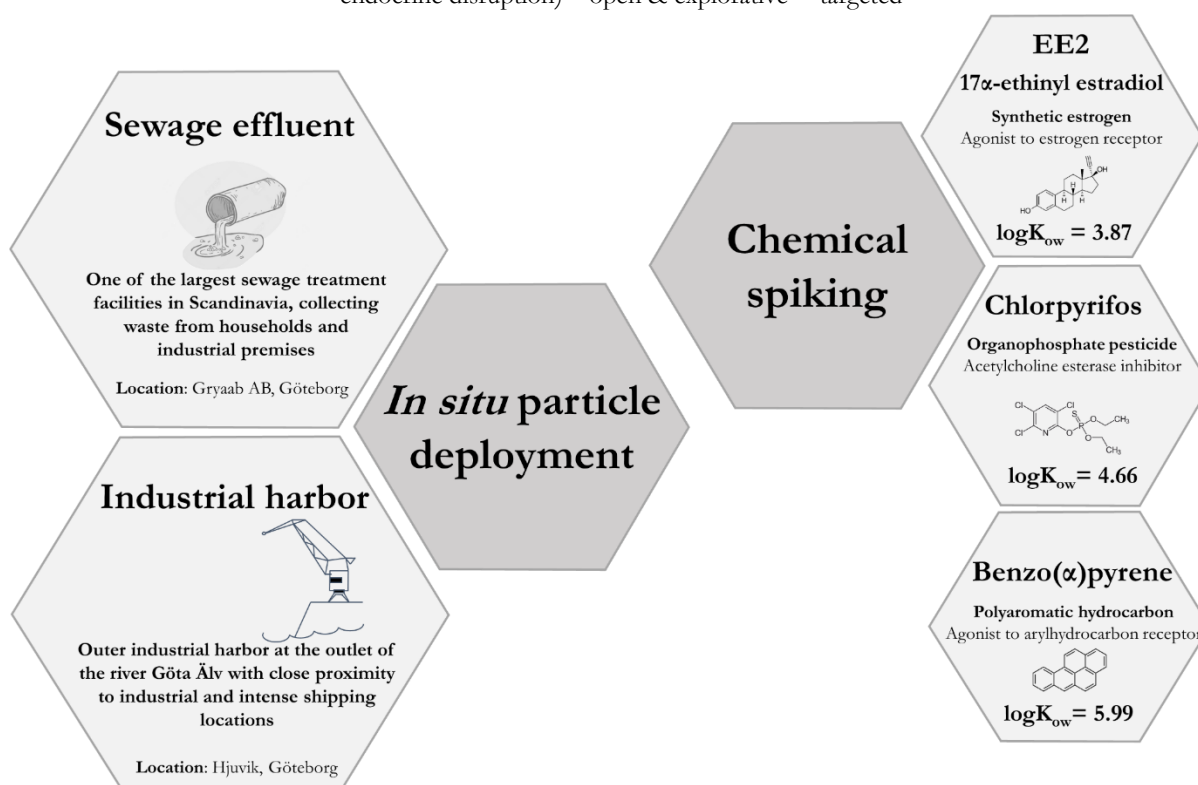
3.6.2 Considerations for experimental design

In **Papers II-IV**, a non-equilibrium (non-steady state) exposure design was used. It allowed establishment of the maximum possible fugacity gradient for chemical migration from contaminated plastic into a (clean) organism⁹². This was essential for achieving the maximum chemical exposure and mass transfer. For exposures, a great focus was placed on exposure via ingestion, which is considered a realistic exposure pathway for aquatic animals. Dietary exposures enabled exposures via this pathway and were accomplished by serving custom-made diets containing MPs. These were specifically developed for these experiments. Reconstituted dry commercial feed pellets were used in feeding trials with rainbow trout [**Papers II-III**], whereas gelatinous pellets, resembling natural prey, were developed for three-spine sticklebacks [**Paper IV**].

Table 1 Summary comparing approaches and associated methods used in **Papers II-IV**. Detailed information about chemical analyses is provided in appended papers.

	<i>IN SITU</i> PARTICLE DEPLOYMENT	CHEMICAL SPIKING
Objective	Explorative approach	Mechanistic approach
Exposure conditions	Environmental relevance	Laboratory conditions
Chemicals	Realistic environmental mixtures	Model hydrophobic organic chemicals
Mixtures	Chemicals in low concentrations (ng/g particles)	Chemicals in high concentrations (µg/g particles)
Particle weathering/aging	Yes	No
Particle type	Polymer (virgin PS MPs)	Polymer (virgin PE, PS MPs) and mineral particles (silica)
Solvent	No	Yes
Chemical analysis	Determination of chemicals associated with virgin and deployed MPs	Chemical sorption quantification
Type of chemical analysis	Non-target chemical analysis (UPLC-HRMS) and targeted 16ΣPAH (HPLC)	Targeted analysis for quantification of specific compounds (LC/MS-MS and HPLC-FLD)
Biomarker approach	Effects (oxidative stress, detoxification and endocrine disruption) – open & explorative	Bioavailability (specific mechanism of action) - targeted

RESEARCH QUESTION



3.7 Fish fillet quality assessment

To address a prevalent hypothesis that MP ingestion by fish can diminish the quality of fish products and affect suitability for human consumption, we investigated potential impacts on the oxidative stability of commercial fish fillets following exposure to MPs (and associated chemicals) [**Paper III**]. This was achieved by using a suite of established nutrition and food science methodologies, such as the determination of peroxide value (lipid peroxides), redness value and sensory assessment¹⁴⁷⁻¹⁴⁹. The assessment of these endpoints allowed the determination of oxidative-stress related impacts on its the oxidative stability and thus on the eating quality of fish fillets.

4 Results and discussion

The work presented in this PhD thesis addressed an array of different questions relating to exposure and potential impacts of MPs in fish. Studies presented here explored the uptake of plastic particles in fish via relevant exposure pathways [**Paper I**], physiological impact of the particles [**Paper II**], chemical transfer [**Papers III-IV**], toxicological effects [**Papers III-IV**] and potential impact of MPs on fish as a human food source [**Paper II**]. Chemical sorption of chemicals from the environment into MPs [**Paper III**] and the relative differences between polymers and other particles and their role as chemical vectors [**Paper IV**] were also addressed. These results are presented and discussed in the following chapters, progressing from a broader discussion of chemical sorption of environmental pollutants onto MPs in the environment to exposure and potential consequences on organism, and lastly food safety.

4.1 Microplastics as vectors for environmental pollutants

The studies presented in this thesis created an opportunity to explore the propensity of MPs to act as **environmental vectors** not only affecting the bioavailability, degradation and distribution of plastic-associated chemicals in the natural environment, but also determining potential chemical exposures to biota. In this section, some findings regarding chemical sorption of HOCs during *in situ* deployment [**Paper III**] and under laboratory conditions [**Paper IV**] are discussed.

4.1.1 Contaminant sorption from the aquatic environment

During a three-week *in situ* deployment, MPs were shown to associate with various hydrophobic environmental contaminants, including PAHs, surfactants (nonylphenol and alcohol ethoxylates, laurylsulphate, cocoamidopropylbetaine) and antimicrobial substances (triclosan) [**Papers II-III**]. MP deployment in distinct environmental matrices (sewage and harbor effluent) revealed different chemical mixtures associated with the plastic particles [**Papers II-III**].

Sewage treatment facilities serve as an important route of entry of MPs into the aquatic environment^{150,151} but are also known to introduce a large diversity of pollutants, such as pharmaceuticals, detergents and components from personal care products¹⁵². As it has been discussed in **Paper III**, an exposure medium-specific chemical profile was identified for MPs deployed in undiluted sewage effluent. The majority of the identified compounds associated with MPs, were the known components of detergents and

surfactants released from personal care products and household applications. Such findings suggest that MPs can obtain a quite specific chemical imprinting specific to this point-source and can act as environmental vectors for these contaminants. This finding was in accordance with the literature, suggesting that chemical composition and diversity of contaminants associated with MPs in the natural environment inherently reflect the spatial and temporal variability of background chemical pollution⁴⁹ and different chemical pollution sources¹⁵³.

Gothenburg harbor is northern Europe's largest port and is subjected to intense shipping pressure. The harbor area is also adjacent to industrial and refinery facilities, thus encompassing background pollution from PAHs, PCBs, dioxins and heavy metals^{154–156}. Plastic collected from highly industrialized and urban areas tend to contain higher concentrations and higher diversity of chemical pollutants compared to those from more remote places^{48,157}. Unexpectedly, the MPs deployed in the outer-harbor site did not acquire a chemical profile that was specific for the deployment medium [**Paper III**]. Attenuated chemical partitioning of various PAHs has been previously reported onto MPs deployed in other harbor environments^{108,153}, but it was not observed in the particles deployed in the Gothenburg harbor region.

4.1.2 Conditions affecting chemical sorption

Chemical partitioning during *in situ* deployment could be influenced by the number of biotic and abiotic factors, including the temporal and spatial conditions in the selected deployment site (i.e., pH, salinity, temperature, runoff or rainfall deposition, tidal and wind activity) or biotic ecosystem components (i.e., primary production)¹⁵⁸. In model predictions, elevated levels of phytoplankton are expected to reduce chemical partitioning onto MPs⁹⁵. With this in mind, it could be speculated that when MPs were deployed in the seawater during spring [**Papers II-III**], the particulate organic matter (due to the increase of primary production) could represent competing entities for chemical sorption, diminishing the sorption of HOCs onto deployed MPs. Biofouling has been shown to decrease the uptake of hydrophobic pollutants in passive samplers¹⁵⁹. A time period of a few weeks is known to be sufficient for the initiation of colonization or settlement of bacterial and algal communities onto deployed MPs¹⁶⁰, potentially diminishing sorption onto MPs. In regard to water chemistry, salinity has been identified as an important mediator of chemical partitioning in laboratory experiments. While some studies show enhanced sorption with increasing salinity due to the “salting-out” effect (reduction of solubility of HOCs with increasing salinity)^{57,59}, others indicate otherwise¹⁶¹.

Another important factor influencing chemical sorption in the aquatic environment is the residence or deployment time¹⁶². A considerably short *in situ* deployment duration (21 days) was used in **Papers II-III** compared to the timescale during which plastic debris resides in aquatic waterways. According to estimations, 80% percent of oceanic plastic debris is older than four years⁹². The residence time determines the sorption equilibrium in the deployed polymer particles. It has been suggested that it would take two years for plastic particles (0.5-2 mm) to reach chemical equilibrium in the ocean⁹². Whether MPs have reached equilibrium from surrounding media during *in situ* deployment remains unclear [**Papers II-III**]. In contrast to earlier studies, that deployed preproduction millimeter-sized pellets or plastic films^{108,163} in environmental medium to facilitate chemical sorption, in **Paper III**, considerably smaller PS particles (100-400 µm) were directly deployed in the ambient waters to facilitate chemical sorption. Chemical partitioning into polymer particles is size-dependent, and increases with the decreasing pellet diameter¹⁶⁴. Knowing that the sorbtion of PAHs to micro-sized particles is faster process compared to sorbtion to larger millimeter-sized plastic pellets¹⁶⁵, it could be possible that the deployment duration of three weeks was sufficient to achieve chemical equilibrium of the deployed particles [**Papers II-III**]. In fact, deployment duration of one month of PS pellets in aquatic environments was already suspected to lead to partitioning equilibrium for parent PAHs¹⁵³. However, for more hydrophobic compounds, a longer residence time could be needed, as the partitioning kinetics are slower for more hydrophobic compounds¹⁶⁴.

In summary, there are a number of factors that can influence the chemical sorption (kinetics and chemical constitution) of HOCs onto MPs in aquatic environments and should be considered when addressing MPs as environmental vectors of pollutants.

4.1.3 Weathering and chemical sorption

In situ MP deployment not only resulted in chemical ab/adsorption of environmental pollutants onto plastic but also led to the initiation of plastic weathering [**Paper III**]. The formation of carboxylic acids due to oxidation was observed in particles deployed *in situ*, as well as color transition [**Papers II-III**]. Yellowing of material has been associated with polymer degradation and is achieved via oxidation of phenolic additives to quinone-type of additives¹⁶⁶. Color change from white to yellow has been associated with an increase of carbonyl bands, increased oxygen content, and reduction of molecular weight as a result of chain scission process¹²⁶. Although the chemical properties of plastic are readily stable, and the degradation process is slow⁵⁹, we could observe indications of the initiation of chemical degradation of PS MPs after three weeks of *in situ* deployment [**Paper III**].

The role of plastic aging on chemical sorption remains inconclusive¹²⁶. In some studies, differences in HOC sorption behavior between virgin pellets and weathered MPs have been observed, suggesting that age and integrity of material can be important factors for chemical sorption. For example, higher levels of POPs have been found in yellowed or weathered pellets (PE) collected from the field^{51,167}. It has been argued that weathering can increase the rate of absorption of pollutants onto plastic and could increase the sorptive capacity⁴⁹. This is achieved by increasing the surface area, widening the pores in the pellets, which makes the effective diffusivity greater. On the other hand, in laboratory experiments, weathered PS showed a lower sorption coefficient for HOCs than pristine MPs¹⁶¹. Oxidizing weathering introduces oxygen-containing groups that subsequently increase the polarity of the surface¹⁶¹. In this way, oxidation reduces the amorphous phase in the polymer matrix to which sorbate molecules bind⁵⁵. Oxidative chain scission and subsequent rearrangement of amorphous (non-crystalline) regions causes crystallization in the polymer¹²⁶, diminishing sorption potential. Additionally, it has been proposed that plastic weather-degraded MPs have increased distribution coefficients and slower chemical diffusions than virgin plastic particles¹⁶⁸. As contradicting views exist, resolving the importance of plastic weathering on sorption HOCs remains a prospect of future investigations.

As mentioned above, during plastic aging, the formation of oxygen-containing groups in the polymer lowers its hydrophobicity and subsequently diminishes sorption of HOCs onto MPs¹⁶¹. As a result, an increased polarity of MP surfaces could also enhance the sorption of hydrophilic contaminants¹⁶¹. In **Paper III**, surfactants containing polar groups (i.e., hydrophilic heads) were detected on deployed (and weathered) MPs, which is consistent with this assumption. In the literature, it is becoming evident that sorption onto MPs is not limited to hydrophobic contaminants, and various polar compounds can associate with MPs^{161,169}. Currently, the majority of discussions involving hydrophobic contaminant sorption onto plastics address chemical partitioning of persistent hydrophobic anthropogenic contaminants, such as PCBs, PAHs and PBDEs, whereas the sorption of polar substances (surfactants, pharmaceuticals, or biocides) is considerably less documented and understood in the context of chemical partitioning^{54,169}.

To better understand particle-pollutant interactions in the natural environment and discuss the role of MPs as vectors for environmental contaminants, we need to improve the current understanding of the processes governing weathering and elucidate the associated sorption behaviors and mechanisms. The role of plastic weathering should be undoubtedly considered when addressing sorption of environmental pollutants onto MPs under environmentally relevant conditions. Additionally, as the research of MPs

advances, it becomes important to expand the current understanding of the existence of complex mixtures in association with MPs, made of both absorbed legacy and emerging contaminants, and address the prevalent complexity in particle-contaminant interactions.

4.1.4 Weathering

During plastic degradation, the polymer matrix disintegrates, leading to the loss of chemical constituents from the solid phase into the water phase (i.e., into the aquatic environment). The loss of chemical additives, specifically PAHs from micro-sized virgin PS MPs, was observed in **Paper III**. Over the course of a few weeks, PS MPs (1 g) of particles leached up to a hundred nanograms of PAHs (e.g., fenanthrene, fluoranthrene, naphthalene). Similarly, a decrease in certain PAHs was observed in virgin preproduction PS pellets (2 mm) deployed in San Diego Bay¹⁵³. In **Paper III**, the greatest chemical leaching was observed for naphthalene, which had the lowest molecular mass among all tested PAHs. This could comply with the proposed notion that low-molecular weight compounds are more prone to escaping the polymer matrix during environmental degradation¹⁵³. The loss of PAHs also could also be attributed to biodegradation, as hydrocarbon degrading bacteria have been found colonizing marine MPs¹⁷⁰. Based on the abovementioned observations, it is important to address that a broad spectrum of monomers and functional additives or chemical residuals found in plastic polymers have capacity to leach and could significantly contribute to overall chemical pollution in the environment. Contamination of beaches by styrene mono- or oligomers has been denoted as a potential consequence of global plastic pollution³². Additionally, plastic additives are commonly found not only in the aquatic environment but also accumulated in animal tissues^{171–173}, potentially due to degradation of plastics in the environment.

4.1.5 Sorption of contaminants onto glassy and rubbery polymers

Field and experimental studies document differential sorption affinities between different types of polymers^{50,163}. In **Paper IV**, a comparative approach was taken to investigate the sorption affinity of three model HOCs onto glassy (PS) and rubbery (PE) amorphous polymers. According to the glassy-rubbery state hypothesis, glassy polymers are thought to have more condensed and cross-linked amorphous regions, limiting chemical absorption into the polymer, while rubbery polymers have greater flexibility in these regions. Under this assumption, rubbery polymers are expected to absorb more HOCs. In **Paper IV**, chemical spiking experiments showed that glassy PS sorbed considerably more than rubbery PE (ng/g). PS, as other glassy polymers, have dual

sorption mechanisms: hole filling and adsorption. Thus, the magnitude of adsorption to the polymer could be quite tightly related to the surface properties of the material. PS MPs used in **Paper IV** had relatively high surface irregularities; hence, a larger surface area (than that of spherical PE) was thought to be an important driver attenuating chemical partitioning onto the particles. On the other hand, PE showed greater sorption per surface area or particle volume, which, in fact, supports the glassy-rubbery hypothesis.

In addition to the physical and chemical nature of the plastic sorbent, the properties of the chemical (i.e., hydrophobicity, molecular weight or volume) play a role in chemical sorption¹⁷⁴. In **Paper IV**, the hydrophobicity of a compound ($\log K_{ow}$) was shown to be an important determinant for sorption. An inverse correlation between the $\log K_{ow}$ and sorption efficacy was documented for the model compounds with differing hydrophobicity (EE2>CPS>B α P). Synthetic estrogen EE2, having moderate hydrophobicity ($\log K_{ow}$ =3.87), showed the highest absorption onto synthetic particles. Hydroxyl groups of EE2 can facilitate rapid chemical sorption onto polymer matrixes¹⁷⁵; thus, the higher levels of EE2 associated with MPs could reflect the faster absorption rate during the spiking experiment. Lower-level sorption of more hydrophobic contaminants, such as chlorpyrifos - CPS ($\log K_{ow}$ =4.66) and benzo(α)pyrene - B α P ($\log K_{ow}$ =5.99), could be explained by slower sorption kinetics¹⁶⁴ and/or the potential partitioning onto other phases during the spiking experiment. While polymers possess differential sorption capacities and sorption mechanisms, and hold different affinities to different HOCs, it could also be important to consider the physical properties of synthetic polymer particles. MP properties, such as size, shape, surface area may be important factors for particle-chemical interactions [**Paper IV**] and thus should be adequately reported in experimental sorption studies, or in MPs research in general.

4.2 Interactions with particles: exposure pathway matters

Aquatic organisms are known to physically encounter MPs in the natural environment. It is becoming increasingly important to understand the influence of exposure route for particle-organism interactions, especially for the smallest particles, such as NNPs. The following section presents findings from **Paper I** and explores the interplay between NNP exposure and biological fate.

4.2.1 Gastrointestinal tract – site for nanoparticle accumulation

In **Paper I**, the major aim was to visualize particle-organism interactions with nanoplastics at the organism level following both water and dietary exposure routes to nanoplastics and to determine particle intake pathways and sites of particle localization, using fluorescence light sheet microscopy (LSM) (**Figure 13**). The present work suggests that the gastrointestinal tract (intestinal bulb) is a prominent site for particle accumulation of polystyrene nanoplastics (PS NNPs), regardless of the route of particle administration: exposure via prey and water has led to particle deposition in the gastrointestinal region [**Paper I**]. Over time (up to 7 days), the gastrointestinal region retained or even attenuated the fluorescence signal (**Figure 13 c, f**), suggesting that particles accumulated in this compartment. Herein, no fluorescence signal was detected outside of the intestinal lumen, suggesting no or minimal particle translocation via intestinal epithelia. Due to resolution restrictions of LSM, it was not feasible to confirm whether NNPs were able to translocate beyond the intestinal lumen, or remained associated with luminal contents (**Figure 13**).

Using gold nanoparticles (Au NPs) as a proxy for inert insoluble particles, a time-dependent accumulation of non-dissolving nanoparticles in the body of the fish was demonstrated following dietary exposures [**Paper I**]. Elemental analysis (ICP-MS) showed an increase in the body burden of Au, while the fluorescence imaging did not demonstrate higher fluorescence in the fish body (or gastrointestinal region). As the accumulation of PS NNPs could not be directly quantified with fluorescence, it remains inconclusive whether body burden of plastic particles increased, similarly to Au NPs. Additionally, because the experimental setup did not include a depuration phase to allow animals to eliminate ingested particles, it remains speculative whether particles residing in the gastrointestinal tract would accumulate in an organism or would be readily egested over time.

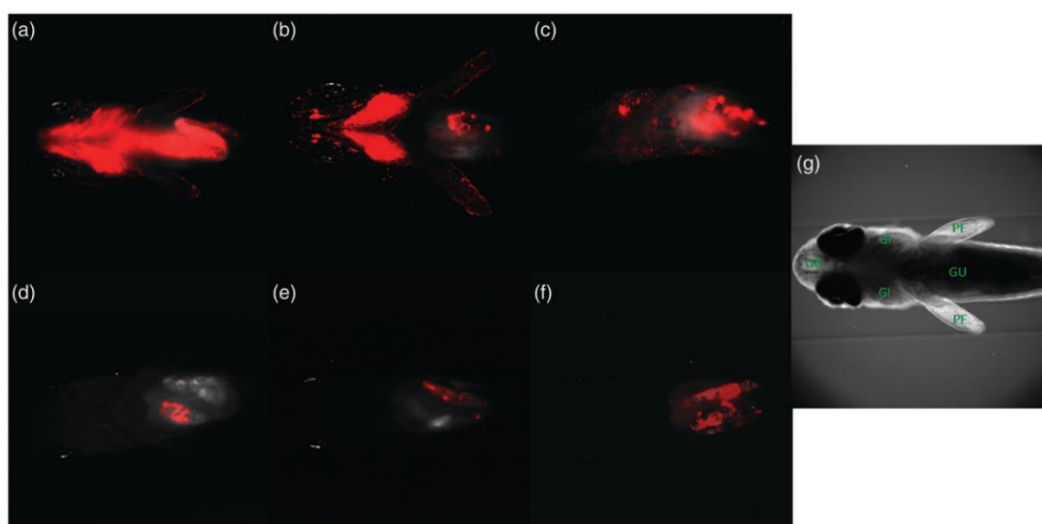


Figure 13 Maximum-intensity projection light sheet microscopy images of zebrafish exposed to fluorescent PS NNPs for 1, 3 and 7 days through an aqueous phase (a, b and c) and pre-exposed brine shrimp (d, e and f) as diet. Transmission image of a control fish for orientation (g). The gray signal corresponds to the autofluorescence of the zebrafish, and the red signal corresponds to fluorescent PS NNPs. OR - olfactory region, GI - gills, PF - pectoral fin and GU- gastrointestinal tract [**Paper I**].

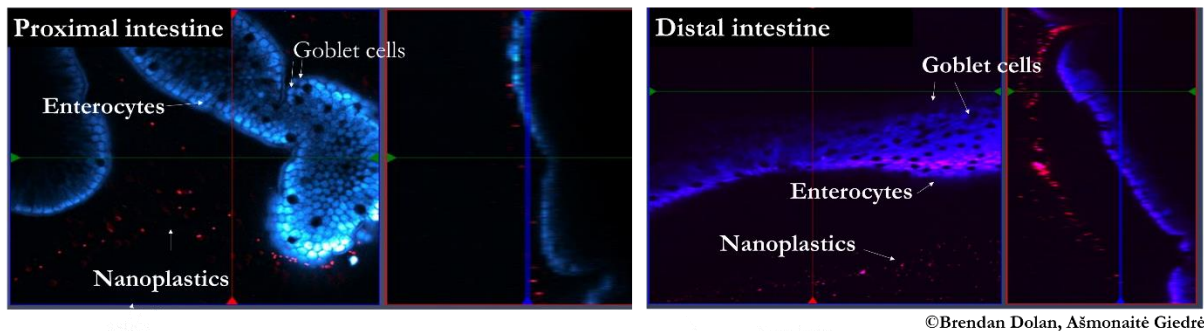
4.2.2 Particle uptake and translocation

The qualitative description of biological uptake across different taxa has been a stepping stone in MP research¹³², but the fate of particles following ingestion in various aquatic organisms remains largely unknown¹⁷⁶. Potential scenarios of biological fate of ingested plastic particles in an organism include: translocation, accumulation (retention) and/or elimination (egestion)¹⁷⁷. To date, it remains not fully established whether ingested MPs translocate, accumulate or are predominately excreted from organisms¹⁷⁸. Relatively little is known about plastic particulate transfer in fish⁸⁶.

As the gastrointestinal tract was documented as a potential biological compartment for particle accumulation [**Paper I**], it was important to address whether PS NNPs can cross the mucus layer and are taken up by enterocytes to be further translocated in organismal tissues. While it was not feasible to accomplish this in **Paper I** due to technical limitations, a pilot investigation was conducted addressing this question. In an exploratory study, two distinct segments of fish intestinal epithelia from rainbow trout were exposed to PS NNPs *ex vivo*. While the majority of MPs remained contained in the mucus layer, some MPs permeated the mucus and adhered to the apical side of the epithelia (**Figure 14**). While these observations serve as preliminary descriptive insights, particle-lumen interactions need to be further studied, especially addressing NNP interactions with mucus, as it could serve as a physical barrier against particle movement into an organism, subsequently limiting exposures to the cells and/or translocation to tissues.

In an attempt to demonstrate possibility for NNP internalization by intestinal cells, PS NNPs exposures were conducted using fish intestinal cell monolayer (RTgutGC). We could document transcellular particle uptake *in vitro* (with potential co-localization of MPs with lysosomes inside the cells) (**Figure 14**). While the ability of nano-sized plastic particles to cross biological barriers and to penetrate and accumulate in tissues has been documented¹³⁰, the magnitude and mechanisms underlying particle uptake and translocation in fish remain largely unknown.

Fish intestinal epithelia (*ex vivo*)



Fish intestinal cell monolayer (*in vitro*)

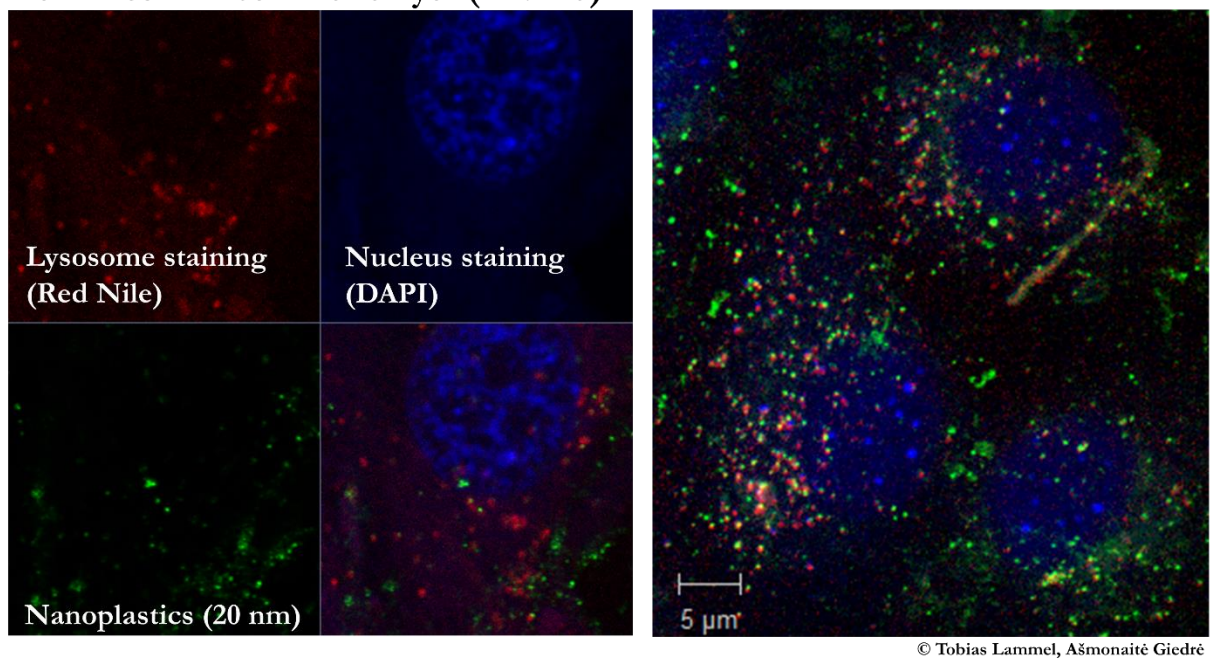


Figure 14 Nanoplastic interactions with fish intestinal epithelia in proximal and distal segments (top) and cellular uptake of nanoplastics by fish intestinal cells (bottom). In the top, orthogonal cross-section images demonstrate nanoplastic permeation into mucus and particles adhering to the epithelia (**nanoplastics** - red color; nominal size 500 nm, **tissue** - blue color). In the bottom, the internalization of particles by cells is presented. It can be seen that nanoplastics (green color; nominal size 20 nm) enter cells and colocalize with lysosomes (stained red) (**unpublished data**).

4.2.3 Exposures to nanoplastics via water

Ingestion and/or intake to the gastrointestinal tract via drinking is a principal, but not an ultimate exposure route for plastic nanoparticles in fish. Adherence to gills, body surfaces and potential intake via the olfactory nerve could be other potential mechanisms by which nanoplastics can interact with an organism and could facilitate the pathway of entry these particles not only into an organism [**Paper I**] but also in aquatic food chains.

In waterborne exposures, gills were described as a potential gateway for suspended nanoparticles to enter an organism [**Paper I**] (**Figure 13 a-b**). This finding was in line with previous experimental studies demonstrating particle accumulation in the digestive and respiratory organs of exposed aquatic animals^{103,177}. However, MPs associate with gills following waterborne exposures, but the particles do not permanently accumulate in gill filaments¹⁷⁹. It was shown that particles adhere to the mucus layer and are later excreted¹⁷⁹, which could partially explain the time-dependent decrease of MPs (fluorescence) in gill lamellae observed in **Paper I**.

Particle adhesion to fins and body surfaces was described as another pathway for biological interactions between suspended NNPs and fish [**Paper I**] (**Figure 13 a-c**). The adherence of particles onto the skin surface could be explained by particle association with components of the mucus layer¹⁸⁰ and/or particle internalization by epidermal cells^{181,182}. NNPs can permeate the fish skin mucus layer and bind mucoproteins¹⁸⁰. Additionally, plastic particle internalization in Malpighian cells, which constitute the epidermis, was demonstrated in fish *in vitro*^{181,182}, suggesting that particles can be recognized and taken up by skin cells via phagocytic activity. Although the cells demonstrated the ability to eliminate engulfed particles, such a mechanism could facilitate particle intake into an organism or simply prolong the retention time on skin epithelia.

In waterborne exposures, a fluorescence signal in the head region was observed and suggested the potential for localization/accumulation of particles therein. Consideration of the ability of NNPs to cross the brain-blood barrier and transfer into the brain is likely^{130,183}; however, in **Paper I**, it remains solely speculative. Furthermore, association of plastic particles with the olfactory bulb could be possible, and olfactory uptake could serve as another point of entry into fish [**Paper I**], leading to attenuation of the fluorescence signal in the brain region (**Figure 13 a-b**). Olfactory channel is known entry for waterborne chemicals^{184,185} and has been suggested as an entry pathway for nanoparticles¹⁸⁶. On the other hand, even though nanoparticles are generally considered to be too large to be directly taken up via the olfactory nerve, the exposure could lead

to interference with receptors in the epithelium^{187,188}. Behavioral analysis conducted in **Paper I** did not reveal any significant swimming disturbances in fish exposed to PS NNPs via both aqueous and dietary routes. Locomotion-based endpoints can serve as integrative indicators for neurotoxicity and are commonly used to detect visual-motor dysfunction or anxiety-like behaviors¹⁸⁹⁻¹⁹¹. However, these endpoints do not capture the full-repertoire of animal behaviors; thus, more subtle behavioral implications may prevail. For example, behavioral alterations related to feeding and shoaling in fish have been reported as a consequence of PS NNP exposure^{183,192}.

Based on PS NNP characterization data, nanoplastics were shown to aggregate over time [**Paper I**]. Particle agglomeration in the exposure medium inferred that PS NNPs were not bioavailable in their nominal size range and that animals were exposed to larger aggregates of particles or that exposure concentration was reduced due to sedimentation. Thus, this observation could suggest that particle stability and behavior in the exposure medium could be an important factor determining particle exposure and its biological fate in an organism.

4.2.4 Fluorescence as a tool to investigate particle uptake

Fluorescence microscopy has been at the forefront of investigations of the uptake and translocation of fluorescently-labeled MPs (or NNPs)⁸⁶. Advanced fluorescence microscopy techniques, such as LSM, have been considered a strong and valuable tool for the *in vivo* imaging of the uptake and localization of MPs in real-time [**Paper I**]. Organismal uptake and localization of nanoparticles in living zebrafish was imaged non-invasively, retaining integrity on the whole-organism level with minimal sample preparation [**Paper I**].

Fluorescence-based imaging is a versatile technique for visualization. However, it also has some disadvantages, which can lead to under- or overestimation of particle uptake or translocation. Some limitations arise from the application of fluorescent tracers. Probe degradation and/or leaching of the fluorescent dyes that can subsequently accumulate in fatty tissues, can lead to artefacts and misidentification⁸⁶. Additionally, imaging of individual nanoparticles (< 200-250 nm) with fluorescence (confocal) microscopy is impossible due to the optical diffraction limit¹⁹³. An approach deploying the approximation of light intensity for particle uptake [**Paper I**], is commonly used¹³¹. It, however, does not allow direct quantification of particle uptake, which can be seen as one of the major disadvantages when studying the uptake and translocation of particles in complex biological systems. While the use of fluorescence for quantification of fluorescent polymer particles in digested animal tissues has been shown to be problematic (due to loss of the signal during sample digestion)¹⁴³, the development of

quantitative approaches for MP studies is essential for validation and quantification of occurrence of this biological phenomenon.

4.3 Impacts associated with ingestion of microplastics

In nature, ingestion of MPs is known as an important exposure pathway for fish^{83,85,115}. The gastrointestinal tract has been pointed out as potential site for plastic particle exposure and accumulation [**Paper I**]. However, the prevalence and adversity of biological effects associated with dietary exposure to MPs (and associated chemicals) still remains largely unknown¹⁹⁴. Thus, in **Paper II**, potential negative consequences associated with MP ingestion on intestinal physiology were investigated and are overviewed in the following section.

4.3.1 Hypothesis formulation

To address physiological impacts and underlying mechanisms in an integrative way, we formulated a mechanism-based hypothesis, linking biological responses across different levels of biological complexity [**Papers II-III**], similarly as implied by the adverse outcome pathway (AOP) framework^{109,195}. In **Paper II**, the primary emphasis was placed on investigating the impacts of PS MP ingestion at the organ level and conjointly linking it with molecular and functional endpoints (**Figure 15**). It was hypothesized that upon the passage of MPs (and associated chemicals) in the alimentary tract, the structural and functional integrity of fish intestinal epithelia becomes compromised, exhibiting local or systemic inflammation, and/or leading to chemical stress (**Figure 15**).

While the physiological and molecular mechanisms for disrupted intestinal barrier function are inherently complex (and not fully resolved), a few hypothetical ways in which MP exposure could affect the integrity and function of fish intestine were identified and were experimentally investigated (**Figure 15**). The described interplay between inflammation, increased epithelial permeability and intestinal barrier dysfunction served as the main premise for hypothesis formulation in **Paper II**¹⁹⁶. The growing body of literature suggests pro-inflammatory effects inflicted by MP exposure^{105-107,197}, for example, the alteration of inflammatory cytokine regulation¹⁰⁵. Elevated expression of inflammatory cytokines has been shown to negatively affect tight junction (TJ) proteins, playing a key role in intestinal paracellular permeability regulation¹⁹⁶.

Complementarily, some polymers, nanoparticles and plastic-associated chemicals, including non-ionic surfactants, identified associated with MPs in **Paper III**, can act as paracellular permeability enhancers¹⁹⁸, and can disrupt the integrity of the physical barrier. Molecular mechanisms of contaminant-induced permeation involve an increased transport of molecules transcellularly (change membrane fluidity or structure, inhibit ABC efflux pumps) or paracellularly (affecting TJ) or alterations in rheology of the mucus layer¹⁹⁸. Certain chemicals may induce opening or weakening of TJ¹⁹⁹, promoting particle internalization (or paracellular kneading) that could subsequently not only promote inflammation in the epithelial tissue but also can lead to enhanced passage of potentially harmful substances or agents into the circulatory system.

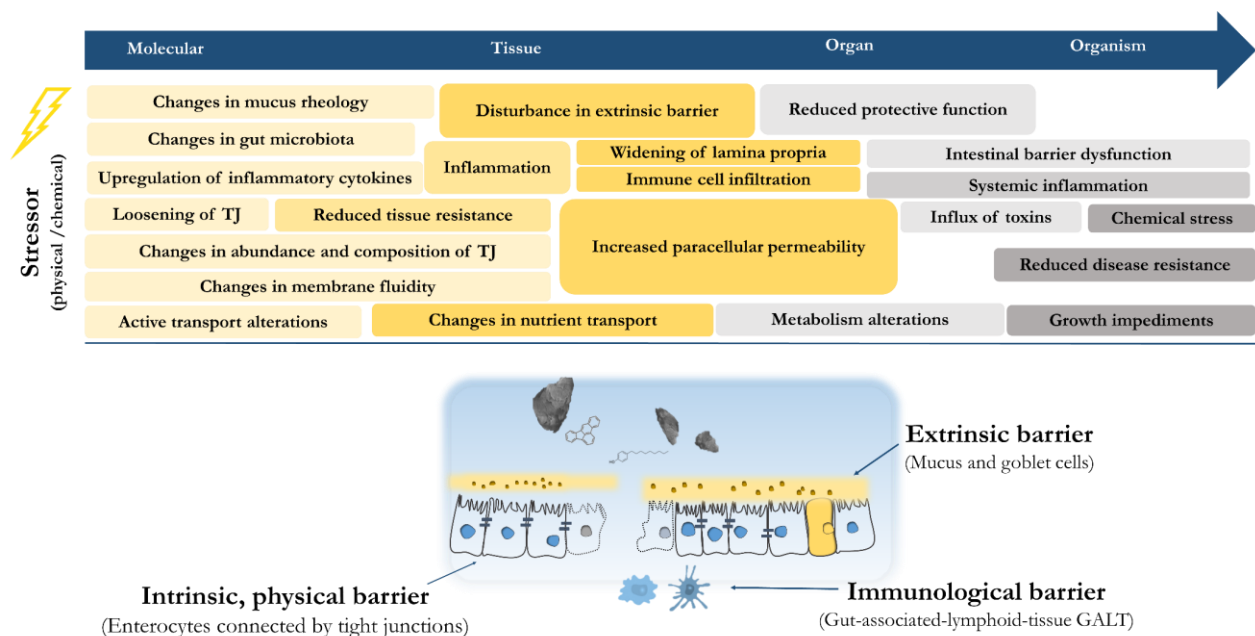


Figure 15 Graphical summary depicting potential impacts associated with MP exposure in relation to the tested hypothesis in **Papers II-III**

4.3.2 Direct impacts on fish intestinal physiology

In **Paper II**, we investigated three barriers, constituting the intestinal epithelia: extrinsic, intrinsic or physical, and immunological, in search for the potential impacts induced by MP exposure (**Figure 15**). However, contrary to our initial hypothesis, we could not confirm that exposure to high levels of PS MPs (100-400 μm ; more than 2000 particles/fish/day) can act as mechanical, chemical or pro-inflammatory hazards in fish upon ingestion. The exposure to MPs did not disrupt the integrity and functioning of intestinal epithelia in two functionally different intestinal regions of the fish, following one month of dietary exposure to PS MPs (**Figures 15-16**). Based on histological assessment, exposure to MPs did not disturb the extrinsic barrier, i.e., alter mucus-producing goblet cell abundance, nor affected the structural integrity of tissue, such as swelling or leukocyte infiltrations. The analysis of the expression of TJ proteins (ZO-1, tricellulin and occludin) accompanied by transepithelial resistance measurements, conjointly strengthened these findings (**Figure 16**). While no evident disruption in tissue morphology was observed, functional endpoints (paracellular permeability (P_{app}) or active transport) were suspected to be affected, as they are commonly used as sensitive indicators to detect barrier deficiencies prior to morphological changes being manifested²⁰⁰. However, herein, no functional impairment was observed, confirming the absence of direct impacts in the gastrointestinal tract upon ingestion.

One of the most important functions of the intestinal epithelia is to maintain selective permeability, i.e., allow the uptake of essential nutrients, yet preventing the transfer of harmful agents, such as bacteria or toxins²⁰¹. Our results demonstrated that the structural and functional integrity of the physical intrinsic barrier was retained, thereby prohibiting particle uptake across the epithelia and subsequent interference with the mucosal immune system [**Paper II**]. Exposure to MPs did not induce inflammation in the proximal or distal parts of the intestine, nor demonstrated signs of systemic inflammation (**Table 2**). This suggested that there was no translocation of relatively large PS MPs, or at least that PS MPs did not activate the mucosal immune system.

PROXIMAL INTESTINE

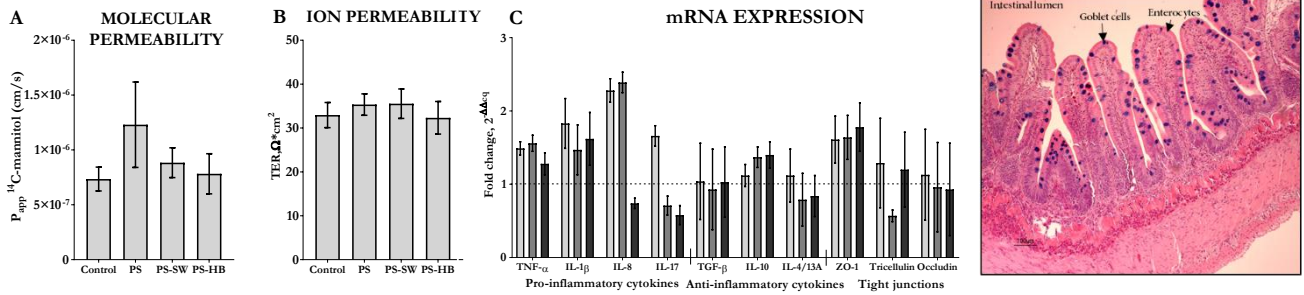


Figure 16 Effects of dietary exposure to PS MPs on intestinal molecular paracellular permeability (P_{app} coefficient, (A, D)), ion permeability (TER, transepithelial electrical resistance (B, E)), and mRNA expression (C, F) in proximal and distal intestines of rainbow trout ($n = 10-12$). Data presented as the means \pm SE; mRNA expression data are presented as fold change ($2^{-\Delta\Delta\text{Ct}}$) relative to the control, which is indicated as a dashed line (- -), ($n = 9-11$) [Paper II]. Histological sections of proximal and distal segments of rainbow trout intestine exposed to virgin PS MPs are provided in the panel on the right.

These findings suggest that the intact intestinal barrier provides a boundary for particulate (and chemical) exposure. Multiple barriers, extrinsic, physical and immunological, along with multiple mechanisms, ensure homeostatic regulation in the intestine in a coordinated manner (Figure 16). Although we could not demonstrate adverse impacts on the fish gastrointestinal tract, we observed some changes in goblet cell abundance in an extrinsic barrier of fish exposed to virgin and sewage-deployed MPs [Paper II]. A tendency towards goblet cell hyperplasia presumably indicated a protective response against particulate exposure in fish intestines (Table 2). Similarly, changes in goblet cell morphology and abundance in response to MP exposure have been described by other authors^{108,202}. An increased cell abundance leading to attenuated mucus production can serve as an important housekeeping mechanism for eliminating ingested particles²⁰³. An increase in mucus production has been proposed as a potential protective mechanism against indigestible particle exposure¹⁰⁸. The mucociliary clearance and incorporation of MPs into feces has been demonstrated for organisms from different taxa^{130,177}, suggesting that synthetic and non-degradable particles pass the alimentary tract and are surely egested if ingested items do not impose physical blockage^{108,178,203}.

The egestion of particles may depend on many factors, such as nutritional intake, frequency of the incoming meals, the physicochemical properties of ingested particles and intrinsic motoric functions of the alimentary tract²⁰⁴. In fish, gastric evacuation seems to be dependent on the size of indigestible objects and increases with decreasing dimensions (2-5 cm range)²⁰⁴, and it seems to be greater for non-nutritious dietary content²⁰⁴⁻²⁰⁶. On the other hand, longer retention times are anticipated for smaller-sized MPs, or NNPs,^{131,207-209} as they have the capacity to tightly adhere to tissues^{210,211}, or to be internalized [Paper I]. Longer retention times of smaller-sized MPs could prolong residence time and exposure duration in organisms. Thus, size could be considered as an important factor determining the retention and translocation of MPs in organisms but could also be viewed as a predictor for biological effects. Owing to the smaller size and larger surface area, MPs (i.e., < 10-20 μm) have higher capacity to induce oxidative stress and inflammation²⁰⁷. However, larger particles are not able to interact with biological receptors and/or efficiently cross physiological barriers (i.e., intact intestinal barrier) [Paper II].

Table 2 Goblet cell abundance in proximal and distal segments of rainbow trout intestines (top) and innate immune responses, including lysozyme activity and hematological parameters in peripheral blood (bottom) from different exposure groups: control, fish exposed to virgin PS MPs (PS-V), fish exposed to MPs deployed in sewage effluent (PS-SW) and fish exposed to MPs deployed in harbor effluent (PS-HB) [Paper I].

Goblet cell abundance (n=6-10)	Control	PS-V	PS-SW	PS-HB
Proximal intestine (cells/epithelia length)	91.91 \pm 4.72	97.49 \pm 2.70	97.92 \pm 3.58	88.43 \pm 2.43
Distal intestine (cells/epithelia length)	66.80 \pm 9.98	70.86 \pm 6.25	75.75 \pm 8.90	60.45 \pm 6.59
Innate immune responses (n=10-11)				
Lysozyme activity (units mL ⁻¹ plasma)	12.72 \pm 1.19	14.17 \pm 2.02	13.58 \pm 1.97	9.89 \pm 0.69
Hematological analysis (n=11-12)				
Immature erythrocytes (%TBC)	0.49 \pm 0.07	0.68 \pm 0.08	0.54 \pm 0.06	0.57 \pm 0.06
White blood cells (%TBC)	4.18 \pm 0.23	3.83 \pm 0.34	4.32 \pm 0.28	4.01 \pm 0.21
Lymphocytes (%TBC)	2.10 \pm 0.16	1.82 \pm 0.14	1.99 \pm 0.14	1.91 \pm 0.12
Granulocytes (%TBC)	0.63 \pm 0.08	0.50 \pm 0.07	0.58 \pm 0.07	0.66 \pm 0.08
Thrombocytes (%TBC)	1.44 \pm 0.14	1.51 \pm 0.2	1.75 \pm 0.16	1.44 \pm 0.17
Lymphocytes (%WBC)	50.04 \pm 1.94	48.68 \pm 2.33	46.42 \pm 2.00	48.34 \pm 2.96
Granulocytes (%WBC)	15.27 \pm 1.68	12.73 \pm 1.11	13.25 \pm 1.15	16.65 \pm 1.92
Thrombocytes (%WBC)	34.69 \pm 2.48	38.60 \pm 2.09	40.33 \pm 2.60	35.01 \pm 3.25

4.4 Microplastics as organismal vectors for pollutants

As the ability of MPs to act as environmental vectors of anthropogenic hydrophobic contaminants has been demonstrated, the propensity of ingested MPs to act as **organismal vectors**, transferring sorbed contaminants into fish, was further explored [Papers III-IV]. This segment describes main findings regarding the propensity of environmentally contaminated MPs to deliver chemicals and induce biological effects upon ingestion [Paper III], as it addresses chemical desorption, transfer and accumulation of HOCs from artificially spiked particles of different materials in fish [Paper IV].

4.4.1 Gastrointestinal tract – a site for chemical desorption

The gastrointestinal tract represents a physiological interface where not only (plastic) particles can exert physical stress, but also where the particle-bound chemicals can interact with the internal surfaces of an organism [Papers II-III]. The gastrointestinal compartment has received attention in discussions addressing desorption of plastic-associated chemicals and transfer into an organism^{99,165,212,213}. However, the current knowledge underlying desorption, as well as the biological fate of plastic-associated chemicals in an organism following ingestion remains very limited^{55,211}.

In **Paper IV**, using biomarker approach as a proxy for the bioavailability of model HOCs, namely, synthetic estrogen EE2 (estrogen receptor agonist), organophosphate pesticide chlorpyrifos (CPS; acetylcholine esterase inhibitor) and a commonly used model PAH, benzo(α)pyrene (B α P; aryl hydrocarbon agonist), we documented the release of chemicals from ingested synthetic MPs (PS, PE) into fish intestines [Paper IV] (**Figure 17**). The findings demonstrated that dissociated chemicals induced specific biomarker responses (ER and VTG, AChE, CYP1a and EROD) and activated intestinal metabolism (**Figure 17 A**). The observed migration of hydrophobic contaminants into the fish was likely due to equilibrium fugacity, driving chemicals from contaminated plastic into clean organisms. Fugacity is a thermodynamic quantity and describes the “escaping tendency” of a chemical from its medium²¹⁴. It could be expected that high fugacity gradient was established between ingested MPs and intestinal lumen [Paper IV], facilitating chemical desorption of model HOCs.

Furthermore, physiological conditions in the gastrointestinal tract are thought to play an important role in attenuating the chemical desorption of plastic-bound contaminants and facilitating their entry into an organism. The presence of gut surfactants in the gastrointestinal tract can enable chemical solubilization and desorption of HOC chemicals^{158,165,215,216}. For example, sodium taurocholate, a cholesterol-derived salt found

in vertebrates²¹⁶ has been used to demonstrate that surfactants can enhance solubilization and increase bioavailability of MP-bound HOCs^{165,166}. The formation of micelles, having an inner nonpolar region, solubilizes HOCs by forcing the migration of sorbed chemicals from the polymer into the intestinal lumen. In this context, digestive fluids may be an important and underlooked interface for the release of HOCs ab/adsorbed from MPs⁵⁵.

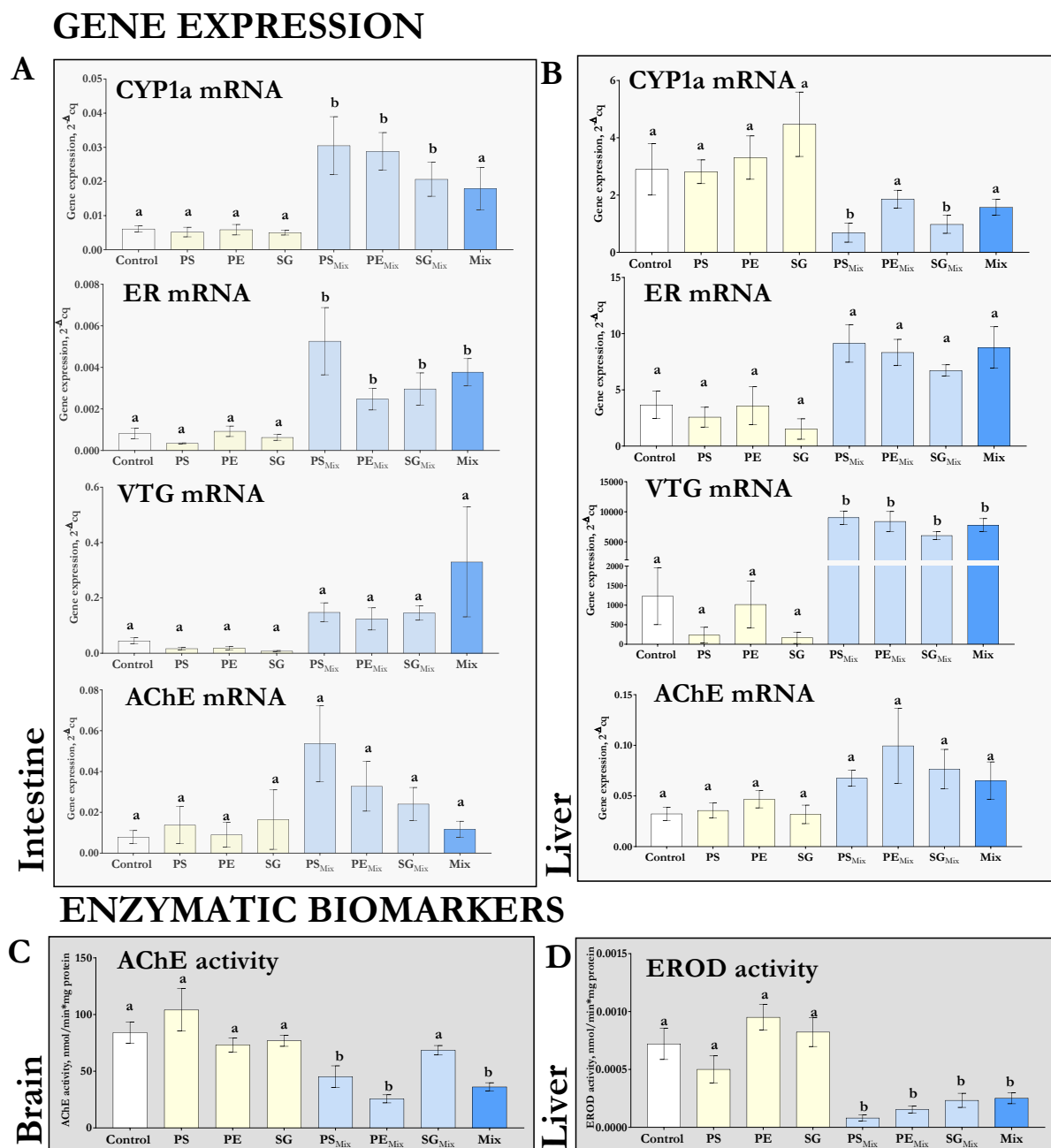


Figure 17 Biomarker responses: gene expression (A-B) and enzymatic activities (C-D) denoting the bioavailability of model HOCs in different organs (intestine (A), liver (A, D) and brain (C)). Data are expressed as the means \pm SE, n=6. Different letters indicate significant differences between the control and exposure treatments (one-way ANOVA, post hoc Dunnett test, $p < 0.05$) [Paper IV].

4.4.2 Biological fate of desorbed hydrophobic contaminants

Desorbed HOCs from MPs are made available for intestinal metabolism and absorption and can reach a whole-organism level distribution. In **Paper IV**, using a biomarker approach and chemical analysis, it was documented that chemicals were desorbed at the site of intake (intestines) and were further distributed to organs of detoxification (liver) or other peripheral organs, such as the brain (**Figure 17**), or the muscle.

As the hydrophobicity of the compound is suspected to determine HOC migration potential from plastic²¹⁷, in **Paper IV**, the role of compound hydrophobicity in governing desorption and subsequent chemical transfer (and accumulation) was addressed. In the study, the focus was set on three model HOCs with differing log K_{ow} values: EE2 (log K_{ow} =3.87), CPS (log K_{ow} =4.66) and B α P (log K_{ow} =5.99), and MP-mediated mass transfer into fish muscle tissue was assessed. The detection of moderately hydrophilic EE2 and pesticide CPS in fish muscle evidently suggested that these chemicals were readily absorbed and distributed in an organism. EE2, due its low hydrophobicity and high metabolic capacity, is unlikely to accumulate in the organism²¹⁸, whereas CPS, having higher hydrophobicity, may accumulate in an animal to a greater extent^{219,220}. Interestingly, benzo(α)pyrene, with even higher hydrophobicity, was not shown to accumulate in fish. It was suspected that benzo(α)pyrene was desorbed from MPs (PS, PE) and was metabolized in the gut epithelial tissue, restraining its further distribution into an organism [**Paper IV**]. Indeed, chemicals with log K_{ow} < 5 are not expected to biomagnify in aquatic food chains, whereas chemicals with log K_{ow} > 5 possess higher bioaccumulation potential²¹⁴. For metabolizable substances with high hydrophobicity, such as B α P, accumulation is negligible. Indeed, the bioaccumulation potential of metabolizable hydrophobic substances, such as PAHs, bound to MPs is considered lower than the accumulation of non-metabolizable persistent compounds²²¹.

In conclusion, the desorption is governed by polymer-contaminant interactions, whereas the chemical transfer and accumulation potential is driven by the chemical properties of the contaminant, such as hydrophobicity. Identification of the physicochemical properties of chemicals that are likely to be desorbed from ingested MPs could enable our understanding about the biological fate of these chemicals in an organism and potential to cause biological impacts.

4.4.3 Searching for ingestion-derived chemical effects

In **Paper III**, indirect chemical effects derived from ingestion of MPs that undergone environmentally chemical exposures were investigated. The biomarker approach was primarily used as a screening tool to detect the prevalence of chemical exposure to unknown yet suspected toxicants and shed light on their prevailing toxicity. Ingestion-derived chemical exposure and effects were examined in hepatic tissue in relation to oxidative stress, detoxification and endocrine regulation. Based on these findings, we could not demonstrate that four week exposure to high levels of environmentally deployed PS MPs promote adverse hepatic stress (**Figure 18**). Nevertheless, a tendency for low-level oxidative stress at both the gene and enzyme levels mediated via the NRF2 pathway could be observed in fish exposed to MPs deployed in sewage effluent. The observed responses were not statistically significant but could speculatively indicate a response to mild chemical stress [**Paper III**]. Studies, addressing the potential chemical toxicity of ingested MPs report instances of hepatic stress, and include effects related to glycogen depletion, fatty vacuolization, and the formation of lesions in the liver⁹⁰. It has also been shown that MPs have a potential to cause endocrine disrupting effects¹⁰¹, however, no indications of estrogenic or androgenic effects were observed here [**Paper III**]. The exposure to deployed MPs has revealed an absence of strong biological responses, potentially indicating low chemical exposure (or transfer) of MP-associated chemicals in fish liver [**Papers II-III**].

The potential of deployed MPs to induce toxicological effects is likely to be dependent on the chemical concentrations, bioavailability and intrinsic toxicity of chemical constituents in the plastic material [**Paper III**]. As non-target chemical analysis provided solely a qualitative description of compounds associated with deployed MPs, the intrinsic concentrations and the availability of the abovementioned contaminants remain largely unknown. In the study examining the potencies of different PAHs associated with deployed MPs, it was suggested that total PAH concentrations in MPs were substantially lower than reported effective concentrations²²². Similarly, the quantities of PAHs (i.e., benzo(α)pyrene, fluoranthene), retrieved from target analysis in **Paper III**, were below internationally recognized assessment criteria in soil/sediment²²³, inferring that the chemical load (and anticipated toxicity) in environmentally deployed MPs generally would be too low to induce adverse biological effects in exposed organisms. In addition to this, strong affinity of chemicals to polymer matrix, high penetration depth of the contaminant in the polymer, or fouling with food or contents of the intestinal lumen, could limit the bioavailability of plastic-bound chemicals for organismal uptake^{55,126}. Finally, the hazard potential of the deployed particles could depend on the composition of the chemicals on the particles. In **Paper III**, anthropogenic contaminants associated with MPs during deployment were relatively

nontoxic and metabolizable substances and thus were not suspected to pose a high chemical risk.

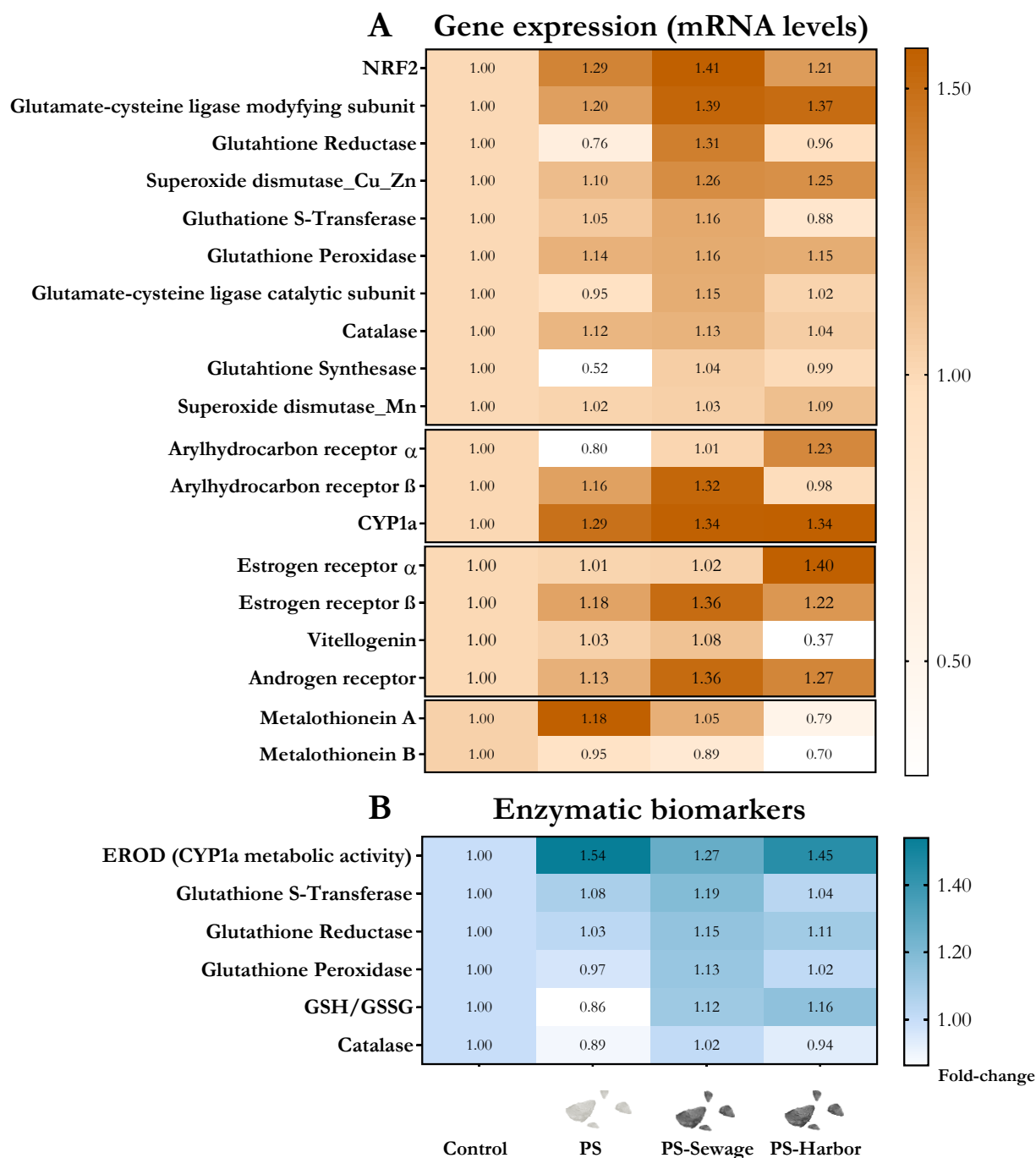


Figure 18 Hepatic gene expression (A) and enzymatic activities of proteins involved in antioxidant defense, CYP450 metabolism and molecular glutathione (B) after dietary exposure (28 days) to PS MPs. Data presented in a colorimetric scale as fold-change (gene expression) and as relative activities (enzymatic biomarkers) compared to control treatment, n=11–12 [Paper III].

Factors such as gut residence time and/or and extraction efficiency of gastrointestinal liquids⁹⁵ can be of importance, affecting release of chemicals from the MPs and uptake in the fish. Gut residence time determines how long plastic particles are retained in an organism, which in turn determines exposure duration to MPs in the gastrointestinal tract. The retention time of MPs in the fish gastrointestinal tract can span from a few hours up to multiple days^{212,217,224}. The gut retention time of MPs in fish species used in **Papers II-III** and **Paper IV** was relatively short (i.e., rainbow trout (< 48 h)²²⁵ and stickleback (< 24 h) (Bour, unpublished data, 2019)) and is thought to inherently limit the release and exposure of MP-bound chemicals into the intestinal lumen. Hence, it could be speculated that gut retention time may not be sufficient to allow for complete chemical desorption²¹⁷. Modeling studies, attempting to predict chemical desorption in artificial gut liquids, suggest a biphasic desorption model consisting first of a fast release of chemicals from ingested MPs (15-30 min), followed by a phase with slow release kinetics²¹². Based on such estimations, in the first phase of chemical desorption, the solubilization could reach up to 14-42% (depending on the kinetic properties of the HOC and polymer), which could facilitate influx of HOCs into an organism. Whether such chemical release would be significant to establish fugacity gradient, facilitating chemical transfer into an organism, remains unknown and more studies are needed to address the potential of chemical desorption in relation to gut residence time, and further elucidate the role of surfactants in this process.

4.4.4 Chemical exposure associated with virgin microplastics

While discussing the propensity of MPs to act as organismal vectors, chemical transfer and potential toxicity deriving from plastic additives or chemical residuals present in plastic formulation also needs to be addressed. In this thesis, much focus was given to PS [**Papers II-IV**]. PS is considered hazardous²³ due to known genotoxicity and carcinogenicity of styrene monomers²²⁶⁻²²⁸. PS material can shed these monomers²²⁹ remaining from the incomplete polymerization processes or during degradation^{30,32,230}. PS is also known to contain potentially hazardous plastic additives, and/or chemical residuals from manufacturing (i.e., solvents, catalysts, processing aids)¹⁵³, which have the capacity to leach out and induce biological effects^{231,232}. While the hazard potential of some plastic monomers and additives is well-studied²³, identification of full array of compounds found in plastic, as well as acquiring associated toxicological information can be challenging²⁴. This was evident in **Paper III**, where it was not only inherently difficult to determine all of plastic-related compounds in virgin PS powder but also to obtain relevant toxicological information for the identified compounds. There were two toxic compounds, namely, DEHP and benzo(α)pyrene, found to be associated with virgin PS MPs [**Paper III**]. DEHP is a suspected endocrine disruptor and potential reproductive hazard²³³. It is recognized as a substance of very high concern (SVHC) with restricted use in consumer products across the EU. As the concentration of DEHP present on PS MPs was not determined [**Paper III**], we could not confirm that whether the restriction limits (0.1% of weight) for this compound were exceeded in the PS formulation. However, it is generally not expected for PS to contain high levels of this additive²³⁴. The second compound of high toxicological relevance was benzo(α)pyrene, and trace amounts of this known carcinogen²³⁵ were found on PS powder (< 1 ng/g particles). The documented presence of plastic additives, residuals, and degradation products does not inherently refer to the hazard potential. The intrinsic chemical load and capacity for leaching are important aspects determining the bioavailability of the toxicants and potential to cause biological effects. Overall, PS is thought to contain considerably less chemical additives compared to other polymer types (e.g. PVC where additive content can be as high as 60-70%⁵), and thus could explain the absence of chemical toxicity. Leachates of plastic additives, such as processing aids and lubricants, that by weight constitute relatively low percentages (0.5-2%⁵), may only marginally contribute to chemical mass transfer and potential biological effects. In support of that, a modeling study aiming to assess the leaching potential of additives (nonylphenol, BPA) into fish, it was demonstrated that ingestion of plastic would be negligible for exposure to these chemicals²³⁶. In summary, it can be concluded that the hazard potential of MPs could be polymer (formulation)-specific and can largely depend on the intrinsic chemical load of the plastic-associated chemicals.

4.5 Environmental relevance of ingestion-derived vector effects

The hypothesis that MPs can act as vectors for various anthropogenic pollutants from the surrounding environment, transferring these contaminants into aquatic organisms, leading to accumulation and inducing biological impacts remains a subject of a discussion. Field observations, as well as experimental studies (including **Paper IV**) demonstrate that the desorption of HOCs from plastics in biota is likely^{56,91,237,238}, however it remains questionable whether MPs are important vectors for mediating the transfer of hazardous chemicals in biota under environmentally relevant conditions. While discussing and evaluating environmental relevance of ingestion-derived MP vector effects, a three main aspects should be considered²¹³: the fugacity gradient, the comparative importance of MP as a source for environmental pollutants and the inherent complexity of chemical interactions associated with MP exposure.

Firstly, the consideration for fugacity gradient is important. Chemical transfer from plastic to an organism takes place only if a fugacity gradient is present between these two media. MP-mediated chemical transfer of pollutants has been shown in numerous experimental studies using a non-equilibrium experimental design, including **Paper IV**, and to some extent **Paper III**. Notably, in **Paper IV**, MPs sorbed substantially high concentrations of hydrophobic compounds ($\mu\text{g/g}$ particles) [**Paper IV**], which established large fugacity gradient in plastic-organism system. Whereas, a considerably lower fugacity gradient has been attained between particles and an organism ingesting particles from an *in situ* particle deployment, since much lower concentrations (ng/g particles) of chemicals (i.e., PAHs) were identified associated with MPs [**Paper III**]. Presence of a fugacity gradient facilitates directional movement of chemicals into an organism, potentially leading to chemical transfer and accumulation of chemicals in tissues of an exposed animal²³⁹. This would undoubtedly increase of the chemical burden in an animal if MPs are the only source of chemicals^{166,213}. While the fugacity gradient in the gastrointestinal tract is imperative for the chemical transfer of plastic-bound chemicals, the prevalence of fugacity gradients in real life remains discussed. It has been argued that in nature, organisms ingesting MPs are expected to be contaminated (i.e., equilibrium with the surrounding environment and MPs), thus, the fugacity gradient should not favor chemical migration from plastic to an organism. It also has been suggested that ingestion of MPs could lead to an opposite “cleaning” effect, reducing animals’ chemical burden^{53,213}, if relatively clean particles are ingested by relatively contaminated organisms. While it has not been possible to experimentally confirm “cleansing effects” in fish¹⁰⁰, and only minor reductions of internal concentrations were predicted in a modeling study²¹⁷, the importance of “cleaning effects” in the context of

MP vector effects remains hypothetical. Although it was experimentally possible to demonstrate MP-mediated transfer of hydrophobic pollutants [**Paper IV**], the chemical transfer from environmentally deployed MPs seems to be low and effects appear to be minor [**Paper III**]. Findings presented in this thesis [**Papers II-IV**] are in line with existing model predictions and experimental studies^{19,240,241}. Given the low incidence of MP ingestion that occurs in the natural environment⁸³, MP-mediated vector effects upon ingestion in fish are suspected to be negligible.

Secondly, for MP ingestion-mediated chemical transfer to be an important pathway, the uptake of HOCs via ingestion should exceed chemical intake via other naturally occurring particulates, such as food, natural organic matter or sediment particles, or exposure via water. In **Paper IV**, the comparative importance between synthetic polymer particles (PS, PE) and one type of naturally occurring particles (silica glass) in mediating chemical transfer was assessed. It has been shown that inorganic silica glass particles not only sorb considerably less than their synthetic polymer counterparts (PS, PE) but also transfer less of hydrophobic contaminants into fish upon ingestion [**Paper IV**]. Although MPs showed a greater propensity for vector effects than inorganic silica particles, the ecological importance of such transfer in the natural environment is expected to be low. MP concentrations currently reported in aquatic environments are considerably lower than naturally occurring particles, and MPs constitute a very small fraction of a broad diversity of particulates in the environment. In addition to this, only a small fraction of persistent organic contaminants (<1%) is expected to be associated with plastic debris compared with other media in the ocean⁹⁵. Modeling studies that consider all exposure pathways, presumably providing environmentally relevant estimations of the importance of MPs as carriers for HOCs, suggest limited importance of MPs as a vector for PBTs to organisms, and that other exposure pathways are of greater importance⁹⁵. Currently, there is no convincing evidence that ingestion of MPs would significantly enhance bioaccumulation of POPs in aquatic organisms⁵³. The exposure to HOCs associated with MPs via ingestion may represent an alternative exposure pathway for these chemicals into biota [**Paper IV**] but its relative importance compared to natural particles or other exposure pathways remains to be further investigated^{92,97}.

And thirdly, it is important to recognize that “the chemical hazard of MP ingestion should relate to all the chemicals in the plastic-organism system, that is, the chemical mixture transferred to or from the organism by ingestion and chemicals should not be considered in isolation”²¹³. Consideration of complexity, derived from MPs themselves and toxicokinetics, is imperative for understanding and assessing the potential importance of MP vector effects. As toxicokinetic interactions between MP-associated chemicals were demonstrated in **Paper IV**, it highlighted not only the inherent

complexity and interconnectedness of biological responses but also inferred to potential challenges for the assessment of potential chemical risks associated with MPs (or chemical mixtures, in general). The phrase ‘death by a thousand’ may be applicable here, as MPs are one of many stressors present in the environment today, and the intricacies of the direct and indirect effects of all these stressors in complex systems are not well understood. There are still too many knowledge gaps and uncertainties surrounding this subject, thus it becomes inherently difficult to evaluate and predict ecotoxicological relevance of MP-mediated vector effects.

4.6 Chemical transfer and impact on human food quality

There is a growing interest in research aimed at assessing the potential impacts of MPs on human food quality and safety. **Paper III** explored the potential of ingested MPs and associated chemicals to reduce the quality of fish products. Maintenance of the oxidative stability of fish fillets is important because it prevents the reduction of nutritional value, the development of unpleasant flavor, and the decrease in commercial value²⁴². It has been hypothesized that chemical exposure to plastic-associated chemicals can induce oxidation of lipids in fatty fish fillets, such as those of rainbow trout, potentially reducing their quality [**Paper III**]. Contrary to this hypothesis, oxidative damage-related effects on fish fillet quality were found to be negligible [**Paper III**]. Although the loss of redness in fish exposed to dietary MPs has been observed, there were no indications for the formation of lipid peroxides or the development of rancid odor in fish fillets (**Figure 19**).

The decrease in redness of fish fillets has been previously associated with oxidation of hemoglobin²⁴³. Auto-oxidation of hemoglobin has been proposed as an important factor propagating the onset of oxidation in the muscle, leading to color changes in the tissue^{149,244}. In that case, loss of redness could be related to biochemical processes governing the conversion of

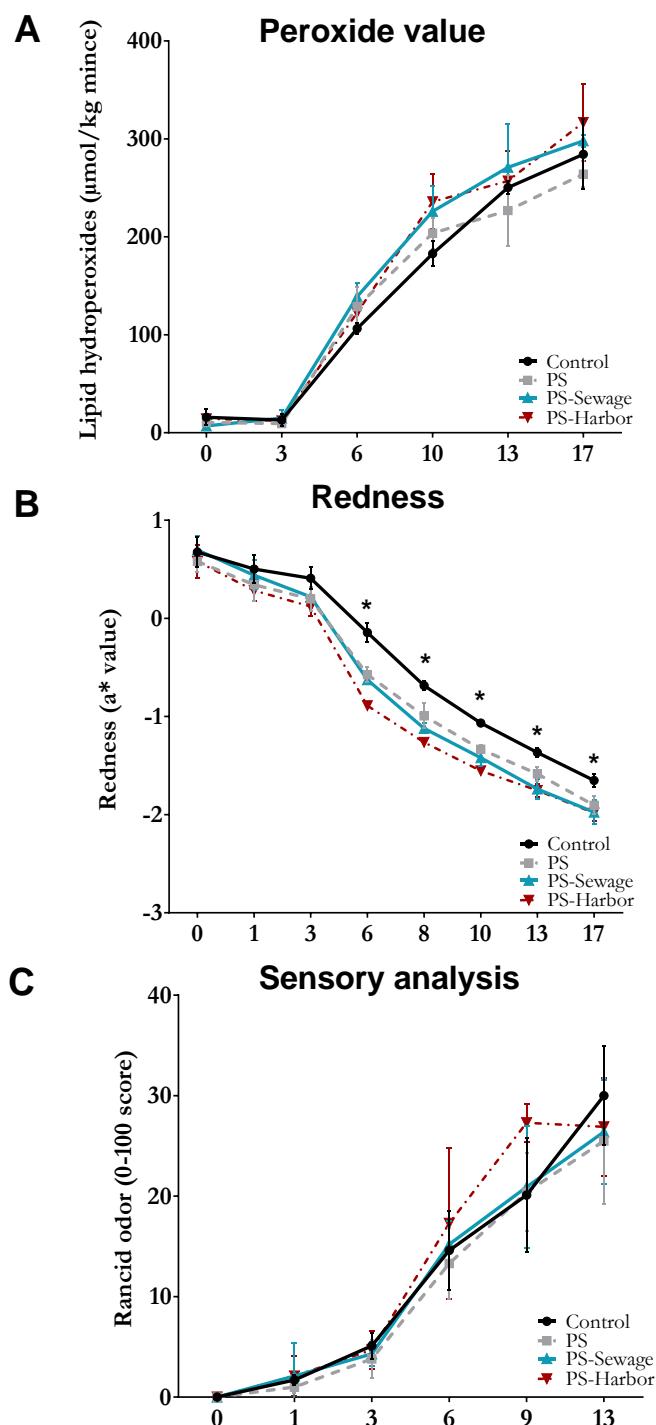


Figure 19 Lipid hydroperoxides (peroxide value, A), redness (a* value, B) and development of rancid odor (C) of pooled minced fish fillets in a storage trial on ice. Data are expressed as means±SD, n=3–4. Significant differences from the control are denoted with (*), p< 0.05 [**Paper III**].

the bright red oxy-myoglobin (Mb) to the brownish met-Mb¹⁴⁹. Another potential reason for the loss of redness in fish fillets could be associated with the levels of astaxanthin in fish fillets. Astaxanthin, which is a carotenoid pigment, can act as a free-radical scavenger or antioxidant, preventing oxidation in fish fillets²⁴⁵. It could be speculated that the loss of redness could be related to the intake/absorption of red pigments, such as astaxanthin, from the diet²⁴⁶.

Herein, potentially lower levels of such pigment could indicate reduced protection against lipid peroxidation. Changes in the coloration of rainbow trout fillets [**Paper III**] could serve as subtle indications of potential MP-induced oxidation in the tissue. With the present knowledge, we suggest that MP-mediated chemical effects on oxidative stability may be limited, and in this scenario, it is unlikely to pose a concern to food quality [**Paper III**]. As the scope of the study addressing potential implications of plastic ingestion on commercial fish fillet quality is novel and the research findings are the first of their kind [**Paper III**], the subject is of potential interest for future investigations.

Public concerns regarding impacts on food quality and human health are linked to the potential of MPs to transfer chemicals into seafood commodities. While investigating the mass transfer of MP-associated chemicals into fish fillets was beyond the scope of the study [**Paper III**], evidence suggests that MP ingestion by commercial species of fish and mussels does not represent an important hazard for human health. Based on some estimations of potential chemical exposure to certain plastic additives and PBTs via MPs, an intake of 7 micrograms of plastic would contribute only 0.1% of total dietary human intake, ingested with mussels¹¹⁴. Nevertheless, monitoring the presence of MP and NNP contamination in seafood commodities has been identified as an emerging issue and is needed to determine potential dietary exposures to these particulates and associated chemicals²⁴⁷. On the other hand, serious direct health implications in humans may occur due to direct exposure to PBT compounds and natural toxins in wild-captured seafood products.

Furthermore, the use of plastic has become ubiquitous in fisheries and aquaculture sectors, with plastic materials being found everywhere from infrastructure elements (buoys, holding tanks, ponds, cages, etc.) and fishing gear (nets, lines) to processing and packaging materials¹¹⁴. Negative aspects of plastic pollution leaking from this sector were initially associated with abandoned fishing gear, leading to “ghost-fishing”. Now, the need for investigations of less imminent and subtler impacts resulting from chemical leaching and wear and tear of plastic has become apparent. In this case, discussions on the potential implication of MP pollution in human food safety and quality can be viewed as an opening for broader-scale plastic pollution questions across different sectors.

4.7 Outlook: from risk perception to risk assessment

Plastic pollution is recognized as an ever-growing environmental problem, and MPs have emerged as novel anthropogenic contaminants in the natural environment. Although MP pollution has become an environmental issue of concern, it remains uncertain whether MPs represent a toxicological hazard in the aquatic environment.

Despite advancements in the research field the current knowledge about exposure and impacts remains fragmented and subsequently impedes an adequate assessment of the prevailing risks of MPs in the environment. Notably, the discrepancies between studies investigating the exposure (occurrence, bioavailability) and biological effects prevail²⁴⁸ and there is an evident mismatch between MPs tested in experimental laboratory studies and *in situ* observations^{19,20}. Field and monitoring investigations continue to increase our understanding of the occurrence of larger-sized MPs (>100-300 μm) occurrence in the environment, with only relatively recent attempts to expand current knowledge about the prevalence of plastic debris at the lowest particle size fractions. Laboratory studies, on the other hand, pursue examining the fate and effects of the smallest particles and have been largely focused on sizes and typologies that are not normally detected in the natural environment. Criticism has been directed towards testing irrelevant types of MPs that are monodisperse, tested in narrow ranges and are small-sized¹⁵. Most MP research is conducted using commercially available reference materials, such as microspheres/microbeads or bulk resin powder or films, which have not been subjected to surface modification, fragmentation and/or aging processes that would take place in a natural environment and may not serve as a fair representation of environmental MPs³⁴. Complementarily, the studies examining the potential impacts of larger MPs particles (> 100 μm), extractable from biological and environmental samples, and which have undergone environmental exposures, remain relatively scarce. Due to the increasing abundance of plastic debris in the natural environment and its persistence and ability to generate micro- or nanoparticles, there is a need to employ realistic exposure pathways to organisms and incorporate environmentally relevant particle types and chemistry gradients in the effect studies. To a large extent, (eco)toxicological studies, investigating the potential impacts of MPs on biota, have employed high exposure concentrations that lack environmental relevance^{104,248,249}. Such studies, primarily addressing adverse effects dominated the peer-reviewed literature, advocating for the precautionary measures for this emerging issue. Conclusions attained from MPs studies are often generalized to facilitate knowledge integration^{11,250}, yet strongly rely on extrapolations across a broad size and chemistry gradients and different biological domains. Though advantageous, this can sometimes lead to inappropriate interpretations of environmental risks of MPs or inaccurate risk communication, leading

to flawed risk perception^{123,250}. In recent years, more neutral, “less alarming” findings have been published in regards to MP exposure²⁵¹, as well as the work presented in this thesis, advocating for the more balanced discussions, regarding the hazard potential of MPs in the natural environment. In moments of rapid knowledge generation, it is crucial to distinguish between the factual and perceived scientific knowledge regarding the impacts of MPs²⁵².

There are many unresolved challenges in the developing field of MP research that relate to generation of robust and transferable scientific knowledge, and its potential applicability for risk assessment. Currently, there is a necessity to bridge the gap between information about exposure, attained from field studies, and data from laboratory hazard testing. As a consequence, the risk assessment of MPs remains premature, and is not sufficient to support policy making¹³².

5 Conclusions and future perspectives

The findings presented in this PhD thesis confirmed the established notion that synthetic polymer particles, or plastics in general, act as a sink and a source of environmental pollutants. MPs can act as environmental vectors and associate with diverse hydrophobic chemicals, including PAHs, nonylphenol and alcohol ethoxylates, and others from the ambient environment [**Paper III**]. Many compounds from these groups are not as persistent and hazardous as other hydrophobic pollutants commonly reported in association with environmentally deployed MPs. MPs are shown to act as carriers for chemicals, such as surfactants and antibacterial agents prevalent in sewage effluent. The chemical sorption of hydrophobic organic chemicals onto MPs is a dynamic process and depends on various factors, such as the hydrophobicity of the contaminant and intrinsic polymer properties [**Paper IV**] or the deployment conditions [**Paper III**]. Morphometric particle properties, such as size, surface area, shape, volume and density, can play important roles in mediating the chemical sorption of HOCs into particles. Thus, comprehensive physical characterization of MPs appears imperative for sorption experiments [**Paper IV**] and is strongly recommended for future MPs studies. In the aquatic environment, microplastics will degrade, leaching of intrinsic chemical constituents into the surrounding medium [**Paper III**]. Therefore, this represents an emerging source of anthropogenic chemicals into the environment.

As plastics degrade, they generate smaller plastic fragments, namely micro- or nanoplastics, which aquatic animals can physically interact with, in this way facilitating their entry into aquatic food webs. Given their small size, nanoplastics can be taken up by aquatic invertebrates at the base of the food chain and can then be passed to higher trophic levels [**Paper I**]. The exposure route, as well as particle behavior in the exposure medium, will determine the organismal uptake pathway and biological compartments, where particle interactions take place [**Paper I**]. The exposure to plastic particles suspended in a water column can lead to particle ingestion (via drinking) and promote particle association with externalities of the body surfaces, such as gills, skin and fins, whereas ingestion nanoplastics in fish via contaminated prey leads to particle accumulation or retention in the gastrointestinal tract. Ingested plastic nanoparticles possess the ability to cross the mucus of the intestinal lumen, and can be internalized by fish intestinal cells. However, the prominence of particle uptake and translocation, as well as mechanisms in fish intestinal epithelia, remains to be fully investigated. Future studies should address mechanisms of uptake of small micro- and nanoplastic particles across epithelial tissues and internal distribution in organisms.

Ingestion is an environmentally realistic particle intake pathway for MPs, and the gastrointestinal tract represents a biological compartment where direct impacts of MP exposure can manifest and where the release of MP-associated chemicals can occur. The results of this work suggested that dietary exposure to high levels of relatively “large” polystyrene microplastics does not induce any adverse effects in fish intestines [**Paper II**]. No signs of mechanical damage or inflammation were observed, and fish retained structural and functional integrity of the intestinal epithelia. Additionally, ingestion of deployed or environmentally relevant MPs was not shown not to induce hepatic stress, suggesting that indirect chemical exposure to MPs is unlikely to cause adverse impacts on organismal health, although MPs were shown to associate with some of hazardous contaminants, which are known carcinogens and endocrine disruptors [**Paper III**]. The findings suggested that ingested microplastics have limited capacity to act as physical and/or chemical hazards and that impacts can depend on the interplay between the intrinsic properties of the synthetic particles (chemical load and bioavailability), exposure conditions (dose, duration) and the capacity of an organism to respond and cope with the stressor. The size of particles should be recognized as an important factor, defining not only the degree of biological uptake [**Papers I-II**], but also subsequent effects and its overall hazard potential [**Papers II-IV**]. Further studies should address the importance of both physical and chemical characteristics in determining the extent of impact on exposed organisms.

The gastrointestinal tract is suggested as an important site where chemical desorption and metabolism of MP-associated chemicals occurs [**Paper IV**]. The hydrophobicity of a chemical, its metabolic capacity, as well as interactions with other contaminants will influence the propensity of chemical transfer and determine the bioaccumulation potential of plastic-bound chemicals in an organism. The ingestion of microplastics depicts an alternative transport pathway of anthropogenic pollutants into fish. Although MP-mediated chemical transfer via ingestion in fish is likely, the chemical exposure and subsequent chemical transfer and effects, are generally expected to be low [**Papers II-IV**]. The environmental importance of MPs as a source for chemical exposure to aquatic wildlife is thought to be minor. Improving the understanding of the uptake of MP-sorbed contaminants upon transition in the digestive system and their further fate in organisms is important, especially concerning plastic additives, which have been predicted to have greater impact on biota than absorbed HOCs and should be addressed in future studies.

In regard to MP-associated chemical leakage into food chains and potential implications for human food quality, MP-ingestion-derived loss of redness in fish fillets could potentially indicate reduced oxidative stability [**Paper III**]. However, it remains inconclusive whether dietary exposure to MPs will have profound impacts on the quality

and shelf life of commercial fish fillets. As plastic pollution increases in the environment, and increasing amounts of aquatic food is produced in aquaculture systems constructed largely of plastic, fish will increasingly be exposed to plastics as they grow, and the impacts of this on food safety and quality should be further investigated.

Given the ever-increasing volume of plastic waste entering the environment and the suspected potential of plastic-associated chemicals to induce toxicological effects, plastic pollution will result in major ecotoxicological challenges in future. As concerns regarding plastic pollution are gaining momentum on the global agenda, it becomes increasingly important to adequately integrate existing knowledge and critically address remaining knowledge gaps and uncertainties while discussing potential risks associated with micro- and nanoplastics.

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