Ecotoxicological Assessment of Microplastics in Limnic Systems with Emphasis on Chemicals Released by Weathering

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Abbreviations

ANSES	French Agency for Food, Environmental and Occupational Health and Safety
ARE	Antioxidant response element
BPA	Bisphenol A
CAS	Chemical Abstracts Service
CBI	Confidential business information
CPPdb	Database for Chemicals associated with Plastic Packaging
DC	Dark control
DEP	Diethyl phthalate
DEHP	Diethylhexyl phthalate
DiBP	Di-isobutyl phthalate
DMP	Dimethyl phthalate
DNBP	Di-n-butyl phthalate
ECHA	European Chemicals Agency
EDA	Effect-directed analysis
EDC	Endocrine-disrupting chemical
EPR	Extended Producer Responsibility
EU	European Union
FTIR	Fourier-transform infrared spectroscopy
GC	Gas chromatography
GR	Glucocorticoid receptor
hAR	Human androgen receptor
HC_5	Hazardous concentration for 5% of species
hER	Human estrogen receptor
HPLC	High-performance liquid chromatography
LCA	Life-cycle assessment
LC-QTOF-MS	High resolution liquid chromatography mass spectrometry
LDPE-R	Recycled low-density polyethylene
NIAS	Non-intentionally added substances
OTC	Organotin compound
PBAT	Polybutylene adipate terephthalate
PBS	Polybutylene succinate
PBT	Persistent, bioaccumulative and toxic
PE	Polyethylene

PEC	Predicted environmental concentration
PET	Polyethylene terephthalate
PLA	Polylactic acid
PNEC	Predicted no effect concentration
PP	Polypropylene
PS	Polystyrene
PUR	Polyurethane
PVC	Polyvinylchloride
REACH	EU law for the Registration, Evaluation, Authorization and Restriction of Chemicals
SB	Starch blend
UV	Ultraviolet
YAAS	Yeast Anti Androgen Screen
YAES	Yeast Anti Estrogen Screen

Abstract

Plastic pollution is a pervasive problem. In the environment, both the physical and chemical aspects of the material contribute to pollution. For instance, discarded plastic is useless waste that is fragmented upon degradation and so-called microplastics <5 mm are formed. Besides, the chemicals added into plastics are usually customized for specific functions, but these can easily transfer from the polymer into an ambient medium. This work examined both of these aspects. Moreover, the question of whether ecotoxicological effects are more likely to appear because of the microparticle properties or the chemicals transferring from the microplastics was addressed. A special focus was laid on the UV-weathering-induced chemical release.

First, conventional and biodegradable plastics made from fossil and bio-based resources were chosen. The different materials (pre-production and recycled pellets as well as final products) were weathered and their leachates evaluated *in vitro*. The leachates were analyzed with non-target screening in order to measure the number of transferred chemicals. Plastics identified as toxic were subjected to further investigations *in vivo*. A biodegradable shampoo bottle was processed to microplastics and the particles' physical and chemical properties were assessed with the freshwater worm *Lumbriculus variegatus*. Here, commonly used endpoints such as mortality, reproduction and weight were tested via different exposure routes. Moreover, the freshwater shrimp *Neocaridina palmata* was exposed to microplastic beads and fragments to clarify if the shape of the particles affects the ingestion and egestion, respectively. Thereafter, two materials that displayed the strongest toxic responses *in vitro* within the first study were weathered and leached. Finally, the shrimps were exposed to the leachates and the locomotor behavior was used as an ecologically relevant but less frequently studied endpoint.

The results of the studies highlight that plastics are chemically complex mixtures, containing a wide range of chemicals in terms of the number and functionality. These chemicals induced oxidative stress, baseline toxicity and endocrine activities. This shows that pellets represent a processing state that comprises chemically heterogenous materials. Moreover, it was shown that a degradation initiator is not necessarily relevant to trigger inherent substances to leach out from plastics. Despite this, the UV-weathering resulted in increasingly released chemicals and exacerbated the *in vitro* toxicities. Even plastics assessed as toxicologically harmless prior to weathering released toxic chemical mixtures once they were weathered. One recycled and all of the biodegradable plastics were toxicologically most concerning. This means that such materials are currently not better than conventional, virgin plastics in terms of their toxicity.

To clarify the source of the microplastic toxicity, *L. variegatus* was exposed to biodegradable microplastics. The particles were ingested by the worms and adversely affected the examined endpoints. In comparison, microplastics that were depleted from their chemicals via a solvent treatment were less toxic. Kaolin as a natural particle control was evaluated alongside and positively affected the weight of the worms. This emphasizes the ecological relevance of fine-sized matter for the test species. The chemicals extracted from the microplastics induced a 100% mortality. A chemical analysis of the material revealed two ecotoxicologically relevant biocides. The physically-mediated effects of the microplastics seemed to be less of a concern for the worms, which is probably linked to their adaptation to high concentrations of naturally occurring particles in the environment. However, the effects related to the chemicals of plastic cannot be ignored, especially for materials that are claimed to be environmentally friendly.

In the third study, the role of the particle shape in the gut passaging of *N. palmata* was studied. While the particle size was a determinant factor for the ingestion, the ingestion and egestion of the beads and fragments did not differ, respectively. The shrimps ingested less fragments when food was provided than in the absence of food. As for the worms, the shrimps are known to ingest many naturally occurring particles. Their unselective feeding behavior towards the particle shape could indicate that microplastics as a physical pollutant are negligible for the shrimps. That is why the chemicals of the two most toxic *in vitro* materials were tested with *N. palmata*. However, no trend towards elevated or reduced movements of the shrimps was observed, even though the leachates contained baseline toxicants. This shows that the *in vitro* toxicities of plastics are not necessarily indicative for effects to occur at the *in vivo* level.

This work highlights that toxicologically safe and unsafe plastic materials exist, but neither a recyclate nor the biodegradable plastics were better surrogates for conventional polymers. Even pellets as the starting materials for consumer products included toxic chemicals. Effects at the suborganism level can be helpful to distinguish toxic from ‘clean’ materials, especially because not every *in vivo* test design has the sensitivity to determine subtle but biologically significant effects. Moreover, the UV-weathering tests resulted in enhanced *in vitro* activities. Accordingly, manufacturers should be held accountable for the entire life-cycle of plastics. To begin with, the number of processed chemicals should be reduced drastically. Otherwise, this could aggravate the exposure situation in the aquatic environment. It is notable that in this work rather negligible effects were ascribed to the physical properties of microplastics with the help of two benthic organisms. However, if plastic production is continuing to grow, this could lead to yet unforeseeable impacts. It is thus important that actors of the entire plastic value chain collaborate with each other and create sustainable and safe plastic uses.

Zusammenfassung

Kunststoffe sind ein integraler Bestandteil unseres modernen und alltäglichen Lebens. Plastik ist leicht, beständig, kostengünstig und mehrfach wieder einsetzbar. Die vorteilhaften Materialeigenschaften sind allerdings ungünstig für die Umwelt. Unsachgemäß entsorgtes Plastik führt zur Verschmutzung von aquatischen Systemen und stellt eine globale Herausforderung dar. Zu den Verunreinigungen in der aquatischen Umwelt tragen sowohl physische als auch chemische Eigenschaften von Plastikmaterialien bei. Einerseits ist das bloße Material in der Umwelt ein nicht nützlicher Wertstoff, welcher durch Verwitterungsprozesse wie UV-Strahlung kontinuierlich fragmentiert, wobei Mikroplastik <5 mm entsteht. Andererseits werden bei der Degradation von Plastik Chemikalien freigesetzt, die dem Material hinzugefügt wurden. Diese Chemikalien können leicht aus dem Material in ein Umgebungsmedium migrieren. Daher untersucht diese Arbeit beide beschriebenen Aspekte und widmet sich der Fragestellung, ob ökotoxikologische Schadwirkungen eher auf die Partikeleigenschaften von Mikroplastik oder auf die freigesetzten Substanzen der Plastikpartikel zurückzuführen sind. Dabei wurde insbesondere die Freisetzung der Chemikalien unter Einfluss einer UV-Bewitterung untersucht.

Für die toxikologischen Untersuchungen wurden konventionelle sowie biologisch abbaubare Kunststoffe ausgewählt, welche aus fossilen bzw. biobasierten Rohstoffen gewonnen werden. Dabei wurden unterschiedliche Verarbeitungszustände der Plastikmaterialien herangezogen, wie zum Beispiel neue und recycelte Pellets. Diese dienen grundsätzlich als Ausgangsmaterial von Kunststoffen für die weitere Verarbeitung zu finalen Produkten. Außerdem wurden zwei Produkte gewählt. Zu den ausgewählten Proben gehören die folgenden Polymertypen: Polypropylen (PP), Polyethylenterephthalat (PET), Polystyrol (PS), Polyethylen (LDPE), Polyvinylchlorid (PVC), Polybutylensuccinat (PBS) und ein Stärkeblend (SB). Die Bewertung dieser unterschiedlichen Kunststoffspezies erfolgte im Hinblick auf ihre Umweltverträglichkeit, vor allem weil die Substitution von synthetischen durch vermeintlich nachhaltigere Polymere wie Rezyklate und „Bioplastik“ aus ökotoxikologischer Sicht unklar war. Diese Kunststoffe wurden vier Bewitterungsszenarien im Labor ausgesetzt und daraufhin in Wasser ausgelaugt. Bei der ersten Behandlung wurden die Proben unter dunklen Lichtverhältnissen ausgelaugt, um einfach migrierende Substanzen aus dem Plastik zu erfassen. Ob ein Umweltstressor wie UV-Strahlung zu einer verstärkten Freisetzung der chemischen Stoffe aus Plastik führt, wurde mithilfe von künstlichem UV-Licht nachgestellt. Die aus dem Plastik ausgelaugten Substanzen wurden aufkonzentriert und mit In-Vitro-Assays auf die Basistoxizität, den oxidativen Stress und antagonistische Aktivitäten an nukleären Hormonrezeptoren getestet. Schließlich wurde

die Anzahl und Konzentration der Chemikalien mithilfe eines Non-Target-Screening-Verfahrens analytisch untersucht.

Die Ergebnisse der ersten Studie (A1) zeigen, dass Plastik unabhängig von der Rohstoffquelle und dem Verarbeitungszustand ein komplexes Chemikaliengemisch ist. Plastik beinhaltet ein breites Spektrum an Chemikalien mit zahlreichen Funktionen. Neben den Additiven, welche beabsichtigt hinzugefügt werden, finden sich außerdem unbeabsichtigt hinzugefügte Substanzen sowie Degradationsprodukte. Es konnten bis zu mehrere tausend Chemikalien detektiert werden, die als Gemisch auslaugen und zu einer zum Teil sehr hohen (un)spezifischen Toxizität in den In-Vitro-Assays führen. Die meisten Positivbefunde gab es im AREc32-Assay zur Bestimmung des oxidativen Stresses (85 %), während 42 % der Proben im Mikrotox-Assay aktiv waren und 40 % anti-östrogene sowie 27 % anti-androgene Wirkungen induzierten. Das UV-Licht war allerdings nicht zwangsläufig notwendig, um die inhärenten chemischen Bestandteile aus dem Plastik zu lösen. Die UV-Strahlung war aber maßgebend dafür, dass eine im Vergleich weitaus höhere Anzahl und Konzentration an Plastikchemikalien auslaugen konnte und gemessen wurde. In den In-Vitro-Assays zeichnete sich durch diese Steigerung der Chemikalien auch eine erhöhte Toxizität ab. Sogar zunächst nach der Dunkelbehandlung als toxikologisch unbedenklich eingestufte Proben setzten nach einer UV-Bewitterung toxische Mixturen frei. Dies verdeutlicht die Relevanz der in der aquatischen Umwelt vorkommenden Prozesse. In dieser Studie wurden zwei besonders toxische Kunststoffe identifiziert: ein recycelter und biodegradierbarer Kunststoff. Nennenswert ist auch, dass es ebenfalls Proben mit geringer Toxizität gab. Das heißt, dass toxikologisch (un)bedenkliche Kunststoffe derzeit vermarktet werden. Nichtsdestotrotz ist es wichtig zu erwähnen, dass vor allem die in dieser Arbeit toxischsten Materialien als nachhaltigere und teilweise sogar sicherere Alternativen zu herkömmlichen Kunststoffen beworben werden. Unsere Experimente zeigen jedoch, dass solche Materialien toxikologisch nicht besser als neu hergestelltes, konventionelles Plastik sind. Ob die Effekte auf suborganismischer Ebene zusätzlich zu In-Vivo-Effekten führen, war eine Frage, die in weiterführenden Versuchen mit den zwei toxischsten Plastikproben und zwei Stellvertreterorganismen aus dem Süßwasser bearbeitet wurde.

In der zweiten Studie (A2) wurde eine biodegradierbare Shampooflasche zu Mikroplastik verarbeitet. Die Effekte des irregulär geformten Mikroplastiks wurden mit dem endobenthischen Oligochaeten *Lumbriculus variegatus* getestet. Zunächst wurde die Partikelaufnahme in den Verdauungstrakt der Würmer bestätigt, um eine enterale Exposition sicherzustellen. Bei den ersten chronischen Versuchen wurde das Mikroplastik (a) auf das Sediment aufgebracht und (b) dem Sediment beigemischt. Weiterhin wurden die Chemikalien aus dem Mikroplastik mit

(c) Wasser und (d) einem Lösemittel gelöst. Die chemische Phase wurde aufkonzentriert und an die Sedimentmatrix gebunden. Da die Würmer in dem Sediment leben und sich wahllos von der darin enthaltenen Organik und den Mineralstoffen ernähren, sind die Tiere den mit Plastikchemikalien besetzten Sedimentpartikeln dermal und enteral ausgesetzt. Zuletzt wurde das (e) mit dem Lösemittel behandelte Mikroplastik dem Sediment beigemischt, um zu klären, ob die von Chemikalien befreiten Partikel zu ähnlichen Effekten führen wie die unbehandelten Partikel. Übergreifend wurden in diesen Versuchen die Auswirkungen der physikalischen und chemischen Eigenschaften des biodegradierbaren Mikroplastiks getestet. Hierfür wurden häufig verwendete Endpunkte wie die Mortalität, Reproduktion und das Gewicht herangezogen. Um die Ursache der Toxizität von Mikroplastik zu klären und auf einen Faktor einzuengen, wurden die Partikel schließlich mit chemischen Analysemethoden untersucht.

Die von *L. variegatus* aufgenommenen Partikel beeinträchtigten die untersuchten Endpunkte. Der Expositionspfad mit den dem Sediment beigemischten Partikeln beeinflusste die Würmer mehr als die Exposition, bei der die Partikel lediglich auf das Sediment appliziert wurden. Im Vergleich zu den ersten Experimenten mit unbehandeltem Mikroplastik verursachte das mit Lösemittel behandelte Mikroplastik weniger negative Effekte. Neben den synthetischen Partikeln wurde eine natürliche Partikelkontrolle (Kaolin) gegenüber den Würmern exponiert. Diese Kontrolle sollte helfen, zwischen physikalisch- und chemisch-vermittelten Effekten des Mikroplastiks zu differenzieren. Da diese Partikelkontrolle jedoch zu einer Erhöhung des Gewichts der Würmer führte, erwies sich Kaolin als eine biologisch aktive Kontrolle ohne negative Auswirkungen. Dies unterstreicht die günstigen Eigenschaften dieser Tonpartikel und damit die ökologische Relevanz von feinkörnigem, mineralischem Material für die Würmer. Dementsprechend war die Testung der mit Lösemittel behandelten Partikel und den Chemikalien selbst notwendig, um aussagekräftige Ergebnisse zu erzielen. Die mit dem Lösemittel freigesetzten Chemikalien verursachten eine 100%ige Mortalität und damit die schädlichsten Wirkungen. Interessanterweise konnten im Material der Shampooflasche zwei ökotoxikologisch relevante Biozide nachgewiesen werden. Mithilfe der parallel durchgeführten Analytik kristallisierte sich heraus, dass die eingebetteten und an dem Produkt haftenden Chemikalien die verantwortlichen Treiber für die beobachtete Toxizität waren. Insgesamt unterstreichen diese Aspekte die Problematik von solchen als umweltverträglich beworbenen Materialien.

In der Studie A3 wurde die epibenthische Süßwassergarnele *Neocaridina palmata* gegenüber sphärischen Partikeln (Beads) und irregulär geformtem Mikroplastik (Fragmente) kurzfristig exponiert. Hier wurde untersucht, ob die Partikelform die Aufnahme sowie Ausscheidung der

Garnelen beeinflusst. Dabei wurde die Anreicherung der Partikel im Verdauungstrakt der Garnelen untersucht. Die Resultate zeigen, dass die Partikelgröße ein entscheidender Faktor für die Aufnahme der Partikel war, jedoch spielte die Partikelform keine signifikante Rolle bei der Darmpassage von *N. palmata*. Sowohl die Beads als auch die Fragmente wurden mit aufsteigenden Konzentrationen im Expositionsmedium von den Garnelen aufgenommen. Nach einer Exkretionsphase wurden 59 % der Beads und 18 % der Fragmente wiedergefunden. Trotz dieser abweichenden Egestionsraten konnte kein signifikanter Unterschied zwischen den mit unterschiedlichen Partikelformen bestückten Behandlungen festgestellt werden. Ähnliche Beobachtungen wurden für die Partikelausscheidungen gemacht. In einem weiteren Versuch wurden Fragmente und Futterpartikel gleichzeitig angeboten. In Gegenwart der Futterpartikel wurde weniger Mikroplastik von den Tieren aufgenommen, jedoch gab es keine signifikanten Unterschiede zur Behandlung ohne Futter. Analog zu den Würmern (A2) ist bei den Garnelen bekannt, dass sie natürlich vorkommende Partikel in der Umwelt in hoher Zahl aufnehmen und an diese angepasst sind. Daher wären beide Arten den in der Umwelt vorkommenden synthetischen Partikeln ebenso ausgesetzt. Aufgrund des eher unselektiven Fressverhaltens der Garnelen in Bezug auf die Partikelform und der Toleranz gegenüber natürlichen Partikeln in der Umwelt könnte dies darauf hindeuten, dass das Mikroplastik zumindest als physikalischer Schadstoff für die Süßwassergarnelen eher unbedenklich ist. Eine finale Schlussfolgerung kann jedoch nur mittels chronischer und realitätsnaher Untersuchungen erfolgen.

Auf den vorherigen Ergebnissen aufbauend wurden weitere Versuche mit *N. palmata* in einer vierten und letzten Studie (A4) durchgeführt. Hier wurden die chemischen Eigenschaften der zwei toxischsten In-Vitro-Materialien aus der ersten Studie untersucht: LDPE-Rezyklat und Stärkeblend. Das Stärkeblend wurde allerdings nicht als Pellets, sondern eine ähnlich toxische Folie als Endprodukt des Ausgangsmaterials verwendet. Da in diesen Versuchen die Chemikalien aus dem Mikroplastik der recycelten Pellets und biodegradierbaren Folie von Interesse waren, wurde aus den Materialien Mikroplastik hergestellt. Ähnlich zum Vorgehen in der ersten Studie wurden die Partikel bewittert und in Wasser eingelegt. Dieses Auslaugmedium wurde aufkonzentriert und in subchronischen In-Vivo-Tests mit *N. palmata* überprüft. Dabei wurde das Schwimmverhalten als ein ökologisch relevanter, aber weniger häufig untersuchter Endpunkt verwendet. Die Schwimmaktivitäten der Garnelen wurden nicht signifikant von den Chemikalien beeinflusst. Ein genereller Trend zu erhöhten oder reduzierten Bewegungen konnte, außer am Tag 14 für das unbewitterte Mikroplastik der Folie, nicht festgestellt werden. Hier wiesen die Bewegungen der Garnelen mit zunehmender Konzentration auf Hyper-

aktivität hin. Analog zu dem In-Vitro-Test auf Basistoxizität in der ersten Studie wurden die Chemikalien aus dem Mikroplastik untersucht. Die unspezifische Toxizität wurde auch hier deutlich induziert. Die beobachteten In-Vitro-Aktivitäten der Plastikchemikalien haben sich nicht in signifikanten Effekten auf der In-Vivo-Ebene widergespiegelt. Vermutlich hatte das Versuchsdesign nicht die notwendige Empfindlichkeit subtile, aber biologisch relevante Effekte zu messen. Weiterhin kann mit den In-Vivo-Versuchen weder bestätigt noch ausgeschlossen werden, dass die beobachtete Variation des Schwimmverhaltens der Garnelen auf die komplexen Chemikalienmischungen aus dem Plastik zurückgeht. Eine chemische Analyse zeigte jedoch, dass die Chemikalien diverse Funktionen haben, welche wiederum verschiedene Mechanismen in den Tieren modulieren und somit zu uneindeutigen Verhaltensmustern führen könnten.

Zusammenfassend konnte in dieser Arbeit gezeigt werden, dass die Toxizitäten der Polymere aufgrund der vielfältigen chemischen Zusammensetzungen jedes einzelnen Kunststoffs nicht pauschalisiert werden können. Die In-Vitro-Assays sind trotzdem hilfreich, um toxische Plastikmaterialien als solche zu identifizieren. Weiterhin wurde gezeigt, dass bereits Pellets als Ausgangsmaterial für Verbraucherprodukte toxische Chemikalien beinhalten. Dabei stellen vermeintlich nachhaltigere und/oder sicherere Alternativen wie Rezyklate und „Bioplastik“ keine toxikologisch bessere Option zu herkömmlichem Plastik dar. Anhand von UV-Bewitterungsversuchen wurde gezeigt, dass Plastikchemikalien vermehrt freigesetzt werden und die In-Vitro-Wirkungen verstärken. Demnach sollte die Verantwortlichkeit der Produzenten auf den gesamten Lebenszyklus ihrer Plastikprodukte erweitert werden. Grundsätzlich muss die Zahl der eingesetzten Chemikalien in der Herstellung und Verarbeitung von Plastik drastisch reduziert werden. Die eingesetzten Chemikalien sollten als Einzelsubstanzen sowie als Mischungen toxikologisch unbedenklich sein. Ein wichtiger Anhaltspunkt für die Entwicklung solcher Kunststoffe sind die in dieser Arbeit als toxikologisch unbedenklich eingestuften Proben. Nennenswert ist ebenso, dass den in dieser Arbeit untersuchten Partikeleigenschaften von Mikroplastik mit den benthischen Organismen eher moderate Effekte zugeschrieben wurden. Zunehmend eingetragene Verschmutzungen in die aquatische Umwelt könnten jedoch zu noch nicht absehbaren Folgen führen. Unter der Annahme, dass die Produktion von Plastik in der Zukunft steigt, könnten sich Belastungsspitzen in der Umwelt bilden. Somit ist es von entscheidender Bedeutung, dass verschiedene Akteure entlang der gesamten Wertschöpfungskette von Plastik kooperieren und eine nachhaltige sowie sichere Nutzung von Plastik schaffen.

1 Introduction

Plastics are a substantial part of modern life. We are surrounded by these diverse but unique materials on a daily basis. They are durable, lightweight, inexpensive and multiply deployable. A brief review demonstrates that traditional materials from natural resources such as metals, ceramics, wood, glass, horn, ivory, shells or amber were replaced with the synthetic materials we are familiar with today. As opposed to celluloid, which is a semi-synthetic or semi-natural material derived from cellulose, Bakelite was the first fully synthetic material discovered by the Belgian chemist Leo Baekeland in 1907. From that point onwards, production focused on the development of new synthetic polymers rather than on processing natural ones (Callapez, 2021; Freinkel, 2011). The large-scale production of diverse plastic products in the 1940s and 50s marks the beginning of our so-called "plastic age" (Thompson et al., 2009). At first, plastic was viewed as an affordable imitation, but fundamentally advanced several fields such as food packaging, transportation and medicine (Madden, 2017). However, the beneficial features of plastic materials are unfavorable in the environment, where a high amount of plastic waste is accumulating and adversely affecting aquatic organisms (Barnes et al., 2009).

1.1 Polymers of natural and artificial origin

Polymers are macromolecules consisting of repetitive monomer units. In contrast to natural polymers (e.g., cellulose, silk, wool or DNA and proteins), synthetic polymers are derived from fossil resources. The admixture of property-modifying chemicals to these polymers results in the formation of plastics, a group of versatile materials with unique properties. Because of its low manufacturing costs, lightweight and longevity, plastics have found infinite applications (Andrady, 2011; Jenkins et al., 1996; Thompson et al., 2009). These materials not only help to keep our food fresh and hygienic, they allow for insulation, fire-retardant coatings in buildings and constructions and enable the transportation of goods and humans. Without a doubt, the man-made material offers considerable technological opportunities and ensures high-quality standards set in our modern society (PlasticsEurope, 2020). Since the mid of the 20th century around 8,300 million metric tons of plastics have been estimated to be manufactured globally, of which 47% were generated in recent years (Geyer et al., 2017). In the European Union (EU), the majority of the plastics raw material is processed by the packaging (39.6%), building and construction (20.4%), automotive (9.6%) and electric (6.2%) industries. The segments need different polymer types, which are listed in descending order of demand: polyethylene (PE), polypropylene (PP), polyvinylchloride (PVC), polyethylene terephthalate (PET), polyurethane (PUR) and polystyrene (PS). The base polymers PE, PP and PET are usually used as packaging

material, whereas a rigid polymer like PVC is useful for construction (PlasticsEurope, 2020). Most of these high-commodity polymers belong to the group of thermoplastics, meaning they are malleable at high temperatures. In contrast, thermosetting plastics cannot be reshaped by heat once they cure, e.g., as for Bakelite or PUR (Callapez, 2021; Madden, 2017).

Unfortunately, the favorable properties of plastics are disadvantageous in the environment. Processed to serve a lifetime, the materials degrade slowly at most under real environmental conditions (Jahnke et al., 2017; Moore, 2008). Littering caused by consumers, producers, the waste disposed of in landfills (Eriksen et al., 2018) and the continuous fragmentation process taking place in the environment result in ubiquitously distributed macro-, micro- and nano-scaled plastic pieces (Gregory, 2009; Horton et al., 2017; Mitrano et al., 2021). Globally, plastic pollution is a major problem and a consequence of mismanaged plastic waste (Borrelle et al., 2020). The majority of plastic waste ever created ended up in landfills or the nature (79%), whereas 12% have been incinerated and only 9% recycled (Geyer et al., 2017). Plastic pieces are accumulating in every imaginable habitat (Bergmann et al., 2017; Brahney et al., 2020; Rochman and Hoellein, 2020) with adverse impacts on wildlife (Derraik, 2002). As a result, plastic pollution is a tangible issue of great concern, one that can only be solved by a collective of different actors stated Wagner (2022).

To address the environmental incompatibility of conventional plastics, bioplastics have been promoted as a promising material that comprise both natural and sustainable features and are considered as alternatives for common plastics. Biological materials should degrade fast and without any (eco)toxicological effects. The plastics currently available on the market can be categorized into four groups: (1) fossil-based and non-biodegradable (e.g., PE, PP and PVC), (2) fossil-based and biodegradable (e.g., polybutylene adipate terephthalate (PBAT)), (3) non-biodegradable but bio-based (e.g., bio-based PE, PET or PP) and (4) bio-based as well as biodegradable (e.g., polylactic acid (PLA), polybutylene succinate (PBS) and starch blends (SBs)). Hence, biodegradable plastics are not *per se* bio-based and *vice versa*. Moreover, the term “bioplastics” is misleading to consumers because it suggests complete degradability and a natural origin (European Bioplastics, 2019; Lambert and Wagner, 2017). Current market shares of such materials are low with 2.11 million tons produced in 2019, which corresponds to less than 1% of the annual plastic production rate. Prospective growth rates are expected to increase in the future (European Bioplastics, 2019). Similar to synthetic plastic materials, bioplastics are chemically modified polymers and comprise different materials (Lambert and Wagner, 2017) that can mimic the characteristics of and replace almost every conventional plastic on the market. The advertised environmental compatibility should be however viewed

critically. Bio-based packaging is in fact not a better or more sustainable alternative compared to fossil-based packaging. For instance, as for greenhouse gas emissions bioplastic performs better but it contributes to acidification and aquatic and terrestrial eutrophication (Detzel et al., 2012). In addition, the crops cultivated to produce bio-based materials are associated with high land and pesticide use (Wagner, 2022). Moreover, the degradation tests conducted for such materials do not really comply with real-world conditions because they are conducted in industrial settings. In the environment, every material is exposed to a set of continuously changing parameters (e.g., microorganisms, temperature and humidity), which disintegrates the material over time and contributes to the formation of microplastics (Haider et al., 2019).

1.2 Chemicals processed into plastic materials

Substances that are intentionally added to plastic materials are called additives and include e.g., plasticizers, fillers, flame retardants, UV and heat stabilizers and antioxidants. Typically, they are incorporated to modify the physical and chemical properties of a polymer in order to obtain useful characteristics. Stabilizers, for instance, enhance the strength and plasticizers increase the plasticity of a material. The amounts of additives used in plastics can range from 0.001−3% w/w to high percentages by mass of 10−70% as for plasticizers (Andrady and Neal, 2009; Bridson et al., 2021; Hahladakis et al., 2018). Geyer et al. (2017) stated that plasticizers, fillers and flame retardants have the highest processing rates. Likewise, other compounds can be processed into plastics such as the non-intentionally added substances (NIAS) that include impurities of the starting molecules, transformation products and breakdown side-products generated during polymerization. Impurities, isomers and intermediates are usually known by the manufacturer, unless they remain technically undetected (Bradley and Coulier, 2007). These chemicals are confidential business information (CBI) and unknown to the public (Groh et al., 2019; Wang et al., 2020a). With the variety of different actors in the supply chain, it is almost impossible to retrieve information on plastic ingredients (van Dijk et al., 2021).

Whether plastics are entirely derived by fossil fuels or not is irrelevant when it comes to the chemicals used for plastics in such a manner that both synthetic plastics and bioplastics are compounded with property-modifying chemicals (Lambert and Wagner, 2017). Wang et al. (2020a) reported over 350,000 chemicals to be registered for production and use worldwide. The number of reported chemicals associated with plastics depends on the study's scope. For instance, Wiesinger et al. (2021) researched plastic monomers, additives and processing aids and summarized 10,500 registered chemicals, of which 2,400 chemicals were of toxicological concern. Groh et al. (2019) focused on plastic packaging and identified 4,200 substances. They

classified 148 substances as persistent, bioaccumulative and toxic (PBT) or having endocrine-disrupting potential. Initiated by ECHA (the European Chemicals Agency), over 400 functional additives or pigments with more than 100 registered tons per year were made public (ECHA, 2021a). The majority of the input substances can transfer from the plastics into a surrounding medium such as water (Lithner et al., 2009, 2012; Teuten et al., 2009) or foodstuff (Muncke et al., 2020). This process is called migration or leaching as opposed to the chemical release, which occurs during degradation. The former includes additives, unpolymerized monomers, catalysts and NIAS, while the latter refers to these substances and additionally to degradation products (Muncke, 2011). Chemicals from plastics are now present in freshwaters (Schmidt et al., 2019, 2020), in oceans (Hermabessiere et al., 2017; Kirchgeorg et al., 2018) and have even been detected bound to airborne particulate matter (Liu et al., 2021).

Bisphenol A (BPA) is probably one of the most prominent substances as it does not follow a typical monotonic dose-response relationship. It has been previously processed for different plastic-related uses, e.g., as a plasticizer in PVC, as monomer for the polycarbonate and epoxy resin production as well as to coat thermal paper (UBA, 2010). Under REACH regulations, that is the EU law for the Registration, Evaluation, Authorization and Restriction of Chemicals, BPA has been restricted for use in consumer products because of its potential to adversely affect the hormone system (referred to as endocrine disruption) (Oehlmann et al., 2008, 2009). The response to an exposure to low levels of endocrine-disrupting chemicals can have different extents that may result in developmental or transgenerational (i.e., reproductive) effects over time (Freinkel, 2011). Infant feeding bottles, plastic bottles and food packaging materials for children under three years are not allowed to contain BPA. At the same time, other materials in contact with food are allowed to contain this substance, if a specified migration level is not exceeded (ECHA, 2021b). European countries can work beyond this regulation as exemplified by ANSES, the French Agency for Food, Environmental and Occupational Health and Safety (EFSA, 2015). The French authorities conducted a separate, more relevant risk assessment in terms of everyday life uses and this resulted in a ban of BPA for all food packaging materials (Whaley et al., 2016).

Only additives that are manufactured and imported with more than one registered ton per year are subject to regulation, i.e., that manufactured/imported chemicals below one ton per year are not assessed in terms of their impact on environmental and human health (Whaley et al., 2016). Polymers are also exempted from registration because of their high molecular weight and therewith the low bioavailability (Mitrano and Wohlleben, 2020). The individual monomers are evaluated though (van Dijk et al., 2021). Moreover, the mixtures of (un)known

substances emerging from plastics complicate classic risk assessments (Fauser et al., 2022). The arising mixture chemicals from plastics aggravate the chemical pollution already present in the environment (Arp et al., 2021). By now, it is well-known that chemicals associated with plastics can become a potential health threat (Capolupo et al., 2020; Muncke et al., 2020). Other chemical-related issues can occur with plastic particles in the environment such as the adsorption of hydrophobic organic pollutants to plastic particles and the desorption of such in the digestive tract of animals (Hartmann et al., 2017; Koelmans et al., 2016). This theory of microplastics acting as a vector for pollutants has been widely discussed, but the hazards of such chemicals attached to plastics were concluded to be limited in environmentally relevant settings (Besseling et al., 2019).

At the start of this thesis, chemical effects from plastics were examined predominantly using marine (Bejgarn et al., 2015; Gandara e Silva et al., 2016; Li et al., 2016a; Nobre et al., 2015) instead of freshwater biota (Lithner et al., 2009; Wagner and Oehlmann, 2009). Only a few of these studies focused on leachates from weathered plastics as e.g., Bejgarn et al. (2015) and Gandara e Silva et al. (2016) and hardly any were known with regard to *in vitro* toxicity. One example is the study by Coffin et al. (2018) that used extracts from UV-irradiated plastics but with leaching conditions adjusted to marine conditions. For this thesis, previous research on plastic food packaging material served as a basis for comparison. Within this specific research field, there is a necessity to generate data and to make this information publicly available as it concerns human health (Muncke et al., 2020). Impacts of elevated temperatures, the solvent type or medium and radiation on the migration and release behavior of chemicals from food packaging plastics have been investigated for a long period (Muncke et al., 2020; Yang et al., 2011). For this purpose, *in vitro* bioassays have been used to investigate multiple toxicological endpoints as described by Groh and Muncke (2017) and Severin et al. (2017). With regard to emerging chemicals from plastics, environmental health has been explored less. Therefore, the plastic leachates and their effects on the (sub)organism level were of specific interest in this work. UV irradiation was used as an initiator for degradation. This way the hazards of many added chemicals, comprising the plastics' chemical mixture, could be assessed (Arp et al., 2021). One of the assumptions in this thesis was that the toxicity of weathered plastics changes in a way that leads to a toxicologically more concerning mixture of leaching chemicals in comparison to the leachates from unweathered plastics. In this work, the entirety of the (non-)intentionally added chemicals and newly emerging degradation products (degradants) will be referred to as plastic chemicals.

1.3 Widespread distribution of microplastics

In the early 1970s, weathered pellet-shaped plastics were discovered floating in the Sargasso Sea (Carpenter and Smith, 1972). Shortly after, Rothstein (1973) detected irregularly shaped plastics in seabird stomachs, one of many findings on plastic debris in seabirds as summarized by Day et al. (1985). Since then, the indigestible plastic items are of increasing concern, having aroused attention based on the entanglement and starvation problems for numerous animals (Derraik, 2002; Gregory, 2009). Microscopically small plastic particles, namely microplastics, have been previously described as particles smaller than 5 mm (Arthur et al., 2009). A more precise size range, i.e., 1–1000 μm, was recently suggested for classification (Hartmann et al., 2019). Once plastics enter the environment, they are going to be broken down gradually into so-called secondary microplastics (fragments). Abiotic and biotic factors play a decisive role through mechanical abrasion, UV irradiation, hydrolysis and microorganisms (Jahnke et al., 2017). Irregularly shaped microplastics are distributed ubiquitously, including the aquatic and terrestrial ecosystems (Horton et al., 2017) as well as the atmosphere, where the particles are transported by aerosols (Allen et al., 2021) such as dust (Rochman and Hoellein, 2020). Particles can be deposited in distal and remote areas (Brahney et al., 2020; Evangeliou et al., 2020), are even detected in ice (Obbard et al., 2014) and deep-sea sediments of the Arctic sea (Bergmann et al., 2017). While secondary microplastics are the result of the fragmentation in nature, primary microplastics (beads) are used and produced for products such as cosmetics, plant protection products, fertilizers and coatings (VCI e.V., 2019). Beads have been detected in wastewater treatment effluents, sewage overflows and industrial outlets (Kalčíková et al., 2017; Mani et al., 2019a) following the application of such particles e.g., as exfoliants in rinse-off cosmetics (Auta et al., 2017). A source for irregularly shaped micro- and nanoplastics are wastewater treatment plants, where even the newest systems release a tremendous amount of particles on a daily basis (Kögel et al., 2020). Microplastics can further arise from use as is the case with fibers from textiles (Sait et al., 2021) and car tire particles (Tamis et al., 2021). Next to natural rubber, tire wear particles include synthetic rubber (Redondo-Hasselerharm et al., 2018a) and are thus defined as plastic debris (Hartmann et al., 2019). Taken together, microplastics are a heterogenous group of particles. They encompass a variety of different traits (e.g., polymer type, size, shape, density, surface and chemicals). This heterogeneity of properties has been detected in the environment. In laboratory studies, the outcome of the experiments is influenced by these diverse plastic features (Lambert et al., 2017; Rochman et al., 2019).

Most polymers have a lower density than water (1 g cm^{-3}), being the main reason why plastics are first found on surface water. Microorganisms colonize the surface of plastics and thereby form biofilms, eventually causing the plastics to sink due to the increment in density (Rummel et al., 2017). Estimates describe that between 31.2 and 51.2 trillion plastic pieces float on the ocean surface, corresponding to a maximum of 236,000 metric tons (van Sebille et al., 2015). The dispersion of the particles leads to varying concentrations along the vertical levels of the aquatic system, i.e., from the surface water to the water column and the sediment. Rivers are transport routes of plastic debris to the oceans (Best, 2019) and contain from 0.17 to 3.45 x 10^5 particles m^{-3} and from 5.15 x 10^2 to 6.49 x 10^7 particles m^{-3} in the water and the sediment phase, respectively (Scherer et al., 2020). These study results highlight sediments as one sink for microplastic pollutants. They further observed that particles within the water phase were composed of PE, PP and PS, whereas sediments contained PE, PS, PP, PVC and many unknown polymer types. In comparison, synthetic fibers were detected to a higher extent in the water, whereas spheres and fragments were more dominant in sediments. Burns and Boxall (2018) reported fibers to be most frequently found in the water column, which was closely followed by fragments. Plastic particles >300 μm have been often sampled in monitoring studies, which is linked to the frequent use of such mesh-sized trawls. However, the plastic particles formed directly in and entering the environment are smaller than this size class. Conkle et al. (2018) demonstrated that this leads to an underestimation of the actual microplastic concentration present in the aquatic environment. Small-sized plastic particles are usually investigated in laboratory toxicity studies, but this cannot be really compared to the exposure data generated thus far (Lusher et al., 2021). Over the years, sampling methods and quantification techniques have improved (Rozman and Kalčikova, 2022). Present studies have detected microplastics >20 μm (Kameda et al., 2021) or even down to 11 μm (Mani et al., 2019b). Kögel et al. (2020) reported that "particle amounts of the size fraction below 10 μm are completely unquantified in all environmental niches, including biota and humans". The analyses are hampered by time-consuming analytical measures, not to mention the lack of standardized methods that prevent an overview of the full scale of plastic pollution as described by Lusher et al. (2021).

At the beginning of this work, few reports were available on the microplastic abundance along European rivers (Wagner et al., 2014). All of them included different mesh sizes for sampling (Klein et al., 2015; Lechner et al., 2014; Mani et al., 2016). Therefore, the actual concentration at the lower microscale was not evident but assumed to be high. Many studies employed high microplastic concentrations, which was often criticized in the microplastic community since it did not reflect the environmentally measured concentrations (Lenz et al., 2016; Phuong et

al., 2016). With that being said, low, medium and high concentrations of a substance are regularly tested in the field of ecotoxicology in order to derive a full dose-response as proof-of-concept (Harris et al., 2014; Huvet et al., 2016). Also, the widespread use of manta trawls displayed a specific size range of microplastics that was not even ingestible by small organisms such as invertebrates. Today, concentrations of the lower microscale are not just assumed but have been proven to reach high concentrations (Mani et al., 2019b; Scherer et al., 2020). Exposure levels including high numbers of microplastics are now seen as beneficial in order to better comprehend potential adverse trends in the future (Lusher et al., 2021).

1.4 From exposure and hazard to risk?

The phenomenon of entanglement and starvation issues due to macroplastic debris has been reported in the 1970s for marine animals. The stomachs of seabirds were observed to be filled with non-nutritious synthetic debris. This can lead to depleted energy and eventually to death (Gregory, 2009). As a consequence, the ingestion itself might be seen as a prerequisite for the induction of adverse effects. While the mere ingestion of bulk debris can already negatively affect animals, other organisms may suffer a similar fate but on a different scale. Thus far, a vast number of ecotoxicological studies has been conducted with microplastics that examined various different organisms and endpoints (Anbumani and Kakkar, 2018; Haegerbaeumer et al., 2019; Thomas et al., 2021; Triebskorn et al., 2019). Suborganism and individual levels such as the oxidative stress, growth, mortality, feeding rate and reproduction as endpoints have been studied to a higher degree than effects on population and community levels (de Ruijter et al., 2020; Lusher et al., 2021). This is equally outlined by a higher percentage of studies focusing on acute rather than on long-term effects (Rozman and Kalčikova, 2022). Effects of ingested microplastics include malnutrition, internal blockages and physical damage in the digestive tract (de Ruijter et al., 2020). Particles may be further taken up by cells via the translocation through an epithelial barrier (Triebskorn et al., 2019) and accumulate in compartments (e.g., organs) (Kögel et al., 2020). The outcomes of microplastic exposure can range from adverse to negligible effects as for freshwater invertebrates, depending on the selected test organism, test material and exposure conditions (Kögel et al., 2020). In the microplastic research field, freshwater and terrestrial species are still underrepresented. In addition, laboratory studies often used PS beads as test material (Burns and Boxall, 2018; Rozman and Kalčikova, 2022), which are provided in solutions including preservatives or surfactants that can overestimate the toxicity of plastic particles (Pikuda et al., 2019). Microbeads represent a small fraction of the particles found in the aquatic environment (Scherer et al., 2020). Under real conditions, animals would encounter different microplastic

compilations (Xu et al., 2020), which depends on the habitat (Haegerbaeumer et al., 2019). The different matrices (e.g., surface water, water column and sediment) in an aquatic ecosystem contain varying numbers of polymers, particle shapes and sizes (Kooi et al., 2021; Mani et al., 2019b). Furthermore, weathered microplastics can lead to a different toxicological outcome as a result of degradation (Wang et al., 2020b). To approach natural conditions, meso- and microcosm studies can be conducted as e.g., by Al-Jaibachi et al. (2020). This study investigated the exposure to 15 μm PS particles and altering stressors and found decreased numbers of daphnids in the first seven weeks, underlying the importance of an environmentally relevant exposure setting.

Next to the physical properties of microplastics (shape, size, density), the intrinsic properties of polymers (chemical composition) can contribute to the ecotoxicity (Lambert et al., 2017). In order to delineate the source of toxicity, an approach to differentiate between physical- and chemical-related effects of microplastics is needed (Ogonowski et al., 2018). Alongside to the microplastic of interest, reference controls such as kaolin, silica, diatomite, ground shells or glass have been used (Gerdes et al., 2019; Ogonowski et al., 2016; Scherer et al., 2019; Schrank et al., 2019; Schwarzer et al., 2022). Reference particles should ideally exhibit equal physical properties as the microplastics. Studies acknowledging this still do not meet all of the needed criteria, e.g., when the size and shape but not the density of the particle control can compare to the microplastic (Schwarzer et al., 2022). While the implementation of such references is necessary (Connors et al., 2017), it generally remains difficult to assess (Phuong et al., 2016). The reference controls often merely hint to plastic chemicals as the key driver of microplastic toxicity. In this thesis, one of the hypotheses was that chemicals associated with microplastics drive the toxicity.

The approaches to characterize the chemical toxicity of microplastic are not standardized yet. Despite this, extraction and leaching procedures are regularly applied and highlight what is embedded in the plastic (extract), what can transfer out of the plastic (migrate and leachate) (Bridson et al., 2021) and whether these chemicals affect aquatic biota (Capolupo et al., 2020; Gardon et al., 2020; Lithner et al., 2009). In the industry, internationally harmonized methods are used to evaluate the extractable and leachable chemicals in contact with foodstuff, medical devices and products and construction materials (Bridson et al., 2021). Plastic particles could be evaluated in products as well before placing them on the market, especially because of the environmental issues and potential issues related to the human health (Vethaak and Legler, 2021). Effects on the environment are not fully resolved yet, whereas research on the effects

of plastic particles on human health has even received less attention than ecological endpoints (Coffin et al., 2021).

A risk assessment evaluates the risk acceptability of a chemical for human and environmental health. For the safety of freshwater organisms, the ratio between the predicted environmental concentration (PEC) and the predicted no effect concentration (PNEC) is calculated. A ratio <1 indicates no unacceptable risk and a ratio ≥1 indicates a potential risk (Oehlmann et al., 2008). Regulatory measures can entail limitations or lead to bans of a substance placed on the market. Such measures have been proposed for microplastics in the EU but address primary microplastics alone (Mitrano and Wohlleben, 2020). However, available studies lack specific quality criteria, which hampers a proper risk assessment for microplastics (de Ruijter et al., 2020). Given the variety of experimental methods in use, the studies lack comparability as stated by Gomes et al. (2022). This leaves scientists with the issue that a risk is difficult to calculate. Nevertheless, several risk assessments were conducted for marine and freshwater ecosystems (Adam et al., 2019; Burns and Boxall, 2018; Everaert et al., 2018, 2020). The findings ranged from no to low risks as well as risks at hotspots. The report by VKM (2019) presented a hazard concentration, which adversely affects 5% of the included species (HC_5), of 71.6 particles L^{-1} for marine and freshwater species exposed to nano- and microplastics. In addition, Gomes et al. (2022) determined a HC_5 of 41.6 particles L^{-1}. Given the microplastic heterogeneity in the used data, the results reflect a somewhat biased picture (Koelmans et al., 2020). At the same time, they deliver meaningful projections for orientation.

In sum, this section described shortcomings in the microplastic field related to the physically- and chemically-mediated issues. The data is currently insufficient for suborganisms, chronic experiments, the drivers of microplastic toxicity, particle reference controls and weathering effects. In addition, the microplastic testing should focus on the influences of various plastic particles and the different life stages of organisms (Lusher et al., 2021). Endpoints relevant for the ecosystem that were less often assessed such as behavioral parameters should receive greater attention (Gomes et al., 2022). After all, adverse behaviors are early-warning signals (Bae and Park, 2014; Faimali et al., 2017) and impacts could affect predator-prey interactions or foraging behaviors and therewith the ecosystem level (Backhaus and Wagner, 2020). The knowledge on microplastic effects we have today is still fragmented. Therefore, Lusher et al. (2021) described: “Despite over a decade of attention and a substantial volume of work, our understanding of the impacts and risk associated with plastic pollution remains in its infancy”.

1.5 Recycling strategies for plastic waste

Solid-waste management refers to the three R's principles: reduce, reuse and recycle. In the waste hierarchy, the prevention, reuse, recycling and recovery are preferred over the disposal (European Bioplastics, 2020). The goal of this order is to reduce impacts on the environment as stated by van Ewijk and Stegemann (2016). Moreover, circular economy promises to solve the plastic waste problem once and for all (Corvellec et al., 2021). Accordingly, the end-of-life-management is more so today's focus than prevention measures (Wagner, 2022; Johansen et al., 2022). The recovery of a high-quality material out of heterogenous plastic waste displays a major challenge, especially in developing countries where logistical problems present first barriers (Hopewell et al., 2009; Madden, 2017). Even when plastic is properly discarded and collected, it is not necessarily remade into a new plastic product and introduced back to the market, which is a common misconception amongst consumers stated Unmüßig (2021). In fact, three major recycling methods are practiced: mechanical recycling (polymers are melted and formed into new products of the same product system as for PET bottles (i.e., a closed-loop system) or into a product with a lower value), chemical or feedstock recycling (polymers are depolymerized to monomers, synthesis gas or oil) and energetic recycling (polymers are used as fuel or to recover energy, which relates to incineration processes) (Schyns and Shaver, 2021; UBA, 2018; van Ewijk and Stegemann, 2016).

Exemplarily on the basis of European countries in the year 2018, 42.6% of the collected post-consumer plastic waste (29.1 million tons) was used for energy recovery, 32.5% were sent to recycling companies and 24.9% were disposed of in landfills. Leading countries in recycling (Switzerland, Austria, the Netherlands and Germany) mainly recover energy (approximately 60%) out of the post-consumer plastic waste and recycle up to 40% (PlasticsEurope, 2020). Van Ewijk and Stegemann (2016) elaborated that "the [waste] hierarchy does not distinguish between different forms of recycling"; these include open-loop recycling practices that have actually low environmental benefit. Environmentally most beneficial are closed-loop systems, especially when a product is remade with minimal, if any, new virgin material input. Down-cycling of a material to produce secondary products of lower quality is another option (Geyer et al., 2016; Schyns and Shaver, 2021). Repeated recycling processes shorten the lifetime of polymers, for instance, due to cross-contaminations (Pivnenko et al., 2015; Shen et al., 2020). Besides, toxicants can accumulate during reprocessing (Crippa et al., 2019), e.g., endocrine-disrupting chemicals (EDCs) like BPA (Dreolin et al., 2019) and DEHP (diethylhexyl phthalate) (Pivnenko et al., 2016). Recycled materials used in contact with foodstuff must still conform with official safety regulations (Freinkel, 2011). In case plastic recyclates contain undesirable

chemicals, the materials may represent so-called regrettable substitutions (Blum et al., 2020; Muncke, 2021). Bioplastics (European Bioplastics, 2020) and recyclates (Geyer et al., 2016) that are marketed as safe and/or sustainable alternatives for conventional and virgin plastics have been studied less in terms of their (eco)toxicity. Because of the chemical compounding (Lambert and Wagner, 2017) and the accumulation of concerning chemicals (Pivnenko et al., 2016, 2017) in such material groups, they are probably not less harmful than plastics made from conventional, new and virgin sources. This will be addressed in this thesis.

1.6 The PlaStrat project

This PhD thesis is part of the research project 'PlaStrat – Solution strategies for the reduction of urban plastic entries into limnic systems', which aimed to develop evaluation criteria for the categorization of environmentally friendly plastics and to establish measures in order to minimize the hazards of plastic residues in limnic systems. Within the field of ecotoxicology, alternatives for conventionally used plastics such as bio-based and/or biodegradable as well as recycled materials should be examined in view of their potential substitutional uses. As a first step, different *in vitro* bioassays were performed in order to evaluate the activation of several suborganism responses by the substances transferred from the synthetic, natural and recycled plastics. The project work further focused on test methods that detect effects on the *in vivo* level, which are triggered by physical and/or chemical properties of microplastics but with a focus on endo- and epibenthic freshwater species. Additionally, ingested microplastics were tracked in the digestive system of a test organism and sublethal endpoints (locomotor behavior) were evaluated in a newly developed test method. The project consortium derived chemical, (eco)toxicological and socio-ecological results during this PhD thesis. The collective work should clarify whether alternatives for plastics are chemically and (eco)toxicologically better options than conventional plastics we use on a daily basis.

1.7 Aims and objectives of the thesis

Given the described limitations in the microplastic research field at the start of this thesis, this work aims to provide a comprehensive overview of the factors that influence the microplastic toxicity on the suborganism and individual levels by evaluating the impact of weathering on the leachate toxicity from plastics (A1 and A4), delineating the source of microplastic toxicity (A2), determining the relevance of particle number and shape in a digestive system (A3) and evaluating the behavior as an ecologically relevant endpoint at the ecosystem level during the exposure to microplastic chemicals (A4). These objectives were driven by the lack of or the limited knowledge on the:

- *in vitro* leachate toxicity of conventional plastics, bioplastics and recyclates
- impacts on plastic leachate toxicity after different weathering scenarios
- leachate toxicity of plastic pellets of different origins
- effects of irregularly shaped microplastics on benthic freshwater organisms
- drivers of microplastic toxicity (physical, chemical or both)
- *in vivo* effects of materials promoted as safe and/or sustainable
- biological influences of a natural particle control such as kaolin
- differences in the ingestion and excretion rates of beads and fragments
- ingestion of low concentrated fragments under co-exposure to food
- locomotor performance as a sublethal endpoint and early-warning sign

1.7.1 Toxicological characterization of chemicals from weathered plastics (A1)

In the first study, different *in vitro* bioassays were performed in order to evaluate the impact potentials of substances leached and released from commonly used plastics, bioplastics and recyclates. Therefore, the baseline toxicity in the Microtox assay, the oxidative stress in the AREc32 assay and the antiestrogenicity as well as antiandrogenicity in yeast-based reporter gene assays were assessed. The plastic materials were selected based on their polymer type and processing state and included pre-production and post-industrial pellets as well as two final products. The plastic samples were artificially irradiated with UV-C and UV-A/B light in dryness and subsequently leached in aqueous medium. Another leaching procedure assessed the impact of UV-A/B irradiation applied directly to the samples when leached in the medium (UV-A/B_{aq}). A dark control served as a reference treatment for the chemicals that migrate out without the application of a degradation initiator such as UV light. Next to the toxicological characterization *in vitro*, the leachates were analyzed with non-target high-resolution liquid chromatography mass spectrometry (LC-QTOF-MS). The combination of the toxicological and chemical analyses enabled this publication to outline the hazards of weathered plastics from a chemical perspective. Since the effects on the *in vitro* level have a lower ecological relevance than effects on the *in vivo* level, the plastics identified as toxicologically most concerning were tested with freshwater invertebrates in the following publications (A2 and A4).

1.7.2 Differentiation of physical and chemical toxicity of microplastics (A2)

In order to delineate the source of microplastic toxicity, the physico-chemical properties of a biodegradable material were studied with the freshwater worm *Lumbriculus variegatus*. In publication A1, both biodegradable materials were shown to induce high *in vitro* toxicity, but the materials could not be processed to a relevant morphological size for the oligochaetes. A

biodegradable material very similar to one sample from A1 containing highly toxicologically active chemicals was used instead and processed to fragments of a morphological relevance. The microplastic toxicity was assessed with different exposure scenarios. The particles were admixed into the sediment and layered on the sediment surface. Kaolin was used as a natural particle control in order to differentiate between physical and chemical microplastic effects. Then, the microplastics were leached in aqueous medium and enriched, which addressed the migrating substances alone. The sediment grains that the worms fed off were coated with the leachate. As the organism dwells in the sediment, it is exposed through the enteral and dermal route to pollutants. The microplastic chemicals were also extracted with a solvent and coated to the sediment grains, reflecting a worst-case scenario with a high chemical load. In the last experiment, solvent-extracted microplastics depleted of their chemicals were admixed to the sediment. The endpoints were dry weight, reproduction and mortality. Finally, the material was analyzed with FTIR, pyrolysis-GC/MS, thermal desorption-GC/MS, GC-QTOF and HPLC. This overarching assessment should help to clarify the source of microplastic toxicity.

1.7.3 Influences of microplastic size and shape on ingestion and egestion (A3)

In order to assess influences of the microplastic size and shape (beads and fragments) on the ingestion and egestion behavior of *Neocaridina palmata*, the freshwater shrimp was exposed to differently shaped (round and irregular) particles. The number of ingested and egested particles was determined and compared to each other. The time in which the particles passed the gastrointestinal tract of the organism was recorded. Moreover, shrimps were exposed to fragments and co-exposed to food to simulate the encounter to synthetic and natural particles that likely occurs in the environment. While in this publication the impact of the particle shape was emphasized, this study should also elucidate whether low concentrations of the particles affect an epibenthic invertebrate in a short-term exposure setting. The focal point of this study was to highlight the gut passage of differently shaped microplastics by using the shrimp as a model organism and to determine whether or not the particles influence the shrimp's feeding behavior.

1.7.4 Evaluation of behavioral performance and microplastic chemicals (A4)

The fourth study was conducted based on the findings in study A3 and the results obtained from publications A1 and A2. Again, *N. palmata* was used as a test organism, but exposed to enriched leachates from recycled pellets and a partly bio-based, but fully biodegradable foil. The foil is a final product of one of the respective pellets examined in A1 and was used instead because the pellets could not be milled to a relevant size class. Despite this, the foil exhibited

similar toxicities as their pellet counterpart. Ecologically relevant endpoints were evaluated. The locomotor behavior (i.e., moved distance and frozen events) was recorded in a 14-day exposure period. The plastics were processed to irregular fragments and similarly treated as in A1 (leaching with UV-A/B irradiation). The enriched chemicals from the materials that are advertised as ecotoxicologically safe and/or sustainable were evaluated *in vivo*. The baseline toxicity of the leachates was further determined in the Microtox assay. This final publication focused on the indirect effects that substances from microplastics could have on shrimps and addressed less frequently examined aspects, i.e., locomotor behavior as a potentially relevant endpoint for interactions in the environment and the safety of plastic alternatives.

2 Discussion

2.1 Key findings of the thesis

The following section outlines a brief overview of the key findings of this PhD thesis. Further details on the experimental design and results are provided in the publications (A1–A4). The main results of the respective publications are the following:

In vitro toxicity of leachates from unweathered and UV-weathered plastics (A1):

- Plastics leach chemicals inducing different *in vitro* activities. Oxidative stress was the most affected endpoint (85%), followed by baseline toxicity (42%), antiestrogenicity (40%) and antiandrogenicity (27%).
- Some plastics triggered several *in vitro* responses, others caused minor to no activities. As plastics of the same base polymer resulted in variable toxicities, it is not advisable to rank their toxicological profiles.
- Aqueous leachates including the chemicals from UV-weathered plastics enhanced the *in vitro* activities when compared to the samples solely treated under dark conditions. Unweathered plastics that were toxicologically harmless emitted hazardous chemicals after the UV irradiation was applied.
- UV-C irradiation of plastics in dryness with a subsequent leaching period revealed the most positive findings (63%), followed by UV-A/B light applied on leached (50%) and dry plastics (48%). No prior UV treatment caused activities in 33% of the cases.
- A recycled and two biodegradable materials proved to be of high toxicological concern prior to and after the UV degradation, demonstrating that sustainable materials such as recyclates and bioplastics are not toxicologically safer alternatives for conventional, virgin plastics.
- Pre-production pellets represent a chemically very heterogeneous material. The count of detected compounds ranged from some few to thousands of chemicals. The majority of the samples had very high chemical counts.
- The released chemicals from the plastics are mostly low-molecular weight substances that are increasingly formed due to the UV irradiation.

Effects of biodegradable microplastics in an endobenthic freshwater worm (A2):

- The oligochaete *L. variegatus* ingests microplastics made of biodegradable materials in a concentration-dependent matter.
- The microplastics were prepared from a shampoo bottle that has been produced with several biodegradable materials in contrast to the provided specification on the label.
- Microplastics that were mixed into the sediment induced stronger adverse effects on the reproduction and dry weight of the worms than the particles that were layered on the sediment surface.
- Kaolin as a natural particle control positively affected the weight of the worms in both particle applications. The natural mineral turned out to be a biologically active control and highlighted the ecological relevance of fine-sized matter for the sediment-dweller.
- Sediments coated with the chemicals from aqueous leachates of microplastics resulted in negligible effects for *L. variegatus*, whereas a high mortality was observed after the exposure to solvent-extracted chemicals bound to the sediment. The solvent-treated microplastics were mixed into the sediment and led to mitigated effects in comparison to their untreated counterpart. The outcome points to plastic-associated chemicals as the main driver for toxicity.
- The extracted chemicals were analyzed and included many degradation products and two biocides. The origin of one of the biocides was ascribed to the plastic bottle, while the other biocide originated from the bottle or the shampoo formulation. Substances from the content of the product thus could migrate and attach to the plastic bottle and likely contribute to the mortality observed in the exposure scenario with the extract.
- A biodegradable plastic material included chemicals determined as ecotoxicologically unsafe, which disagrees with its' environmentally friendly claimed advertisement.

Ingestion and excretion rates of differently shaped microplastics by a freshwater shrimp (A3):

- Both beads and fragments, which are of spherical and irregular shape, are ingested to a similar extent by the epibenthic test organism *N. palmata*. The ingestion rates were not significantly different, indicating an unselective feeding behavior.
- The ingestion of both microplastic shapes increased as a function of concentration. Concentrations that equal low environmental particle numbers were ingested readily.
- Medium- and small-sized beads were more often detected in the digestive tract of the shrimps than bigger-sized beads, highlighting that the morphology of an animal plays a crucial role when assessing ingestion capabilities.

- The shrimps tended to ingest less fragments in the presence of food particles, but this was not significantly different to the fragment exposure in the absence of food.
- Ingested microplastics were partially excreted by *N. palmata* within 4 h. Even though a higher number of beads was found in the digestive tract within the egestion period than for the fragments, the excretion of the two differently shaped microplastics was not statistically different.
- Shrimps are known to feed on high loads of natural particles in order to facilitate the mechanical fragmentation of food in the digestive system. Combined with their rather unselective feeding behavior, this suggests that irregular fragments commonly found in the environment could be at least physically negligible for epibenthic shrimps.

Behavioral analysis of a shrimp exposed to leachates from UV-weathered microplastics (A4):

- Leaching chemicals from non- and UV-weathered microplastics from a biodegradable, partly bio-based starch blend (SB) foil and recycled low-density polyethylene (LDPE-R) pellets did not adversely affect the locomotor activity of *N. palmata*.
- A general trend towards elevated or reduced movements was not observed, except on day 14 in the exposure to the leachable chemicals from the unweathered microplastics of the SB foil. The locomotion of these shrimps indicated hyperactivity with increasing concentrations.
- The concentrated substances from the unweathered and UV-weathered microplastic leachates induced high baseline toxicities in the Microtox assay, highlighting that the examined plastics release *in vitro* toxicants and can be regarded as negative examples in the design of (eco)toxicologically safe and sustainable materials.
- Since the *in vitro* toxicity did not translate to substantial effects at the individual level, it can be argued that the shrimps were not sensitive to the plastic chemicals under the chosen experimental conditions.
- As some test parameters were triggered by the plastic chemicals, manufacturers must be held responsible for the chemicals they put into plastics, starting with the reduction and simplification of such chemicals and transparent disclosures of the chemicals used in the production process of plastics.

2.2 Leaching toxicity from plastics

The substances added into plastics are not necessarily bound to the polymer matrix and thus can leach out from the material. In addition, transformation products and parts of the polymer units (i.e., oligomers and monomers) can be released after a degradation (see section 1.2 for further details). In this work, the entirety of the passively leaching chemicals (additives and NIAS) and the chemicals emerging during the depolymerization processes (degradants) were examined with several *in vitro* bioassays. Therefore, 12 different plastics were leached in water to approach aquatic conditions, enriched via solid-phase extraction and examined as extracts in the bioassays. It is notable that the assessment of the *in vitro* activities serves as a first step to characterize the mixture toxicities arising from the plastic composites. The impact potentials of the *in vitro* results are thus first indicators that were further analyzed on the *in vivo* level in order to record ecologically relevant effects. For now, the mere toxicities of the plastics without the application of a degradation initiator such as UV light will be discussed according to their polymer type, processing state and polymer purity.

2.2.1 Categories to profile the toxicities

One of the sorting criteria for the toxicological assessment of plastics is the polymer type. By this, a list of preferable and unfavorable polymers could be compiled and recommendations made for many actors along the production chain in order to ensure safe uses. It is noteworthy that, while this work focuses on the environmental health of limnic systems, potential human health implications can be derived as well from the generated *in vitro* data in this thesis. After all, the studied assays included a human breast cancer cell line and two screens with human estrogen and androgen receptors. Besides, the use of certain polymers for specific application areas predominantly relates to their physico-chemical traits making the materials unique for the respective needs. Some of the selected polymers in this thesis are used for applications in contact with human food (PlasticsEurope, 2020). In the first publication (A1), such plastics were PET, PS and PP and did not readily leach bioactive compounds, considering all of the examined endpoints. When such materials are processed into food-contact materials, some hazardous substances are regulated but many are not (Muncke, 2021). Chemical safety must be fulfilled regardless of whether the material is newly produced or recycled (Geueke et al., 2018). Moreover, the toxicities of chemical mixtures emerging from plastics are overlooked in regulation (Muncke et al., 2020), meaning that regulatory specifications have to be improved (Daniel et al., 2019). This is stressed by the detection of a variety of chemicals from plastics (Bradley and Coulier, 2007). In reality, “humans and ecosystems are exposed to hundreds of chemicals either individually or in combination from single or multiple sources”

(Aurisano et al., 2021b). This suggests that an investigation of a broadly selected spectrum of plastics is likely to elicit a high rate of positive findings for polymers that otherwise would be ranked as toxicologically inconspicuous. The literature cited below confirms this.

Wagner and Oehlmann (2009) tested the estrogenic potential of mineral water packaged in (non-)reusable PET bottles and detected both no and high estrogenicity. These *in vitro* results were attributed to migrating xenoestrogens coming from the plastic bottles and were further verified using an *in vivo* test with a freshwater snail that is known to be sensitive to estrogens and estrogen-mimicking substances. Interestingly, a point of introduction for such substances could be the production state itself (e.g., plasticizers, detergents and disinfectants), but they delineated the packaging material as the main source for EDCs. Ultimately, their study showed that PET can have varying *in vitro* responses, including no estrogenic potential at all as further shown by Kirchnawy et al. (2014) for 11 PETs. Given these responses, the previous debate about whether or not such compounds leach out of the material is thus not surprising (Bach et al., 2012). Moreover, the study of Kirchnawy et al. (2014) outlined estrogenic activity for one sample each of the polymer's PE, PS and PP. Regarding PE, PS and PP, Schiavo et al. (2018) did not detect any significant bioluminescence inhibition of the bacterium *Aliivibrio fischeri* in the Microtox assay, whereas milled PE, PS, PP and PET pellets as enriched leachates activated the oxidative stress response in the AREc32 assay under dark leaching conditions (Rummel et al., 2019). In our study (A1), a low-density PE (LDPE) induced the *in vitro* endpoints either not at all, slightly or below the detection limit. In contrast, a LDPE recyclate (LDPE-R) was the most potent sample detected in A1. In line with our results, Horodytska et al. (2020) reported that recycled LDPE contained a variety of added compounds.

Furthermore, two biodegradable samples and a virgin as well as a recycled PVC sample were of medium to high toxicological concern (A1). Lithner et al. (2009) examined various plastics, including thermoplastics and thermosets and found plasticized PVC products to induce short-term toxicity in *Daphnia magna*. Lithner et al. (2011) provided the additive amounts used in polymers and highlighted that out of all plastics PVC needs a high amount of additives. They grouped the hazards of the monomers and ranked PVC, next to PUR, as polymers that contain the most hazardous substances. Hence, it is not surprising that chemicals from PVCs have been reported to induce adverse effects *in vitro* (Groh and Muncke, 2017; Völker et al., 2021; Zimmermann et al., 2019) and *in vivo* (Li et al., 2016b; Lithner et al., 2009; Oliviero et al., 2019; Scherer et al., 2019). In a recent multi-criteria framework study, PVC was ranked as the polymer with the highest cumulative risk harm, especially when regarding the monomers and additives used for production and its service life (Senathirajah et al., 2022). Lastly, Yang et al.

(2011) analyzed a spectrum of 455 plastics and observed that most items included estrogenic compounds. Concerning bioplastics, the materials cannot be generalized due to the multiple resources used (European Bioplastics, 2020). Following Zimmermann et al. (2020), they are not expected to be toxicologically less concerning than conventional plastics, which is in fact in accordance with our results. However, this is quite the opposite message of what has been conveyed by European Bioplastics (2020). Taken together, the cited studies emphasize our conclusion made in the publication A1: a ranking of the polymer toxicity is not reasonable because the toxicity of plastics of the same base polymer can vary. This is interlinked with the heterogenous and customized chemical composition of each individual plastic, which is not known by the public. Since the producers have the information available to them, they could disclose (non-)intentionally incorporated plastic ingredients (Aurisano et al., 2021b).

The processing state could be another approach to categorize the toxicity profiles of plastics. This refers to the plastics' raw material (i.e., pre-production pellets), scrap produced during the production (i.e., post-industrial or pre-consumer) and the final plastic product (i.e., post-consumer, once it is discarded). The two latter describe the source of the plastic waste, which comes either from producers or end-users (Huysman et al., 2017; Pivnenko et al., 2016). The differentiation of virgin (newly produced) and recycled materials is another criterion closely interlinked with the processing state. Over the course of the conducted *in vitro* work and the chemical analysis in A1, the plastic samples have been determined to be chemically complex. We expected that plastic additives as well as other processing chemicals accumulate along the production chain and during reprocessing. Accordingly, pre-production pellets were assumed to not leach toxicants or at least exhibit minor toxicity in comparison to finished products or recycled pellets from post-industrial and post-consumer sources. Most pre-production pellets showed no toxicities after mere leaching (PP, PET, PS and LDPE). However, the biodegradable pellets induced high activities. Relative effects of the post-industrial recycled pellets (LDPE-R) and two PVC products (virgin and recycled) were also striking, whereas the post-consumer recycled pellets (PET-R) showed no biological activity. In the appended study A2, *L. variegatus* was examined and exposed to extracted chemicals of a biodegradable PLA-labelled shampoo bottle, which affected both the survival and weight. Again, this could point to finished plastic products containing a higher number of concerning chemicals than the base materials.

Our *in vitro* findings demonstrate that the recycling process does not necessarily introduce hazardous chemicals as was observed for the post-consumer PET-R. However, Eriksen et al. (2020) noted that the chemical properties of PET allow to maintain food-grade quality with the recycling process, while Dreolin et al. (2019) measured the concentration of BPA in virgin

and recycled PET pellets and the recyclates contained higher levels of this endocrine disruptor. This highlights that not every recycling company can preclude factors influencing the purity of the materials. Rummel et al. (2019) used leachates prepared in dark conditions with additive-free pre-production PE, PS, PP and PET pellets and detected oxidative stress *in vitro*. The same pellets (except for PE) neither affected the algae growth nor photosynthetic activity (Rummel et al., 2021). Similar to our study A1, leachates prepared with PE, PP and PS pellets did not include baseline toxicants (Schiavo et al., 2018). Moreover, Zimmermann et al. (2020) ascribed higher *in vitro* activities to 33 bioplastic products compared to a selection of 10 raw materials. Rendell-Bhatti et al. (2021) observed adverse developmental effects in sea urchin embryos due to exposure to leachates of plasticized pre-production PVC pellets. Both of our PVCs were active *in vitro*. Our *in vitro* and *in vivo* data and the literature demonstrate that, while exceptions exist, the processing state of plastics could be a first indicator hinting to whether or not toxicants may be present.

Plastics have been found to vary in their toxicities. Data on the pellets' toxicity are somewhat inconsistent, too. The second conclusion in A1 was that the data indicate that pre-production pellets exhibit toxicity. However, pre-production pellets seemed less harmful than the final plastic products and post-industrial recyclates. One question that needs to be raised is which processing steps allow the entry of undesirable chemicals, including compounds previously assessed as harmless but detected to be toxic in mixtures. For instance, Ubeda et al. (2019) compared the oligomer profiles in PLA pellets and film and revealed no changes between the two. They implied that manufacturing techniques such as a high temperature that is used for extrusion could create a different chemical profile though. Thus, the entire production chain is a potential pathway for various chemicals to be introduced or created, whereas the entirety of these chemicals can be then emitted during a products' life cycle. Pellets are of high importance because they form the basis for a variety of applications. While it is preferable that these raw materials do not contain any toxic compounds, a comprehensive study with virgin high-commodity pellets is lacking. Such a study could identify the pellets evincing chemically pure and toxicologically safe traits and expand on the methods used to produce such materials. At the moment, potentially harmful substances such as polycyclic aromatic hydrocarbons, phthalates, brominated flame retardants and volatile organic compounds have been found by Lowe et al. (2021) in recycled consumer products. They surveyed virgin and recycled consumer products and detected 918 and 587 chemicals, respectively. This was in part explained by the higher sample size for recyclates, but still demonstrated the sheer number of chemicals in recyclates. Despite the hazardous nature of some additives, e.g., as for

brominated flame retardants, these "fulfill essential functions and thus cannot be phased out easily in certain polymeric materials" (Aurisano et al., 2021b). While concerning chemicals should not be emitted to the environment, we may still rely on specific chemicals because no better alternatives currently exist for challenging areas of applications.

To put it concisely: A differentiation between "good" and "bad" polymers in terms of toxicity is hardly feasible. The mere leaching of plastics in water resulted already in elevated activities *in vitro*, but this observation did not include all plastic samples. The data from this work as well as the literature demonstrate that plastics have varying degrees of toxicological safety. Neither the recycled plastics nor the bioplastics have been proven to be better options or alternatives for virgin, conventional plastics. This is disconcerting because the advertisement for biodegradable and recycled materials as safe and/or sustainable for the environment is misleading to consumers. For now, these materials should be handled with caution due to the insufficiently developed chemical safety. Accordingly, multiple criteria need to be considered in order to provide truly better plastic alternatives. For instance, production processes and methods used to produce the raw materials and the design of plastic products must prevent the entry of toxic chemicals and mixtures. At the same time, technologies should promote safe and sustainable chemical alternatives (Aurisano et al., 2021b). In general, toxicological safety should begin with the starting materials. For the production itself, prevention measures must be adopted in order to stop the accumulation of toxic substances along the production chain. Moreover, the mitigation of chemical releases should be addressed prior to the development of a product by designing safe and simple substances combined with inert material properties (Fenner and Scheringer, 2021). The (eco)toxicity of such materials could be easily evaluated by performing fast screens as with the *in vitro* bioassays in this work and many other relevant screens suggested elsewhere (Groh and Muncke, 2017). This ensures a products' chemical safety before introducing it to the market (Muncke et al., 2020). Preventing the entry of toxic chemicals and mixtures into the international market is an essential step towards the 'clean' circularity of chemicals. This should be prioritized instead of having to deal with end-of-life management, which of course is crucial but would safe economic efforts and costs in the long run (Wagner, 2022). Also, clean recycling solutions are essential in order to meet high-quality standards of chemical flows needed for overall acceptable and good health. It is notable that the toxicants already in circularity must be eliminated in any case (Leslie et al., 2016; Muncke et al., 2020) and that if or when plastics enter the environment, only non-toxicants arise upon degradation (Haider et al., 2019). Some of the aspects raised here have been also described by the European Commission in the 'Chemicals Strategy for Sustainability Towards a Toxic-

Free Environment', aiming at "the transition to a safe and sustainable-by-design approach to chemicals". This strategy should enforce stricter regulations that better protect human and environmental health (European Commission, 2020).

2.2.2 Nonspecific and specific toxicities

The *in vitro* bioassays used in this thesis were suitable to assess the toxicity of plastic chemical mixtures. The plastics leached chemicals both with nonspecific (baseline toxicants, oxidative stress inducing chemicals) and specific modes of action (endocrine disruptors such as anti-estrogens/-androgens). This highlights the variety of chemicals in plastics and what transfers out of the material. One major advantage of these bioassays is the fast, simple and reproducible testing, especially for mixtures (Groh and Muncke, 2017; Muncke, 2021; Muncke et al., 2017). Considering all of the conducted weathering scenarios in A1, most of the positive findings were discovered in the AREc32 (85.4%) and Microtox assay (41.7%), followed by the YAES (39.6%) and YAAS (27.1%). However, relative activities were very similar in the AREc32 (38.1%) and Microtox assay (37.0%), while slightly less activities were found in the YAES (21.1%) than in the YAAS (25.4%). This underpins the prevalence of unspecifically acting chemicals in the selected plastic samples and is likely attributed to the high sensitivity of such assays towards many chemicals (Escher et al., 2014, 2013). Over the course of this PhD thesis, Rummel et al. (2019) published a similar study to A1. They also tested leachates from UV-weathered microplastics with several *in vitro* bioassays, e.g., in the PPARγ (peroxisome proliferator-activated receptor) and AREc32 assay. While both tests were highly responsive, all of the plastic leachates induced oxidative stress. In a study conducted by Zimmermann et al. (2019), the Nrf2/ARE pathway was triggered by only 41% of the 34 extracts prepared with plastic products. As for raw materials and final bioplastic products, 42% of the extracts resulted in an elevated oxidative stress response (Zimmermann et al., 2020). Both of their studies reported a higher percentage of samples having baseline toxicity, which is in contrast to our findings. Qiu et al. (2022) exposed *Escherichia coli* to enriched leachates from 15 plastic bags composed of PE or PP. To approach environmental conditions, they performed the leaching in artificial seawater. The analytical measures presented diverse additive contents in bags. In total, 240 chemicals were identified, e.g., classified as plasticizers, antioxidants, flame retardants, lubricants and other unclassified additives. Here, significant inhibition of luminescence was detected for 40% of the leachates, mirroring our results of A1. These findings suggest either (a) that final plastic products have more chemical inputs indicative of unspecific toxicity than their raw materials, as reported by Zimmermann et al. (2020), or (b) that different extraction methods play a decisive role in the observed responses

(Abbas et al., 2019; Bridson et al., 2021). Both factors seem to contribute to the chemical and toxicological outcomes. Also, additional aspects such as the immersion duration, temperature, pH, medium or solvent, salinity and surface area can influence the findings (Qiu et al., 2022; Tsochatzis et al., 2020; Yang et al., 2011). To exemplify negative results, Piccardo et al. (2021) leached 16 plastic packagings for 28 days in filtered, natural seawater and none of them (PE, PP, PET, PS, composites thereof and cellophane) affected the bioluminescent bacteria in the Microtox assay above the significant threshold. The same applies for a study conducted by Schiavo et al. (2018), where no relevant inhibitions in *A. fischeri* for virgin plastic pellets were detected. Once again, this highlights that leachables and extractables differ and that there is an urgent need to standardize the assessments of plastic chemicals transferring out of plastic materials.

Furthermore, plastics have been reported to contain and transfer chemicals having endocrine activity by many authors (Bach et al., 2012; Coffin et al., 2018; Qiu et al., 2022; Severin et al., 2017; Szczepańska et al., 2016; Yang et al., 2011; Zimmermann et al., 2019). The studies found are often limited to food contact materials and estrogenicity. However, antagonistic activities of antiestrogenic and antiandrogenic compounds have been less studied (Severin et al., 2017). While this could indicate that producers are more likely to process estrogen- or androgen-like substances instead of antagonistic compounds, the underlying mechanistic mode of the receptor-mediated bioassay rather explains this observation. Chemical mixtures can contain agonistic and antagonistic active chemicals, which can compete with the respective receptor. Therefore, antagonistic effects could be masked "by competing agonistic compounds" and *vice versa* (Itzel et al., 2018). This means that negative activities in the YAAS and YAES in A1 could reflect androgen and estrogen receptor agonists in the plastic mixtures. This can be resolved by separating antagonists from agonists via fractionation and thereby reducing the potential masking effects. Recently, Sakuragi et al. (2021) measured concentration levels of nine benzotriazole UV stabilizers in plastic bottle caps, food packaging and shopping bags and found that several of them act as (ant)agonists at the human estrogen (hER α/β) and androgen receptor (hAR). With regard to the health of organisms in the environment and humans, this is highly relevant as these were products of daily use that could enter the aquatic environment through intentional or accidental dumping. Beyond that, plastic chemicals could affect health in ways we are not currently aware of nor able to understand. Völker et al. (2021) underpins this statement with their investigation on the adipogenic activity of extractables from plastic products, a previously unconsidered mechanism of toxicity. Altogether, EDCs are highly relevant based on their untypical monotonic dose-response relationship and multitude

of potentially induced effects. Next to antagonistic activities, the chemicals emerging from the raw and final plastics can exhibit additive and synergistic effects due to the complex mixtures (Coffin et al., 2021). Therefore, producers should simplify the chemicals put into plastics in order to pave the way to a toxic-free environment (Fenner and Scheringer, 2021).

2.2.3 Chemically complex formulations

The high resolution untargeted LC-MS analysis performed in this thesis classified the selected plastics as complex chemical mixtures due to the many released and detected substances. This was observed following the extraction procedure with methanol and microplastics (A2) as well as the mild leaching experiments with water and pellets (A1) or microplastics (A4). At the same time, some of the selected plastic pellets released some few chemicals. For example, the LDPE-R, PVC and both biodegradable materials emitted most chemicals and induced high toxicities. A positive trend was observed between elevated toxicities and a high number of chemicals but a statistically significant correlation could not be proven for each endpoint and treatment. This means that toxicologically bioactive plastics can also comprise low chemical counts, e.g., as for the PS samples in the antagonistic screens. However, none of the chemically very complex plastics triggered no toxicities at all (A1). Also, it was not surprising that the recycled materials released high counts because of the many constituents usually going into reprocessing (Gerassimidou et al., 2022), yet this does not apply for every recyclate (Lowe et al., 2021). The same is true for biodegradable materials. Such materials are made to degrade fast and generate many degradants (e.g., oligomers) (Tisler and Christensen, 2022), but not every material of this category includes a multitude of chemicals. Interestingly, bioplastics have been shown to be chemically just as complex as conventional plastics (Zimmermann et al., 2020). Irrespective of the feedstock, our plastics released some few to several thousands of substances. The lowest count with 42 substances was detected for a PP sample, whereas the LDPE-R sample released most features with 2,804 substances after a mere leaching treatment in water (A1). In the study A4, the SB foil even released 2,984 compounds after a similar treatment. Along that line, many other studies report that plastics include and release hundreds to thousands of chemicals (Bradley and Coulier, 2007; Gewert et al., 2018; Li et al., 2021; Lowe et al., 2021; Qian et al., 2018; Tisler and Christensen, 2022; Völker et al., 2021; Wagner et al., 2013; Zimmermann et al., 2020). It should be emphasized that the different methods used to extract these plastic chemicals (Bridson et al., 2021) and the instrumental differences impair a proper comparison. In this work, a LC-based approach was applied, which assesses in contrast to GC-MS (semi-)volatile and nonpolar organic substances (Bradley and Coulier, 2007). In their study, the authors used a comprehensive set of analytical

tools in order to identify ingredients from six polymers typically in contact with human food. Taking PVC as an example, they determined 3 to 145 (non)polar substances, the detection of this specific range depended on the application of different methods. As referenced in section 1.2, chemicals processed into plastics may be unintentionally added but are usually known by the producer if they are technically detectable. Analytical labs could be however confronted with the issue of not being able to resolve every impurity and reaction- or side-product. As a consequence, some NIAS remain truly unknown. Previous studies have mostly focused on the chemical release from plastic products and now on recycled plastic items. In our study (A1), we demonstrated that the high number of chemicals including in and coming out from plastics starts at the beginning of the production process, namely with virgin, raw materials and delays in the reprocessing phase for recyclates. Based on this, the blended formulations and further processing steps introduce concerning chemicals and add up to the complexity of mixtures. The leachables investigated in our studies represent relevant exposure scenarios for organisms as opposed to the extractable chemicals that transfer by means of a solvent. In the laboratory, environmentally realistic conditions can be approached by leaching plastics in marine and freshwater media (Capolupo et al., 2020). Future studies should pay particular attention to this, especially on the effects of salinity on the migration of substances in plastics (Tsochatzis et al., 2020).

Taken together, the findings demonstrate that raw materials of plastics, final plastic products as well as their recyclates leach (migration) and release (formation of new chemicals) a broad number of chemicals. Although exceptions do exist, meaning that plastics of low toxicity and chemical complexity are equally available, the sheer number of the chemicals that cannot be easily identified is worrisome. The chemical mixtures are not currently assessed as a whole as described in the EU communication towards a toxic-free environment. Chemical safety is being evaluated with single substances and thereby this approach disregards hazards posed by the exposure to “multiple chemicals from different sources and over time” as described by the European Commission (2020). A tremendous amount of hazard assessments is available for registered substances (van Dijk et al., 2021), but the whole mixtures or chemical cocktails that have cumulative and even potential synergistic effects remain unknown. This displays a major health concern (European Commission, 2020). The observed toxicities in A1, A4 (*in vitro*) and in A2 (*in vivo*) have been most likely driven by combination effects, which is one of the main reasons regulatory laws need to be updated. Fenner and Scheringer (2021) commented that chemical simplification should be “a logical consequence of ever-increasing chemical pollution”. The identification of chemicals was not a focus in this thesis, but

clarifying which chemicals are causing toxicity is a relevant step towards the elimination of concerning substances from the production process. Aurisano et al. (2021b) noted that the identification and quantification of every plastic chemical is not really feasible; it is "highly resource-intensive", alluding to effect-directed analysis (EDA). The method reduces the complexity of a sample by fractionation and combines it with bioassays and a final identification step (Muncke et al., 2020). However, even with EDA truly unknown chemicals cannot be resolved due to poor spectral database records (Wagner, 2017). In light of moving towards a circular economy, extensive analytical methods will be necessary to differentiate between the virgin and recycled plastics (Chen et al., 2022) and thus to ensure 'clean' material streams. A substantial problem is the lack in transparency among the stakeholders (Gerassimidou et al., 2022). Confidentiality claims can be put to use for privacy and security reasons (e.g., to conceal personnel involved in animal testing) and to protect trade and business information such as production sites, toxicologically non-relevant impurities and starting materials that otherwise could be taken advantage of by other firms and result in high economic losses. This leaves "scientists and regulators with a Sisyphean task" (Coffin et al., 2021). By disclosing the data available to the plastic industry, this would allow to estimate actual environmental concentrations (van Dijk et al., 2021) and pave the way to progress so that actual sustainable and safe plastic alternatives could be developed. Moreover, plastic could be labelled with a list of additives in order to allow consumers to make an informed decision "on the plastic additives they are willing to expose themselves and potentially the environment to" (Burrows et al., 2022). However, a full disclosure of chemical ingredients is only feasible to a limited extent because of competitive disadvantages. It has been proposed that independent institutes could evaluate the toxicities of plastic chemicals. Also, white and black lists with toxicologically (un)acceptable chemicals could be beneficial to facilitate the shift towards the development of safer plastics. To achieve this, the transfer of knowledge along the entire production chain needs improvement (Sattlegger et al., 2020).

2.3 Weathering effects on the plastic leachates

Weathering takes place continuously in the environment. Plastic can be subjected to UV light, changing temperatures, mechanical abrasion, hydrolysis and biofouling (Jahnke et al., 2017). Obviously, this results in chemical as well as physical changes of the original plastic material. One major focus in this thesis was the leaching of (micro)plastics in ultrapure water that were weathered with UV light as an initiator for degradation (A1 and A4). This was performed in order to clarify whether or not released chemicals subsequent to the UV irradiation treatment have a different toxicity pattern than chemicals leached in a dark reference treatment. The

latter was discussed in section 2.2, whereas the toxicological and chemical profiles of the UV-weathered plastics will be discussed in the following section. It is notable that the weathering experiments were conducted under laboratory (i.e., artificial and controlled) conditions and that in nature several different factors come together. The UV-weathering of plastics and the related release of chemicals is a current matter of concern, especially for aquatic systems (Arp et al., 2021; Rillig et al., 2021). Therefore, both *in vitro* (A1) and *in vitro* as well as *in vivo* (A4) studies focused on the chemical evaluation from (un)weathered plastics that add to the poorly understood impacts on environmental health.

2.3.1 UV-induced changes of the leachate toxicity

The concentrated leachates of the differently UV-weathered plastics resulted in the following activities: 62.5% of the UV-C treated plastic samples were bioactive, 50% were bioactive after the UV-A/B_{aq} treatment (UV-A/B light and a concurrent leaching phase), which was followed by 47.9% after the UV-A/B only and 33.4% were bioactive after the dark control (DC). This means that the *in vitro* toxicities enhanced after the UV treatments or in other words, the toxicity of the UV-weathered plastics exacerbated. This was not surprising since Yang et al. (2011) reported similar results, namely that stresses such as irradiation, moist, heat and UV light increased the release of estrogenic active compounds from plastic consumer products. Likewise, Coffin et al. (2018) presented higher estrogenicity of UV-irradiated plastics in comparison to their virgin counterparts and artificially weathered plastics were also toxic but less concerning than polluted samples recovered from estuaries. This points to the UV light as an influencing factor for the chemical release from plastics. In the aquatic environment, the chemicals attaching on the surface of plastic particles play an additional role. For instance, Chen et al. (2019) collected micro- and mesoplastics from marine waters and leached these under common stresses. They showed that natural solar irradiation increases the desorption of plastic-attached EDCs, whilst microwaving and autoclaving decreased the concentrations of EDCs. They described that strong UV irradiation such as UV-C can be destructive to EDCs. Moreover, Rummel et al. (2019) observed leachates from UV-weathered plastics, except for PET, to induce a higher oxidative stress response in the AREc32 assay than from unweathered samples. In a more recent study, Rummel et al. (2021) exposed algae to the same samples and detected only the leachates from weathered PE to be baseline toxic.

The findings in A1 showed that a degradation initiator is not necessarily relevant for plastics to leach *in vitro* detectable toxicants. This means that plastics that have not been treated with any kind of stressor can leach bioactive substances as well. For instance, one third of the

plastic samples has leached toxicity after the dark control (A1). This is in accordance with the above-cited literature, since concerning chemicals have been shown to migrate or leach from unstressed or unweathered plastics. We further highlighted that leachates of UV-weathered plastics can induce slight or no toxicological changes at all (A1 and A4). Completely new *in vitro* activities were detected as well. Unweathered plastics that were inactive after leaching in the dark control were triggered to release or form harmful substances after the UV light was applied. This was not exclusive to the worst-case scenario with UV-C. This finding mostly included plastics with a small count of chemicals. Following this logic, plastics of low chemical complexity are not necessarily safer than chemically complex samples because new chemical mixtures can arise after the UV degradation. As a consequence, assessments must ensure the safety of a plastic material or product during its lifetime as well as "in terms of their future release to the environment" (Rillig et al., 2021). With regard to environmental health, plastics should be assessed under environmentally realistic conditions. This could be done e.g., by simulating the degradation in artificial (Capolupo et al., 2020) or natural seawater as leaching medium (Sait et al., 2021). The study A1 stressed the need for future ecotoxicological studies to evaluate the impact of weathered (micro)plastics as well as their leachates. Otherwise, the plastic chemical toxicity of leachates might be underestimated. In support of this notion, Arp et al. (2021) suggested that weathering impacts should be prioritized as research subject to better understand the hazards posed by the continuous weathering in the environment.

The chemical hazards from plastics to wildlife have been described many years ago by Teuten et al. (2009) and Oehlmann et al. (2009). In the latter review, biological effects on wildlife after the exposure to BPA, that can be found to this day in high-commodity plastic (Dreolin et al., 2019), have been reported. Furthermore, ecotoxicological effects from plastic leachates have been examined early on under laboratory conditions. One study that has been cited very often was conducted with *D. magna* by Lithner et al. (2009). In their work, PVC and PUR have been identified as one of the most acutely toxic samples. Several years later, these specific polymers are still one of the most commonly identified materials to induce toxicity (Völker et al., 2021; Zimmermann et al., 2019). In order to elucidate the origin of the toxicity, Lithner et al. (2009) used several solid-phase extractions and separated hydrophobic pollutants, cations or metals and tested the different phases in a procedure of exclusion. As for the PVC products, the hydrophobic pollutants were the driver for the observed toxicity. Coming back to effects of leachates from weathered plastics, Bejgarn et al. (2015) exposed the marine copepod *Nitocra spinipes* to 22 leachates from UV-weathered plastics and observed no consistent trend for the toxicity with the increase in irradiation time. After the exposure to artificial sunlight,

the samples' toxicities either decreased, increased or remained unchanged. In the publications A1 and A4, negligible reductions of the *in vitro* toxicity could be observed after the weathering. Gewert et al. (2021) also exposed *N. spinipes* to such leachates and "observed higher toxicity for leachates after plastic was exposed to UV light compared to leaching in darkness". The polymer types they have assessed were not indicative for a specific toxicity pattern. They identified PVC and PP as one of the most toxic leachates, whereas leachates from PET and PS induced low toxicities. Moreover, Xu et al. (2020) weathered single-use plastics composed of different polymer types (PE, PET, PP, PE and nylon) in one batch under sunlight for 20 days. The prepared leachate contained plastic chemicals as well as secondary MPs (<8 μm) and NPs, which resulted in significant reductions of the thoracic appendage curling rate in daphnids. However, chronic sublethal effects were attributed to the concurrent exposure to micro- and nanoplastics and the analytically detected hydrophobic chemicals and metals. A recent study not only examined the bioluminescence inhibitions for plastic bag leachates (Qiu et al., 2022), but further tested the swimming behavior of marine medaka larvae *Oryzias melastigma*. Since altered behavior is considered as an early warning sign, it is an important endpoint gaining increasing interest in microplastic toxicity testing as reviewed by Sun et al. (2021). Qiu et al. (2022) observed both hyperactivity and hypoactivity, when the fish larvae were exposed to the leachates. Since they conducted a comprehensive identification for leaching additives and performed a redundancy analysis, they were able to determine specific groups of additives as influencing factors on the movements of the larvae. Antioxidants, fatty acids and by-products positively influenced the swimming behavior, whereas flame retardants and dyes negatively impacted the movements of the larvae. In the publication A4, both hyper- and hypoactivities were observed in the shrimp *N. palmata*. These behaviors were not really conclusive at that time. Given that the plastics used in A4 included and leached chemical mixtures (see section 2.3.3), it is reasonable that different mechanisms could have been modulated and resulted in the varied locomotory behaviors of the shrimps that we have observed. Moreover, decreased swimming activities were observed in the positive control with NaCl, whereas the chemicals leaching from the tested microplastics (SB foil and LDPE-R) did not result in adverse effects. In general, the exposure of the shrimps to the leachates from (un)weathered LDPE-R and SB microplastics resulted in varied swimming activities, which is in accordance with the results by Qiu et al. (2022) but in contrast to what has been reported by Gewert et al. (2021). Finally, Sun et al. (2021) performed a meta-analysis on locomotor behaviors for aquatic organisms that were exposed to plastic particles and their chemicals. They did not differentiate between the particle properties and the chemicals and reported that the speed and moved distance was significantly inhibited. Our *in*

vivo data obtained for the leachates from UV-weathered microplastics highlight the following: The samples were highly baseline toxic *in vitro*, but this did not translate to an ecological relevance at the *in vivo* level.

In sum, the *in vitro* and *in vivo* findings for plastic leachates after weathering can differ. Effects at the *in vitro* level do not have to translate to substantial effects at the *in vivo* level, but the *in vitro* effects were still indicative of toxic compounds. Given the selection of 12 plastic samples and the four treatments, 48 samples were tested in total in study A1. This enabled a general statement for the impact of UV-weathering on the plastic leachate toxicity. Under the chosen conditions, an increase in the leachate toxicity was demonstrated. However, leachates of UV-weathered plastics seem to result in diverse *in vivo* findings. Both the effects of new and aged plastics should be considered. On a side note, weathered microplastics themselves were not ecotoxicologically assessed in this thesis. Several studies focusing on effects of weathered or aged microplastics are available (Arp et al., 2021). In short, the toxicity of weathered particles may decrease or remain unchanged in comparison to their pristine particles. Arp et al. (2021) reported that in one study a reduced effect was observed over time, which was explained by the depletion of BPA. The toxicity can increase as well as described by Wang et al. (2020b), who reported photodegraded PS plastic particles to inhibit the growth of juvenile groupers. In any case, this demonstrates that it is important to not underestimate the toxicity of plastics under environmental settings (Rillig et al., 2021) and to include a reference treatment for the plastic leachates or the plastic particles themselves in parallel to a weathering treatment.

2.3.2 Release of chemicals after UV-weathering

Inherent chemicals that leach from plastics transfer from one matrix to another one (e.g., from the polymer matrix to an ambient medium). In contrast to this migration process of chemicals, the release of chemicals is solely initiated by degradation processes (Muncke, 2011). In the studies A1 and A4, the plastics were leached in water. The substances thus migrated from the polymer matrix into a leaching medium and/or the water hydrolytically initiated the release. A combination of both processes here is very likely. The same is true when UV irradiation is applied. According to Gewert et al. (2015), plastics with a carbon-carbon backbone such as PE, PP, PS and PVC are susceptible to UV light. Polymers with heteroatoms in the main chain (e.g., PET and PUR) degrade via hydrolysis. In A1 and A4, the migration due to not covalently bound additives, the hydrolysis and the UV irradiation probably provoked the chemicals to leach. In combination with UV light, temperature is another aspect responsible for accelerated degradation (Andrady et al., 1998). The impacts of weathering on the leaching chemicals are

underlined by the vast number of detected chemicals, especially after the UV treatments. On average, 761 compounds were detected for the leachates from the dark control (A1). After the UV-A/B treatment, the leachates contained 938 compounds. After the UV-C and UV-A/B_{aq} treatments 994 and 998 chemicals were detected, respectively. The identified chemicals are summarized according to their occurrence per plastic sample and weathering treatment (Table A1). The data demonstrate that new chemicals were emerging as breakdown products or degradants were formed. The latter is however not displayed in the Table A1. The PET and biodegradable samples emitted many degradants, but these were not listed for the sake of brevity. Most of the detected chemicals were low-molecular fragments. Besides, not only did the mean number of chemicals increase with the UV-weathering, but the mean intensity (i.e., estimated amount of leachables) of chemicals increased as well.

The degradation of plastics and therewith the release of chemicals depends on many aspects, e.g., polymer type, shape, concentration of additives and environmental conditions (Fauser et al., 2022). In terms of special release patterns of the identified chemicals, not one compound was identified that was exclusive to the dark control treatment. The identified chemicals of this treatment were usually detected after several if not after every UV treatment. Many of the identified chemicals were however specific to one treatment, i.e., only occurring after the UV-A/B or UV-C treatment (Table A1). Besides, more chemicals were identified for the leachates of the UV-C light than after UV-A/B. It can be argued that the UV light accelerated the release of inherent chemicals, but it is not reasonable to ascribe the higher identification rate for UV-C induced chemicals to a trend because only a small fraction of chemicals was identified. Also, the UV-C treatment is considered as the worst-case scenario, but the UV-A/B proved to be sufficient in order to detect diverse chemicals. Chen et al. (2019a) showed that natural solar irradiation enhanced the leaching of EDCs attached to the surfaces of marine microplastics and mesoplastics. They subjected these plastics to common stresses (radiation, heat and UV light) and examined their leachates. While microwaving and autoclaving led to a reduction of EDCs, the plastics leached a higher rate of EDCs after being subjected to UV light. This means that the application of common stresses can provide valuable insights into the added plastic compounds.

In A1 and A4 the release kinetics and degradation behaviors of the identified chemicals were not a key aspect. Other studies measured the release rates of chemicals from plastics and thus could examine whether or not plastic chemicals are readily degradable. For instance, Paluselli et al. (2019) investigated the release of dimethyl phthalate (DMP) and diethyl phthalate (DEP) from PVC electrical cables as well as di-isobutyl phthalate (DiBP) and di-n-butyl phthalate

(DNBP) from LDPE trash bags. The plastics were leached in seawater with light and dark conditions in the laboratory. As for PVC, the light induced the release of two mainly detected phthalates (i.e., the DEP and DMP) by a factor of two when compared to their dark conditions. For PE, no significant difference was observed between the two treatments for each the DiBP and DnBP. Within the first two weeks, the phthalates reached their maximum leaching capacity and then, the concentrations decreased continuously up to 14 weeks. Based on their quantifications and the plastic amount entering the oceans, they estimated 0.32−0.86 million tons of phthalates from plastic bags to "leach in the first two months of their introduction into the oceans every year". This is concerning given the endocrine-disrupting properties of such substances.

In this context, organotin compounds (OTCs) have been used as stabilizers in PVC materials and are known to have endocrine disrupting effects. Chen et al. (2019b) studied the release kinetics of these compounds from PVC under the influence of UV light. Dimethyltin and dibutyltin were detected to be released, whilst two other OTCs photodegraded rapidly. This means that UV light initiates the chemical release, but at the same time such chemicals can readily disappear. Under dark conditions, the OTCs reached a concentration plateau after 24 h. Moreover, they showed that high salinity inhibited the "release and photodegradation of OTCs". Tsochatzis et al. (2020) refers to this phenomenon as the "salting-out effect". This is relevant with regard to the salinity both packaged food and the aquatic environment can have. Another study examined the chemical release from synthetic and natural textile fibers for 56 days during the UV-exposure (Sørensen et al., 2020). They detected increased concentrations for some substances, whereas others "remained at background levels". Therefore, the fibers released UV-resistant and readily degradable chemicals.

The studies cited above provide useful information on the assessment of the chemical release from UV-weathered plastic samples. All of them quantified the release rates of the chemicals added into plastics into seawater. This may be time-consuming, but it helps to contextualize the leaching behavior of intrinsic plastic chemicals in environmentally relevant settings. This could further help to elucidate the issues associated with plastic design and to derive actually safe plastic alternatives. Thus far, the leaching of plastic chemicals in the environment has not been really addressed that much, compared to the extensive research done on the migration behavior of plastics typically in contact with food or drinking water (Rillig et al., 2021). Rillig et al. (2021) stated that long-term consequences of plastic degradation and pollution release have been overlooked. Because of the ongoing plastic pollution in the aquatic systems and the continuous release of chemicals, they noted that plastics should be toxicologically safe during

their life-span and beyond that. Recently, Fauser et al. (2022) even questioned traditional risk assessments. They argue that future studies should develop new methods and approaches to assess the impacts of UV-weathering on plastic under environmentally relevant conditions.

2.3.3 Functionality of identified plastic chemicals

The chemical analysis of the plastic mixtures showed that the samples included a wide range of substances in terms of number and functionality. The chemical identification was not really within the scope of the study A1, but the data was still compiled and summarized in detail for this thesis in Table A1. The table further includes the chemicals of the starch blend foil used in A4. The results of the identification are highlighted in this section because some chemicals were identified after the different weathering treatments. In general, the analyzed leachates included many chemicals, but only a small fraction (57 substances) was identified. The table does not include the many detected degradants of the PET and biodegradable samples for the purpose of brevity and to focus on the chemicals (non-)intentionally added into plastics.

First, different compounds were identified to be present in several leachates (Table A2). PET and the biodegradable samples released e.g., 4-hydroxybenzoic acid (CAS: 99-96-7), which is used for plastic production. The plasticizer and adhesive *n*-butylbenzenesulfonamide (CAS: 3622-84-2) originated from the PS-GP, PP-H, LDPE and PVC samples. This is in contrast to chemicals being specific to one polymer, such as 2-hydroxybenzothiazole (CAS: 934-34-9) for PET, the UV stabilizer drometrizole (CAS: 2440-22-4) for PVC and tributyl phosphate (CAS: 126-73-8) for LDPE (Table A2). Groh et al. (2019) developed a comprehensive database for Chemicals associated with Plastic Packaging (CPPdb). The functions of added chemicals (e.g., as solvents, catalysts, plasticizers, flame retardants, antioxidants, dyes, UV and heat stabilizers, fillers, lubricants, NIAS and breakdown products) have been assigned to the identified chemicals (Table A3) and they have many different purposes. The diversity of the chemicals and their functions is not surprising, considering the versatility of plastic materials. The table further indicates that some chemicals are associated as elements in toys (Aurisano et al., 2021a). With that being said, the remaining chemicals of Table A1 not listed in Table A3 are not typically used or detected in plastic packaging or toys. Among these are several that belong to benzothiazoles (CAS: 934-34-9, 941-57-1), benzotriazoles (CAS: 136-85-6, 94-97-3), benzoic acids (CAS: 99-04-7, 586-89-0) as well as polyethylene and polypropylene glycols. The former has been measured in car tire rubber leaching into freshwater as well as marine medium. It was further detected to leach from PP, PET, PVC and PS, but to a lesser degree than from car tire rubber (Capolupo et al., 2020). Apparently, it is processed in consumer textiles,

although it is often used as a vulcanizer in rubber production (Sørensen et al., 2020). In this thesis, the recycled LDPE was determined to contain this compound, which following Lowe et al. (2021) has a greater occurrence in recycled products than in virgin materials. Sørensen et al. (2020) analyzed natural and synthetic fibers made of wool, PET and PA and exposed them to UV in seawater; they identified phthalates, organophosphorus compounds, benzophenone, benzothiazole, phthalide and phthalimide. *N*-butylbenzenesulfonamide, for instance, leached from all three microfibers with the highest levels in natural wool fibers. In this thesis, it was found in six samples. Benzophenone is a UV absorber and was found in the LDPE-R leachate (2,4-dihydroxybenzophenone, CAS: 131-56-6), whilst benzotriazoles are additional UV absorbers found in the PVC samples and the LDPE-R (Wypych, 2015). Benzotriazole, benzothiazoles and phosphates were detected in PE and PP bags by Qiu et al. (2022). Thus, these are known to be plastic-related (Wypych, 2015). As for the benzoic acids, they are used as preservatives or plasticizer precursors (Lowe et al., 2021) and were released after weathering (Sørensen et al., 2020). Sait et al. (2021) conducted a similar experiment as Sørensen et al. (2020) and detected three benzophenones. They described that the UV stabilizers absorb the UV wavelengths and thereby protect the polymer, but over time the polymer will be depleted of this substance. It is therefore important to study the plastic leaching process to get a general overview about the concentration peaks for toxic substances (see section 2.3.2) in order to estimate the toxicity.

To address the toxicity of some of the chemicals mentioned above, Sakuragi et al. (2021) for instance reported nine individually tested benzotriazoles, which were detected in water and food packaging as well as shopping bags, to have endocrine-activating potential *in vitro*. It is noteworthy that (eco)toxicological data for individual substances of Table A1 are available. However, the toxicity of each substance will not resolve the overall toxicity that researchers are confronted with in chemical mixtures. This has been highlighted in the study A2. Here, the biocide climbazole has been established to be ecotoxicologically relevant; it is typically used for its antidandruff properties in shampoo (Richter et al., 2013) and attached to the shampoo bottle. Climbazole probably transferred from the shampoo content to the surface of the bottle, but this substance is not the main cause for toxicity observed in the worm *L. variegatus* (A2). Other substances were identified (e.g., monomers, series of oligomers and another biocide) and ascribed to the polymers PBAT, PLA and PBS, to ingredients of the shampoo bottle and to the content of the product itself. The product thus confirmed to be a composite material of several blends of biodegradable materials, which was not provided as information on the label of the bottle. Both the content and the material of a product can thus contribute to the

detection of many chemicals that may interact with each other. This means that single toxic substances exist in these chemical mixtures, but the entirety of chemical mixtures can have additive, synergistic and/or antagonistic activities that drive the toxicity. Along that line, it is difficult to fully understand the underlying mechanisms. As of now, the complex mixtures of added chemicals pose unknown hazards. In particular, uncertainties arise for the degradation or the resistance to degradation of plastic chemical compounds. Some compounds could be readily degradable, whereas others outlast realistic environmental conditions (Sørensen et al., 2020). This is discussed in section 2.3.2. To shed light on the drivers of plastic chemical toxicity, an EDA should be performed to reduce the complexity of the sample and complement this by bioassay testing. In this case, the analyst must still know what substances to look out for, which is particularly difficult for some compounds. The chemical analysis carried out for publication A1 identified three known NIAS: 2,6-di-tert-butyl-p-benzoquinone (719-22-2), fenozan (20170-32-5) and an oxaspiro chemical (82304-66-3); these stemmed from the post-industrial recycled LDPE pellets (Table A1). However, not every NIAS can be detected because reference standards are not available and this way no toxicity testing can be performed (Groh and Muncke, 2017). According to the authors, such substances can have "a significant part of the overall migrate".

The chosen samples in this thesis contained typical plastic chemicals, e.g., used for packaging, toys, car tire rubber and textile fibers (A1, A4). This highlights the potential application types of the pre-production pellets (A1). The identification of a biocide was unexpected though as it migrated from a cosmetic product onto the surface of a bottle (A2). This is in contrast to the second detected biocide, which was an integral part of the shampoo bottle. In total, functional plastic additives, NIAS and degradation products (latter not displayed in Table A1, but these were detected especially for PET and biodegradable samples) were identified. A high number of chemicals was detected, but only a small fraction was identified. As a result, the majority of the compounds remains unknown. Despite this, the abundance of different functional plastic chemicals was highlighted for plastic pellets as well as one plastic consumer product. These whole mixtures need to be addressed with new and improved risk assessments (Fauser et al., 2022), especially since this thesis demonstrated the occurrence of newly emerging chemicals once plastic materials are UV-weathered.

2.4 Driving factors of microplastic toxicity

The different factors potentially determining the microplastic toxicity were studied in A2 and A3. In publication A2, *L. variegatus* was exposed to different microplastic applications. At the start of this thesis, the endobenthic freshwater worm *L. variegatus* was not actually used to assess microplastic toxicity, although it is a typical test organism in ecotoxicology. At that time, only two studies have examined the feeding behavior of these worms (Imhof et al., 2013; Scherer et al., 2017). In contrast, *D. magna* was frequently used for microplastic toxicity testing (Triebskorn et al., 2019). Moreover, alternatives for conventional plastics (e.g., bioplastics and recyclates) were not really assessed at that time but questioned in terms of their toxicity. Two of the *in vitro* tested plastic samples were highly bioactive, i.e., the SB pellets and LDPE-R pellets (A1) and SB foil (A4). The results of the biodegradable and partly bio-based material (i.e., the SB) were surprising, considering their advertisement as an environmentally friendly as well as compatible and sustainable material. Therefore, the focus was laid on the assessment of such a material in publication A2. However, the biodegradable material could not be milled to a morphologically relevant size class for *L. variegatus*. For this reason, a shampoo bottle made of a similar biodegradable material, for which the information was provided that it was highly active *in vitro* (sample 'PLA 3' in Zimmermann et al., 2019), was used instead and prepared as microplastics. These microplastics significantly affected the number of worms, whereas solvent-extracted microplastics that were depleted from their associated chemicals induced lower effects. The solvent-extracted chemicals had detrimental effects on the number of worms. Taken together, this study hinted to the chemicals associated with the biodegradable material as the main driver for toxicity.

In general, benthic organisms were previously examined less compared to pelagic organisms (Haegerbaeumer et al., 2019). Next to *L. variegatus*, we examined the feeding behavior of the epibenthic shrimp *N. palmata*. In publication A3, the shrimps' ingestion and egestion rates for differently shaped and sized microplastics (i.e., beads and fragments) were investigated. The irregular fragments were also co-exposed with food so that a conclusion could be drawn as to whether or not the available food influences the ingestion. This was a baseline study in order to evaluate the potential factors that influence the ingestion as well as the egestion. The latter is helpful in resolving the retention or gut passage time of the respective particles. The studies investigating the ingestions were conducted for 24 h, while the excretion time was set for 4 h. Hence, the ingestion experiments with beads and fragments are comparable to acute toxicity tests. However, these did not result in negative outcomes (e.g., in the form of a high mortality or else). For the shrimps, the particle shape obviously did not matter with regard to both the

ingestion and egestion. The bead size was a relevant factor though as medium- and smaller-sized beads were ingested more frequently than bigger-sized beads. When food was present, *N. palmata* ingested less but insignificant amounts of fragments. This study demonstrated that the particle shape does not play a decisive role for the freshwater shrimps. Moreover, it was highlighted that the beads and fragments can be excreted fast by the shrimps. Given that no adverse effects occurred within the period in which the particles passed the gut, this suggests that the particle shape is not relevant for the epibenthic shrimp under the chosen conditions.

2.4.1 Physical versus chemical toxicity

Plastic particles comprise both physical and chemical properties. For this reason, it is difficult to differentiate between chemically- and physically-mediated effects (e.g., due to associated chemicals in or on plastics in contrast to the particle size, shape or density). Plastic chemicals can be leached or extracted and thus evaluated separately from the polymeric material itself. This is one way to assess the effects of whole chemical mixtures containing plastic chemicals, the relevance of which has been shown in sections 2.2 and 2.3. For internal physical damage or blockage to take place, microplastics have to be ingested by animals (Carrasco-Navarro et al., 2022). In order to discern physical and chemical effects of microplastics, several studies included reference particles as a control. Therefore, microparticles with similar properties as the microplastics of interest have been used. Over the years, the lack of adequate controls has been criticized many times. The critique also referred to the fact that organisms are regularly exposed to a multitude of natural particles in the environment (Backhaus and Wagner, 2020; Connors et al., 2017; Lusher et al., 2021; Ogonowski et al., 2018). Other experimental methods for the evaluation of the physical toxicity of microplastics relate to the purification of particles with a solvent. Redondo-Hasselerharm et al. (2018b) cleansed microplastics in methanol and depleted them from their added chemicals. Pikuda et al. (2019) purchased nano-sized plastic particles, which are distributed in solutions containing preservatives or surfactants, purified these and exposed *D. magna* to the analytically verified ‘clean’ particles. Interestingly, results pointed to the antimicrobial preservative sodium azide as the driver for the observed acute toxicity in daphnids.

In publication A2, an overarching assessment was used to elucidate the source of microplastic toxicity. As a reference treatment, the natural mineral kaolin was used and long-term effects of the microplastics were compared to the ones of the particle control. In a first step, however, the microplastic ingestion was verified. The number of ingested microplastics increased with the increment in concentration. The worms were then exposed to microplastics via different

exposure routes (i.e., enteral through ingestion of the sediment grains and the pore water and dermal through the uptake of chemicals from pore and overlying water). Besides, the particles were applied once as a layer onto the sediment surface and once mixed into the sediment. The latter significantly reduced the number of worms after 28-days of exposure to 8.4% of microplastic particles sediment dry weight^{-1}. This is reasonable because the organism ingests particles whilst burrowed in the sediment (Carrasco-Navarro et al., 2022). Interestingly, our kaolin treatment proved to be a biologically active control, as demonstrated by the increased dry weight of the worms after both particle applications. Other reports with *L. variegatus* have observed a lack in toxicity for e.g., tire wear particles (Carrasco-Navarro et al., 2022; Redondo-Hasselerharm et al., 2018a), PS (Redondo-Hasselerharm et al., 2018b) and PE microplastics (Silva et al., 2021). These studies are the only reports published over the course of this thesis that focus on the microplastic toxicity and *L. variegatus*. However, these studies had different experimental conditions in comparison to our study. Silva et al. (2021) tested a maximum concentration of 2% of differently sized PE microplastics and concluded that the reproduction was not affected by the microplastics' size or concentration. A slight decrease in the number of worms was observed only for 32–64 μm PE particles at 0.13% in the tested sediment. This demonstrates that *L. variegatus* was not sensitive to these specific microplastics. Extremely high concentrations (i.e., 40% of sediment dry weight) of PS particles also did not affect the number or growth of the worms (Redondo-Hasselerharm et al., 2018b). In this case, the particles were washed three times with a solvent to remove any additive chemicals. This way only the physical toxicity of this specific polymer was tested and emphasized that (a) a particle control is not necessarily relevant if hazardous compounds are not embedded in the polymer matrix anymore and (b) that the endobenthic worm copes very well with a high load of synthetic microparticles. In our study A2, the biodegradable particles were shaken for 24 h in methanol and the treatment with the highest concentration (i.e., 8.4% sediment dry weight^{-1}) of solvent-treated particles still led to significant reproductive effects. This impact was lower compared to the impacts caused by the untreated microplastics. This indicates that either chemicals associated with the bottle packaging or the content were still present after the treatment with the solvent or that the particles themselves caused the effect. Given the exceedingly high concentrations used in Redondo-Hasselerharm et al. (2018b), the effects rather point to the processed chemicals as the driver for toxicity. This was supported by the high mortality and decreased dry weight, when exposed to extractable chemicals of the bottle made of biodegradable materials. Negligible effects were observed for the aqueous leachates of these microplastics. In sum, the results demonstrated that even though the particle control was biologically active, extracted chemicals that were coated to the sediment grains severely

affected the test organism. This represented a worst-case scenario in terms of chemicals from plastics accumulating in sediments. If we consider the lowest concentration (i.e., 1% of plastic chemicals sediment dry weight^{-1}) as environmentally relevant, only little biological impact is seen. Under environmentally relevant conditions (e.g., 1% untreated microplastics sediment dry weight^{-1}), no biological impact of microplastics has been detected. Our extraction method enabled this publication to shed light on the toxic chemical mixtures arising from a cosmetic product. Furthermore, two ecotoxicologically relevant biocides from the shampoo bottle and content were identified and assumed to be one of the key components to drive the toxicity. In this context, it is noteworthy that the worms of the other studies as a test organism were not insensitive towards the respective particles or that our test organism was overly too sensitive. The results are rather case-specific, which could be linked to the polymer types, the individual chemical composition and the selected test species. Carrasco-Navarro et al. (2022) stated that *L. variegatus* is an adequate model organism for testing of microplastic toxicity, although they could not detect effects following the exposure to microrubber particles. Their "experimental design was [however] able to discern differences between the growth" in two different tested sediments. Silva et al. (2021) also detected biochemical alterations in *L. variegatus*, although no changes on apical parameters (such as reproduction or mortality) were observed. This demonstrates the importance of the surveyed endpoints.

Overall, not all microplastics are without impacts on the worms. The particles themselves are likely negligible for *L. variegatus* as reported by Redondo-Hasselerharm et al. (2018b), when considering the worms high sediment reprocessing rate leading to a short residence time of the microplastics within the gastrointestinal tract (Carrasco-Navarro et al., 2022). It is thus difficult to ascribe an observed effect to microplastics for organisms that are naturally surrounded by a high load of particulate matter (Ogonowski et al., 2018). Furthermore, single factors or a combination of factors can influence the physical toxicity of microplastics. Kögel et al. (2020) conducted a research review and found nine determinants, i.e., exposure time, concentration, particle size, shape, condition, polymer type, species, developmental stage as well as sex and environmental conditions (e.g., food availability). In the study A2, the physical effect of the microplastics seemed to be less of a concern. However, the shampoo bottle made of several biodegradable blends included toxic chemicals that could be released over time in reality and was represented in the publication by the extraction method. Along that line, such materials should not be claimed to be environmentally friendly/compatible. At the same time, it is noteworthy that less harmful materials of the same material category are available on the market (Zimmermann et al., 2020).

In publication A3, the ingestion and egestion behavior of differently shaped microplastics by the shrimps suggested that the invertebrate does not feed selectively on one specific particle shape. This was supported by the study of Korez et al. (2020), who showed that brown shrimp (*Crangon crangon*) cope well with different inorganic particles such as quartz grains, bivalve shells and aragonite fragments. The study A3 has not been greatly discussed in this thesis as no apparent microplastic toxicity was detected. This is most likely interlinked with the acute exposure and the rather low particle concentrations used. In this context, this means that an effect or response observed depends on the experimental conditions chosen. While we could not observe any obvious response, this does not necessarily mean that no effects occurred at all in *N. palmata* (see the changes at the biochemical level for *L. variegatus* by Silva et al., 2021). Regardless of whether microplastics induce an obvious effect (A2) or not (A3), the materials put on the global market must be designed on the premise of (eco)toxicological safety. Finally, publication A3 served as a baseline study to obtain information about influencing factors (e.g., particle shape, size or food availability) on the gut processing time in a freshwater crustacean species, because the increasing number of plastic particles in the environment could potentially become a health threat. However, neither the shape nor the food was an influencing factor. Future studies need to take a closer look into accumulating ingested particles that are processed in the gut of Crustacea. In light of the evidence demonstrated by Dawson et al. (2018), Antarctic krill are able to fragment microplastics into nanoplastics. This could become a health threat because of the potential of nanoparticles to translocate physical barriers and accumulate in tissues. Therefore, it is important for future studies to look further into synthetic particles that remain within the gastrointestinal tract of an organism.

2.4.2 Adequate reference particle controls

None of the studies with *L. variegatus* mentioned above included a reference particle control. Given the negative outcome for chronic endpoints, the question arises whether such a control is necessary or not. Even if microplastics have been depleted of their additive chemicals, it is still possible that polymer-specific effects could occur. In A2, an overarching assessment was performed to find the origin of microplastic toxicity. We acknowledged the fact that a particle control could be helpful and included kaolin. However, this control proved to be a biologically active control. The particles were observed to be of beneficial value for the dry weight of the worms, which made it difficult to derive a definite statement with regard to microplastic toxicity. To work around this issue, the study further examined the effects from the shampoo bottle made of several biodegradable material blends as aqueous leachates, solvent extracts

and solvent-treated microplastics. Following Schwarzer et al. (2022), a particle control should have similar physical properties as the microplastics, but this is difficult to obtain due to the heterogenous features of microplastics (Lambert et al., 2017). Ogonowski et al. (2018) stated that it "is not likely that experimenters will ever find ideally matching reference particles". In the following some examples will be given to highlight how other studies have dealt with this problem. For instance, Schrank et al. (2019) examined effects of plasticized and unplasticized PVC particles on daphnids and compared them to glass beads. Obviously, the spherical beads lacked the irregular shape of the microplastics. Since the glass control had affected the growth of the daphnids, they explained that this could be related to the species' high processing rate of spherically shaped particles and therewith to a reduced food intake. Other studies included several particle controls as done by Scherer et al. (2019), who exposed chironomids to PVC particles and applied diatomite and kaolin as references. This way they were able to state that at least the shape of the PVC particles could be excluded as a driver for toxicity. Furthermore, Schwarzer et al. (2022) examined the impacts of three different PS shapes (beads, fragments and fibers) on *D. magna* and observed sublethal effects. They used glass, cellulose (acetate), kaolin and ground mussel shells as references. None of these controls elicited adverse effects. Therefore, they concluded that the effects were material-specific.

Putting the study examples aside, it is important to mention that natural particles can provoke adverse effects as well (Ogonowski et al., 2018). This thesis summarized that the observed responses can range from positive effects as in our study A2 to no or negative effects as noted with the studies above. This can be caused by decreased food intake and therewith a caloric deficit. In this case, the nature of the particles (synthetic or natural) does not matter as long as high quantities are available. De Ruijter et al. (2020) stated that most microplastic studies with aquatic biota suggested or demonstrated that the toxicity mechanism was related to the "inhibited food assimilation". This emphasizes that the individual exposure conditions (e.g., concentration, exposure time or available food) play an important role. This also means that the use and selection of a reference particle control in microplastic toxicity testing depends on the individual exposure conditions, including the test species and microplastic used. This warrants further investigations so that future microplastic testing ultimately will be aligned. In our study A3, there was no need to include a particle control as we did not focus on chronic effects. However, we included procedural blanks to account for particles that are introduced e.g., by air or equipment into the analysis. Taken together, controls are crucial when assessing microplastics.

2.4.3 Underlying toxicity mechanisms

To understand the factors and mechanisms of action that influence and drive microplastic toxicity, different experimental measures need to be taken into account (Lusher et al., 2021). De Ruijter et al. (2020) summarized the modes of action that were frequently used to describe an observed microplastic effect. The main explanations were a reduced food intake, physical damage and leaching chemicals. Lusher et al. (2021) stated that "research needs to move from describing toxic effects of particles towards understanding the underlying mechanisms of action and toxicity pathways affected". This refers to the main explanations used that are no longer sufficient. A recently published study will be highlighted, where the authors described an effect more in detail. Völker et al. (2021) demonstrated that extracts from plastic consumer products include chemicals that induced adipogenic activity in murine 3T3-L1 cells. Hence, the products contain metabolism-disrupting chemicals. Because this is a specific response of the cells, they further tested the activities of the extractable chemicals at two other receptors related to the adipogenesis (i.e., at the PPARγ and glucocorticoid receptor = GR). Two plastic samples triggered a "high PPARγ activity" and "a strong induction of lipid droplet formation". Three other samples were active at the PPARγ but inactive in the adipogenic assay. None of the extracts activated the GR assay. On these grounds, they concluded that the specific plastic chemicals extracted are "not mediated via PPARγ and GR" but that these are probably drivers of several mechanisms of action. This emphasizes the complexity of the chemical mixtures of plastics, but more so it underlines the complexity of the toxicity pathways involved.

2.5 Solutions and mitigation strategies for plastic pollution

Due to littering and mismanaged waste plastics can enter the aquatic ecosystems, where they break down into microplastics subsequent to the weathering processes. It is thus important to reflect on solution strategies and mitigation measures to control such plastic emissions into nature. The discussion thus far covered the main topics of the publications of this thesis: (a) the chemicals leached (or released) from (weathered) plastics of conventional, biodegradable and recyclable origin, (b) the factors influencing the toxicity of microplastics and (c) the subtle behaviors of organisms (ingestion and egestion and swimming activity) during the exposure to microplastics or their chemicals. Accordingly, the studies only investigated the effects of or the influencing factors on microplastic toxicity. The means required to mitigate such effects have not been discussed therein. The following sections address the strategies developed for the global plastic waste problem, the reduction of total plastic emissions to the environment, the definition of a circular economy and the challenges we have to deal with as plastic moves through various stages.

2.5.1 A set of different measures for a systemic problem

Plastic pollution is one of the world's major pressing environmental issues. In order to achieve meaningful reductions of plastic emissions into the aquatic system (rivers, lakes and oceans) and thereby reducing the number of emerging microplastics, the entire plastic system needs a drastic change (Borrelle et al., 2020). Based on the expected growth rate of produced plastic, Borrelle et al. (2020) estimated the amount of plastic waste entering the aquatic systems by 2030. Under the circumstances that waste management will not improve in the near future and the *business* runs *as usual*, a total of 90 million metric tons will reach the aquatic system. Moreover, they estimated the means required to reach an emission target of 8 million metric tons by 2030 and highly recommended to combine different strategies (e.g., limiting the virgin plastic production, reducing the generated plastic waste, a properly functioning management of waste and the recovery of the waste already present in the environment through cleanups). Along that way, the substitution of certain materials and the reduction of unnecessary items like single-use products would help (Borrelle et al., 2020; Prata et al., 2019). Wagner (2022) noted that plastic pollution is not solely a waste problem, but added that it is also a resource issue. He explained that an increased production of biodegradable materials would at least allow to reduce the amount of persistent plastics in the environment. However, Burrows et al. (2022) stated that it "is debatable if they [bioplastics] are better for the environment over conventional plastics and if they add to, or solve existing problems". It could be argued that bioplastics are currently mitigating the plastic waste problem and do not relieve pressure on the environment, which is in line with Ribba et al. (2022). Based on the reported findings in this thesis, i.e., the (eco)toxicity of such materials *in vitro* (A1) and *in vivo* (A2), such materials first need to be better designed in terms of their chemical additives and the biodegradability to really outperform high-commodity plastics. Wagner (2022) further noted that plastics are valuable, which is often overlooked. This could be taken into account through levies on single-use product and taxes for producers. Producers could be also held accountable for the entire life-cycle of the products they manufacture (Freinkel, 2011). This is known as the concept of Extended Producer Responsibility (EPR). Thereby, producers could be encouraged to design recyclable products according to a recycling activist in Freinkel's book. In my opinion, the EPR concept should apply for the release of toxic chemicals as well, so that producers solely use and add safe chemicals to plastics we consume on a daily basis. After all, they should ensure that the plastic products they place on the market are safe during and after use. This should be tested by independent laboratories (Sattlegger et al., 2020). Finally, life-cycle assessments (LCAs) could be applied, too. In short, this is a tool to measure the environmental impact of a

product over its' entire lifetime. This enables producers to find eco-friendly alternatives or to identify the factors needed for improvement (Prata et al., 2019).

The plastic waste can be viewed as a societal problem. Wagner (2022) described the societies' excessive consumerism as one major driver of the waste problem. This implies that there is a lack in education, wherefore awareness must be raised on the following aspects: how to use and discard plastics properly, which plastics might be avoidable (e.g., single-use plastics and often for food packaging), what kind of environmental and human implications can emerge due to our daily use of plastic (e.g., due to microplastics) and lastly, how to recover the plastic waste from the environment. People might then start to look for plastic alternatives, which must be placed on the markets (Prata et al., 2019). After all, our dependency on the synthetics will not simply vanish (Freinkel, 2011). Freinkel outlined that the synthetic materials are not *per se* the problem, but our mismanaged relationship with the material is (considering that a commodity with a certain value is thrown away, improperly managed and not recycled back to the plastic system). The current linear economy (produce, use and discard) enhances all of the environmental issues we have to face today. To conclude, Wagner (2022) described that plastic pollution is "the result of multiple failures at multiple levels". Thus, it becomes obvious that we are dealing with a systemic problem that only a collective of different actors is able to solve. In other words, there is no one-size-fits-all solution. The proposed solution strategies are individually great and efficient, but only alleviate the symptom of plastic pollution when used individually. Consequently, combined actions and the cooperation between authorities, the plastic industry, scientists and the public are key to transform this system (Borrelle et al., 2020; Burrows et al., 2022).

2.5.2 Transitioning towards a circular economy

One of the key actions necessary to transform the plastic system is the transition from a linear to a circular economy (Wagner, 2022). According to van Buren et al. (2016), a circular system includes far more options than just the "reduction of waste through recycling". The definition they proposed relates to the creation of economic, social and environmental value. Moreover, a circular economy addresses all of the phases within the value chain (i.e., design, production, use and end-of-life management) (Syberg et al., 2021). The gradations of circularity van Buren et al. (2016) have listed include 9 R's instead of the three commonly known (see section 1.5). In the following, these are listed in descending order of importance: refuse (prevent), reduce, reuse, repair, refurbish, remanufacture, repurpose, recycle and recover energy. Whereas the linear system or a system with feedback loops generates waste, open loops do not exist in a

circular economy. In a "circular system materials are applied in products in such a way that they can be recovered and reused almost endlessly" (van Buren et al., 2016). Because fossil resources are limited and not sustainable, the transition towards circularity is crucial. One of the great advantages of material circularity would be the reduction of the plastic waste that enters and pollutes the environment (van Buren et al., 2016). It should be kept in mind that a combination of different efforts must be put into practice at the different stages of the plastic value chain. However, research often studied end-of-life management rather than tackling the challenges at the initial phases of plastics (1st: the design, 2nd: the production and 3rd: the use) (Johansen et al., 2022). Johansen et al. (2022) reviewed studies on the plastic value chain and reported most of them had focused on how to reduce the plastic material used for packaging products and how to integrate the recycled plastic into a product. In terms of toxicological safety, these are important aspects to consider because food packaging should ensure a high-quality grade material that does not transfer toxicants to foodstuff (Muncke, 2021). Johansen et al. (2022) described that it is difficult to use recycled materials for such purposes, because these are usually mixed with other polymers and chemical additives. This would complicate the proposal by Burrows et al. (2022), who suggested to disclose the additive composition as a list labelled on sold products as thousands of chemicals can be detected in recyclates (A1). The third phase of the plastic value chain is the use, i.e., the purchasing, using and disposing of a product (Johansen et al., 2022). As mentioned before, people must be better educated about the implications of the plastics they use daily. The three cited studies here (Wagner, 2022; Johansen et al., 2022; van Buren et al., 2016) agree that society needs a fundamental transition (change in behavior) as well in order for a circular economy to succeed. Johansen et al. (2022) further elaborated on end-of-life solutions for the plastic waste (including the collection, sorting, recycling, LCAs or mass flow analyses and regulations), for which a higher number of studies is available than for the remaining phases of the value chain. Reprocessing is complicated because of the different polymers that have optimal methods for reprocessing and the sheer number of incorporated chemicals (Burrows et al., 2022). The studies reviewed by Johansen et al. (2022) identified the contamination of plastic waste as a major problem and overarching aspect that challenged more than one phase of the value chain. They stated that we first have to take a few steps back in order to move forward; this means that we must rethink "the way we design, produce and use plastic products". Future research should thus focus on the initial phases that plastic has to undergo. This could open new opportunities in terms of collaborations, since the phases of the value chain are highly interconnected.

2.5.3 Regulatory interventions along the plastic value chain

The key policy initiatives that have been introduced to tackle the plastic waste from a circular economy viewpoint have been recently outlined by Syberg et al. (2021). The regulatory work conducted (inter)nationally and even locally will be summarized in the following section. For instance, regulatory measures are helpful to interfere with the production process of specific products such as for plastic bags that are often thrown away after only a single use. Therefore, bans and levies have been used to target the plastic bags worldwide and thus to reduce plastic emissions into the environment. With regard to end-of-life management, there are initiatives to increase the collection and reprocessing of plastic waste, e.g., with deposit-fund systems as for PET bottles or containers. This way plastics can be kept in circularity, when they are joined with a specific price (Freinkel, 2011). To address the initial steps of the plastic value chain (i.e., the design), legislations have been introduced in the EU to regulate the packaging design and the packaging waste. For plastics to remain in circularity, the materials have to be drafted for full recyclability (Syberg et al., 2021). This could impede the toxicological safety of a plastic product, considering the toxicants in recycled materials that have to be removed first (Leslie et al., 2016). Syberg et al. (2021) explained that the regulatory measures shifted from the initial bans and directives for waste management towards circularity aspects. This means that the policy initiatives now can occur at every phase of the plastic value chain. There are still some areas that are not regulated yet as for the pre-production plastic pellets, for which this thesis has demonstrated that the plastics' raw material already includes a high number of chemicals that as mixtures are toxic (A1). Accordingly, the production processes must be monitored. For recycled materials, concerning chemicals in circularity have been demonstrated in this thesis, which is supported by other reports (Leslie et al., 2016; Lowe et al., 2021). Regulatory actions should thus be prepared for such materials, so that material and chemical streams are 'clean' (Aurisano et al., 2021b). Because the entire plastic value chain is highly interconnected, it is indispensable that all actors come together to solve the global plastic waste problem (Wagner, 2022). Along this way, regulators could help to facilitate the transition to a circular economy. This may require governmental authorities to take a more precautionary than a risk-based approach (Coffin et al., 2021).

In conclusion, the vast amount of plastic waste in the environment cannot be fully recovered. It is thus important that all of the measures summarized above are taken seriously by the actors along the entire plastic value chain. Only as a collaborative effort, safe and sustainable uses that are equally recyclable can be developed and thereby would promote plastic emissions to be mitigated (Wagner, 2022). Therefore, safety, sustainability and circularity

(recyclability) aspects must be designed into the materials that are marketed globally. In this work, it was demonstrated that neither bioplastics nor recyclates performed better than conventional, virgin plastics in terms of their (eco)toxicity. For the moment, these materials are marketed as better alternative options to newly produced plastic materials, but they lack technological sophistication. Their supposedly environmental compatibility (especially for bioplastics) and greater sustainability (recycled plastics) in comparison to the virgin, fossil-based plastics is misleading. This does not mean that the production of fossil-based plastics should continue as is. However, bioplastics and recyclates are advertised as environmentally more conscious materials, when in fact every plastic material produced needs resources and thus cannot be sustainable *per se* (Freinkel, 2011). Detzel et al. (2012) already summarized that bio-based packaging is not more sustainable compared to fossil-based packaging. Moreover, this work showed that the plastic raw material (i.e., the pellets) can also not be classified as safe as the processed chemicals induced several *in vitro* responses. At the same time, it is noteworthy that toxic-free plastic materials exist as well. The design and production phases of such 'clean' materials should be used as a reference and could facilitate the transition towards a safer and circular system. After all, the toxicological safety is one of the key principles for such a type of economy to function successfully (Johansen et al., 2022). Designing an ideal plastic material would comprise all of the favorable features conventional plastic has, but the new materials should dematerialize regardless of the surrounding conditions and without leaving any toxic residues (Haider et al., 2019). Future research should improve the shortcomings of current plastic materials and their alternatives with emphasis on environmental and human safety, sustainability and circularity.

3 Conclusion

This PhD thesis provides the following conclusions (A1–A4):

- Plastics can leach some few or up to several thousands of chemicals, depending on the customized chemical composition of each plastic.
- The leaching chemicals induced several *in vitro* responses, including baseline toxicity, oxidative stress and endocrine activities, highlighting the variety of plastic chemicals.
- A categorization of the polymer toxicity is not reasonable because plastics of the same base polymer can result in different toxicities.
- The raw material of plastics (pre-production pellets) includes a concerning number of chemicals, which means that the initial stages of the plastic value chain require special attention in order to design safe materials.
- Plastics are chemical mixtures with known and unknown chemicals. The detected and identified substances were typical plastic chemicals such as functional additives, NIAS and degradation products.
- Bioplastics and recyclates are not better options for virgin, conventional plastics with specific regard to the chemicals included in such materials.
- Recycled plastics usually comprise more chemicals than virgin plastics, many of which can be hazardous accumulating substances.
- Toxicologically negligible plastics are available and sold on the market as well.
- UV-weathering exacerbated the plastic toxicity, but a degradation initiator is not really necessary for plastics to leach toxicants.
- The mean number and intensity of released chemicals from the plastics increased with the UV-weathering process.
- Plastics of low toxicity and chemical complexity can emit toxic chemicals after the UV-weathering process. Therefore, it should be guaranteed that plastic materials are safe during and after their lifetime, considering a potential emission to the environment.
- The physical toxicity of microplastics was less of a concern for the worm *L. variegatus*, but the chemicals from microplastics of a biodegradable bottle adversely affected the number and dry weight of the worms.
- Kaolin as the particle control was a biologically active control in a way that it positively affected the weight of the worms. This highlights that a particle reference control must be selected carefully based on the experimental conditions chosen.
- The microplastic size mattered for the shrimp *N. palmata* in terms of ingestion, but the particle shape seemed to be an irrelevant factor for the gut passaging.

- The examined endobenthic and epibenthic species seem to cope well with the physical properties of microplastics, which is not surprising given that benthic organisms are exposed to and also take up a high load of natural particles in the environment.
- Activities of plastic chemicals detected *in vitro* may but do not necessarily translate to substantial effects at the *in vivo* level.
- The swimming activity is an ecologically relevant endpoint but difficult to assess with regard to potential effects of microplastics because plastic leachates contain diverse chemicals that trigger different toxicity pathways.

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5 Annex

A1 Enhanced *in vitro* toxicity of plastic leachates after UV irradiation

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(2) Conducting tests and experiments	
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Enhanced *in vitro* toxicity of plastic leachates after UV irradiation

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ABSTRACT

Plastics can release numerous chemicals and thereby, contribute to the chemical pollution in aquatic systems. To which extent environmental degradation processes influence the release of plastic chemicals, is currently unknown and subject of research. We therefore evaluated aqueous leachates of 12 differently formulated plastics (e.g., pre-production, post-industrial and recycled pellets as well as final products) using *in vitro* bioassays and chemical analysis via LC-HRMS nontarget approach. We weathered these plastics by UV irradiation (UV-C and UV-A/B) under laboratory conditions in dryness and a subsequent leaching period in ultrapure water ('atmospheric' weathering) or directly in water ('aquatic' weathering, UV-A/B_{aq}). A dark control (DC) without UV light served as a reference treatment. Some plastics triggered several toxicological endpoints (low-density polyethylene recyclate (LDPE-R), starch blend (SB), bio-based polybutylene succinate (Bio-PBS) and polyvinyl chloride (PVC)), whereas others caused little to no effects (polyethylene terephthalate (PET), polystyrene (PS), polypropylene (PP) and LDPE). UV irradiation enhanced the plastics' toxicity, even for samples initially evaluated as toxicologically inconspicuous. The plastic samples caused oxidative stress (85%), baseline toxicity (42%), antiestrogenicity (40%) and antiandrogenicity (27%). Positive findings were measured after UV-C (63%) and UV-A/B_{aq} (50%) treatments, followed by UV-A/B (48%) and DC (33%). Overall, we detected between 42 (DC) and 2896 (UV-A/B_{aq}) chemical compounds. Our study demonstrates that differently formulated plastics leach toxic chemicals. UV exacerbates the plastics' toxicity by either generating active compounds and/or by facilitating their release. UV light even leads to the release of bioactive compounds from plastics of low chemical complexity. To prevent the exposure to plastic-associated chemicals, the application of chemicals could be reduced to a minimum, while on a regulatory level the evaluation of plastic eluates could be another focal point next to singular compounds.

1. Introduction

Plastic pollution is a global challenge as approximately 1.15–2.41 million tons annually enter the ocean through riverine systems (Lebreton et al., 2017). In the environment, plastics are subject to weathering such as UV irradiation, abrasion, hydrolysis and biofouling (Geyer et al., 2017; Jahnke et al., 2017). Degradation processes initiate the release of incorporated chemicals (e.g., plasticizers, antioxidants, stabilizers, slipping agents and residues) and small polymer units (i.e., monomers and oligomers) (Gewert et al., 2015; Hahladakis et al., 2018). Plastic-associated chemicals contribute likely to the existing chemical pollution (Persson et al., 2013), whereas low concentrations of e.g., plasticizers can induce adverse biological effects (Oehlmann et al., 2009; Teuten et al., 2009). In this context, our understanding for plastic-mediated effects has to be improved on a chemical level (Fauser et al., 2020).

Previous studies investigated the toxicity of virgin plastic pellets (Gandara e Silva et al., 2016; Nobre et al., 2015; Schiavo et al., 2018) and commercially available plastic products (Li et al., 2016; Lithner et al., 2009; Oliviero et al., 2019) for a variety of aquatic organisms. Adverse effects were generally attributed to leached chemicals from virgin (i.e., supposedly free from added chemicals) and processed or recycled (i.e., high additive content) plastics. However, only a few studies so far addressed the toxicological effects of leachates from UV-weathered plastic materials (Bejgarn et al., 2015), especially with *in vitro* bioassays (Coffin et al., 2018; Rummel et al., 2019; Yang et al., 2011). As a consequence, potential environmental hazards from UV-induced

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Table 1
Selected plastics for the experiments.

Type	Sample	Description
1	PP-H	pristine pellets (homopolymer)
	PP-C	pristine pellets (copolymer)
2	PET-A	pristine pellets (amorphous)
	PET-R	crystalline, post-consumer (recycled) pellets
3	PS-GP	pristine pellets (general purpose)
	PS-HI	pristine pellets (high impact)
4	LDPE	pristine, crystalline pellets
	LDPE-R	post-industrial (recycled) pellets
5	PVC-A	cuts of transparent plate (amorphous)
	PVC-R	cuts of sheet pile (recycled)
6	Bio-PBS	pristine pellets (bio-based and biodegradable)
	SB	pellets (petroleum-/bio-based and biodegradable)

plastic degradation products have been studied to a lesser degree (Gewert et al., 2018).

The present study focused on these aspects by examining *in vitro* toxicities of plastic leachates following artificial weathering scenarios. Therefore, we selected a diverse range of plastics including seven petroleum-based plastics, three recyclates as well as two biodegradable and fully or partly bio-based materials. The plastics were chosen dependent on their high production volume, increasing recycling rates (PlasticsEurope, 2019) and social perception as 'clean' alternatives. Because property-modifying chemicals are added to plastics derived from renewable feedstocks (Lambert & Wagner, 2017) and to reused plastics (Bodar et al., 2018; Pivnenko et al., 2016; Schyns & Shaver, 2020), examinations on adverse effects of such materials are just as important as for conventional plastics.

The main objective of this study was to evaluate the influence of UV on the leaching behavior of chemicals as integral components of plastic material and the related toxicological impact. We simulated weathering by either 'atmospheric' UV irradiation with a subsequent leaching period or directly under 'aquatic' conditions. Leached chemicals were concentrated and screened in *in vitro* bioassays to assess the baseline toxicity (Microtox assay), oxidative stress response (AREc32 assay) and antagonistic activities at nuclear sex hormone receptors (yeast-based reporter gene assays). Finally, we analyzed the released plastic-associated compounds by high performance liquid chromatography coupled to high-resolution mass spectrometry (LC-QTOF-MS).

2. Material and methods

2.1. Test materials

The following polymer types were chosen: polypropylene (PP), polyethylene terephthalate (PET), polystyrene (PS), low-density polyethylene (LDPE) and polyvinylchloride (PVC) as petroleum-based plastics; PET, LDPE and PVC were also tested as recyclates (R) and bio-based polybutylene succinate (Bio-PBS) and a starch blend (SB) as biodegradable plastics. The SB contains polybutylene adipate terephthalate (PBAT), thermoplastic starch, glycerin and polylactic acid (PLA) and thus, represents a petroleum- and bio-based material (Table 1). Each plastic category comprised two materials (Table 1), which were obtained from project partners as pellets (<5 mm), except for two PVC products (Fig. S1). The PVC samples (transparent plate and sheet pile) were cut to pellet-like pieces and thereby, increased in surface area to achieve similar leaching conditions. To record the leachable surface area, 100 pellets of every plastic sample were measured on each dimension (± 0.001 mm) using a stereo microscope (Olympus SZ40) and extrapolated to the tested mass (Table S1).

2.2. Artificial weathering

100 g of each plastic were evenly distributed in 2 L DURAN® crystallizing dishes (190 mm in diameter). All glass products were rinsed prior to use with acetone and annealed at 200°C for at least 3 h. Test vessels were placed in a chamber with ventilation holes, constructed with two UV lamps attached on each level (Fig. S2, Table S2). Under laboratory conditions, the test materials were irradiated for 24 h by either UV-C at 250 nm (treatment 2 (T2): worst-case scenario at 45.2°C as ambient temperature) or UV-A/B at 280–400 nm (T3: daylight scenario at 40.7°C) and subsequently leached for 24 h in 1.1 L of ultrapure water. Both treatments represent 'atmospheric' exposure conditions. To cover potential volatile compounds, the test materials were leached for 24 h in 1.1 L of ultrapure water during UV-A/B irradiation (UV-A/B_{aq} at 33.4°C) (T4: 'aquatic' scenario). A corresponding dark control (T1) was not irradiated (darkness at 23.9°C) but treated identically. Temperatures were recorded in 5-minute intervals using data loggers (HOBO Pendant®, Onset Computer Corporation, Bourne, USA) (Table S5); the listed temperatures relate to the temperature during the UV procedures. Since the UV-lamps were deactivated for the leaching procedure in T2 and T3, the temperatures naturally decreased (T2: 25.7°C and T3: 24.3°C). In the dark control, the temperature remained similar during the leaching (23.1°C). Procedural blanks with water only were included for each treatment to assess background contaminations. UV intensities (Radiometer RM-12, Opsytec Dr. Göbel GmbH, Ettlingen, Germany) accounted for 19.1 ± 2.3 W m^{-2} (UV-C) and 2.3 ± 0.3 W m^{-2} (UV-A, including 0.25 W m^{-2} of UV-B) (Table S3). According to Gewert et al. (2018), the applied UV-A irradiation simulated 8.16 h of European sun exposure (Table S4 in Supporting Information (SI)) and thus, displayed mild exposure conditions compared to Gewert et al. (2018). Leachates were then separated from test materials via Büchner funnels into Erlenmeyer flasks, of which 100 mL were taken and measured (pH and conductivity) with a portable multimeter (HQ40D, Hach Lange GmbH, Düsseldorf, Germany) (Table S6).

2.3. Solid-phase extraction

1 L of each leachate was acidified to pH 2.5 with sulfuric acid (3.5 mol L^{-1}) and enriched by solid-phase extraction (SPE) with Telos C18/ENV cartridges (Kinesis GmbH, Wertheim, Germany) to retain hydrophobic compounds (Abbas et al., 2019). Before sample loading, columns were conditioned with 2 mL heptane, 2 mL acetone, 6 mL methanol (pro analysis) and 8 mL ultrapure water. A SPE blank was prepared with 1 L ultrapure water. Columns were dried under a stream of nitrogen and eluted with 5 mL acetone and 5 mL methanol, of which 200 µL were stored at -20°C for chemical analysis. The remaining eluates contained 89.1 g of plastic-equivalents (EQs). 200 µL of dimethyl sulfoxide (DMSO) was added to the plastic eluates and concentrated under a stream of nitrogen to 200 µL as final extract volume. These 5000-fold concentrated extracts were stored at -20°C prior to testing.

2.4. In vitro *bioassays*

2.4.1. Microtox assay

The Microtox assay with the bioluminescent bacterium *Aliivibrio fischeri* assessed the baseline toxicity (ISO 11348-3, 2007). The assay was performed according to Völker et al. (2017) with minor modifications. Control samples (negative and solvent controls, SPE blank, procedural blanks), a reference compound (3,5-dichlorophenol; Table S7, Fig. S3a) and plastic extracts were tested in duplicates, reaching 1% (v/v) as solvent concentration per well. Considering a 0.30 to 37.5-fold enrichment, the tested plastic extracts are equivalent to 5.22–668 mg plastic. For a better under-

standing, the results were expressed in plastic-EQs, where 1 mg of plastic-EQs corresponds to all leachables from 1 mg of plastic pellets. In total, three to four independent experiments were conducted.

2.4.2. AREc32 assay

The AREc32 assay with the human breast cancer cell line MCF7 assessed the oxidative stress response, i.e., the cellular detoxification mechanism (Wang et al., 2006). The cell line, obtained from Signosis Inc. (Santa Clara, CA, USA), included the Nrf2-ARE signaling pathway coupled to a luciferase reporter gene. Following the procedure described by Völker et al. (2017), the assay was conducted with minor modifications. Control samples (comp. 2.4.1, reference compound (*tert*-butylhydroquinone); Table S7, Fig. S3b) and plastic extracts were examined in duplicates per independent experiment (three to five in total), not exceeding a final solvent concentration of 0.5% (v/v). The plastic samples corresponded to 1.74–222 mg plastic-EQs, which resulted in 0.10 to 12.5-fold concentrated extracts.

2.4.3. Yeast-based reporter gene assays

Yeast-based reporter gene (*lacZ* encoding for β-galactosidase) assays were conducted to assess the endocrine activity. As preliminary screens revealed few estrogenic and androgenic findings, but resulted in high antagonistic activities, we examined the antagonistic activity at the human estrogen receptor α (hERα) (Routledge & Sumpter, 1996) and human androgen receptor (hAR) (Sohoni & Sumpter, 1998), respectively. Therefore, the Yeast Anti-Estrogen Screen (YAES) and Yeast Anti-Androgen Screen (YAAS) were conducted as in Abbas et al. (2019) with minor modifications. Cell densities were adjusted to 250 ± 25 (YAES) and 500 ± 50 (YAAS) of formazine attenuation units. Assays were performed in 96-well plates with eight technical replicates for each sample (controls: comp. 2.4.1, reference compounds: 4-hydroxytamoxifen (YAES) and flutamide (YAAS); Table S7, Fig. S3c, d), not exceeding 0.2% (v/v) as solvent concentration. The plastic samples contained 111 mg plastic-EQs; this means that 6.2-fold concentrated extracts were tested. To detect antagonistic activities, 17β-estradiol (YAES) and testosterone (YAAS) were added as agonists to the medium. Hence, antagonistic compounds such as antiestrogens or antiandrogens inhibit the corresponding agonist from receptor activation and reduce the reporter gene activity. In total, three to five independent experiments were conducted.

2.5. Data analysis

To derive concentration-response curves and effect concentrations (ECs), nonlinear regressions were performed using a four-parameter logistic function (GraphPad Prism® 5 and 8, GraphPad Software Inc., San Diego, CA, USA). Cytotoxic samples were generally excluded from analysis and independent experiments were only considered for analysis when the half maximal response (EC_{50}) was next to the listed concentrations in Table S7.

For the Microtox assay, the luminescence inhibition of procedural blanks was subtracted from the respective samples. EC_{20} values were derived for samples exceeding the 20% inhibition threshold and describe the plastic-EQ inducing this inhibition. Therefore, the minimum and maximum effect was constrained to 0 and 100% inhibition, respectively. For the AREc32 assay, solvent controls and procedural blanks were pooled due to no significant differences (Kruskal-Wallis test followed by Dunn's multiple comparison test, $\alpha = 0.05$). The minimum effect was constrained to an induction ratio (IR) of 1 and derived $EC_{IR1.5}$ values refer to the plastic-EQ inducing a 1.5-fold elevated signaling pathway. For yeast screens, antagonistic activities were normalized to a minimum and maximum effect, represented by a control with agonist (0% receptor inhibition) and without agonist (100% receptor inhibition). Negative and solvent controls as well as procedural blanks were pooled as described before and used to derive the limit of detection (LOD). Activities above the LOD, calculated with pooled controls plus three times the standard deviation, were considered significant. To identify toxicity patterns, data were normalized as relative activities and compared in a heat map. Therefore, the highest EC value from either the Microtox or AREc32 assay corresponded to an activity of 0%, while 100% was set to 0.0001 mg plastic-EQs.

2.6. Chemical analysis

The SPE eluates (section 2.3) were measured via HPLC-MS/MS as described previously in detail (Nürenberg et al., 2015). Analysis was performed using a TripleTOF 6600 (SCIEX) coupled via an ESI source to a binary HPLC instrument (1260 Infinity, Agilent) equipped with a reversed phase C18 column (Zorbax Eclipse Plus, 2.1 mm x 150 mm, 3.5 µm, Agilent). As eluent a water-acetonitril gradient was used, buffered with 0.1vol% formic acid at a flow of 300 µL min^{-1}. The method used both ESI(+) and ESI(-) ionization mode (scan mode 100–1200 Da). Samples were diluted 1:1 with ultrapure water and injected at 50 µL. The data analysis was processed according a non-target approach as described by Jewell et al. (2020) and Köppe et al. (2020). After data acquisition, peak picking, componentization, alignment of the components and blank correction, the components and their intensity were summed for each sample and ionization mode. The data are summarized in Table S9. The total count and intensity of all detected components were normalized according to the highest and lowest values and displayed in the heat map. The intensity is used as an indicator for the approximate amount of chemicals in one sample, derived from the most intense feature of the component.

3. Results

3.1. Microtox assay

The SPE blank induced negligible luminescence inhibitions with 4.40 ± 1.55% (Table S8). Solvent controls did not inhibit the luminescence, resulting in non-derivable EC_{20} values (not listed in Table S8). Procedural blanks as background contaminations were subtracted from the respective samples (Table S8). Seven out of the 12 plastics induced baseline toxicity (Fig. 1). PVC (PVC-A, PVC-R) and PS samples (PS-GP, PS-HI) as well as the Bio-PBS, SB and the LDPE-R resulted in low EC_{20} values (high effect) (Fig. 1, Fig. 5). Efficacies ranged from the lowest effect with 341 ± 90.1 mg (T2: PS-GP) to the highest effect with 7.32 ± 3.59 mg (T2: LDPE-R) (Fig. 1, Table S8). The LDPE-R induced the highest observed effect in the Microtox assay. Both biodegradable plastics and the LDPE-R were the most potent plastics for every weathering treatment (Fig. S4). The toxicities generally depended on the applied weathering condition. For instance, UV-C provoked elevated toxicities for PS samples, whereas no effect was observed in the dark control (Fig. 1, Fig. 5). No observed effects correspond to 750 mg plastic-EQs and were also found for PP-H, PP-C, PET-A, PET-R and LDPE (Fig. 1, Fig. 5). The toxicity order for treatments was the following in the Microtox assay: UV-C > UV-A/B and UV-A/B_{aq} > DC (Fig. S5). Moreover, we observed that this assay had one of the highest relative positive findings (Fig. S5).

3.2. AREc32 assay

Few samples did not activate the Nrf2-ARE signaling pathway, which corresponds to 250 mg plastic-EQs. The SPE blank (not displayed in Fig. 2) induced the oxidative stress response with 206 ±

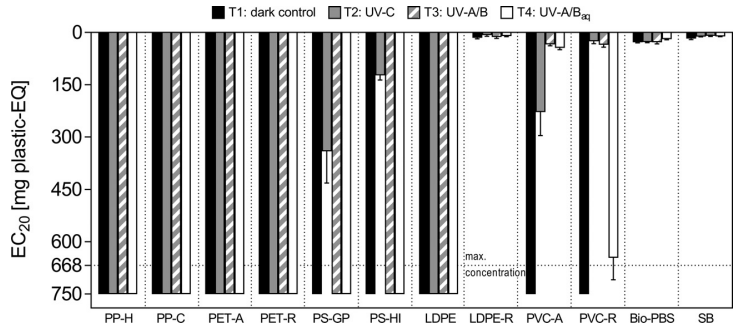

Fig. 1. Microtox assay. Baseline toxicity (mean EC_{20} ± SEM) of plastic extracts [mg plastic-EQ]. 668 mg plastic was the maximum tested concentration. 750 mg indicates that extracts did not exceed 20% luminescence inhibition.

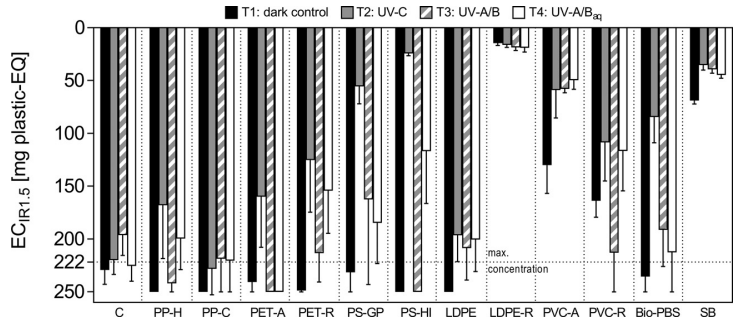

Fig. 2. AREc32 assay. Oxidative stress response (mean $EC_{IR1.5}$ ± SEM) of plastic extracts [mg plastic-EQ]. 222 mg plastic was the maximum tested concentration. 250 mg indicates that extracts did not induce a 1.5-fold elevated signaling pathway. C = control.

20.6 mg (Table S8). Similar minor inductions can be observed for pooled background contaminations and solvent controls (abbreviated C) from 229 ± 13.6 (T1) to 196 ± 19.3 mg (T3) (Fig. 2). Compared to the Microtox assay, the response is more differentiated in the human cell line (Fig. 2, Fig. 5). Every plastic material but not every tested weathering scenario induced a 1.5-fold elevated response. For instance, specific weathering types induced no effects for six out of 12 plastics (e.g., for T3 and T4: PET-A or T1 and T3: PS-HI). The most potent samples were LDPE-R (T1: 14.7 ± 2.11 mg) and SB (lowest $EC_{IR1.5}$ in T2: 35.2 ± 4.90 mg), followed by PVC-A (T4: 49.6 ± 8.72 mg) (Fig. 5, Table S8). As in the Microtox assay (Fig. S4), LDPE-R and SB again induced the highest observed effects. Overall, the weathering toxicity resulted in the following order for the AREc32 assay: UV-C > UV-A/B_{aq} > UV-A/B > DC. This assay had the highest rate of positive findings (Fig. S5).

3.3. Yeast-based reporter gene assays

In the yeast anti-estrogen screen, the SPE blank inhibited the human estrogen receptor hERα by 7.41 ± 1.41% (not displayed in Fig. 3, see Table S8). Pooled controls had minor effects with 1.56 ± 0.74% inhibition (T2: C) (Fig. 3, Table S8). Samples >20.2% (LOD) of antiestrogenicity were considered as significant (Fig. 3). Every plastic material inhibited the receptor and thus, leached antiestrogenic compounds (Fig. 3, Table S8). Significantly increased activities were observed for seven out of the 12 plastics (i.e., LDPE-R, PVC-A, SB, Bio-PBS, PVC-R, PS-HI and PS-GP) for either every weathering scenario (LDPE-R and Bio-BPS) or only specific treatments. The strongest antiestrogenic activity was observed for T3: LDPE-R with 75.4 ± 1.97% (Fig. 3, Table S8). Comparable inhibitions were achieved with the PVC-A (T2: 62.0 ± 1.85%) and PVC-R sample (T2: 54.2 ± 2.23%) (Fig. 3, Table S8). In general, UV-C irradiation (T2) led to pronounced effects for eight out of 12 plastics (Fig. 5), resulting in the following weathering toxicity order in the YAES: UV-C > UV-A/B > UV-A/B_{aq} and DC (Fig. S5). The YAES resulted in a lower positive finding rate than the Microtox and AREc32 assay.

Regarding the yeast anti-androgen screen, we observed inhibitions of the human androgen receptor hAR for the SPE blank with 13.0 ± 2.52% (not displayed in Fig. 4, see Table S8). Here, activities >31.3% (LOD) were considered significant. Pooled background contaminations and solvent controls (C) had their highest impact for T3: 2.91 ± 1.64% (Fig. 4, Table S8). All plastic samples inhibited the hAR (Fig. 4), but activities depended on the applied weathering scenario. The following samples led to a significant increase in antiandrogenic activity: LDPE-R, SB, Bio-PBS, PVC-A, PS-HI and PS-GP (Fig. 4, Table S8). Hence, six out of 12 plastics resulted in significant antiandrogenicity. Compared to the YAES (Fig. 3), we observed lower activities for PVC samples (Fig. 4). High activities were again detected for LDPE-R and SB (Fig. 5). Here, UV-A/B irradiation during leaching (T4) revealed to be the most affecting treatment with 96.2 ± 0.44% for LDPE-R and 86.3 ± 0.11% for SB (Fig. 5, Table S8). In contrast to the YAES (Fig. 3), only four plastics revealed pronounced antiandrogenic activities after UV-C irradiation (Fig. 5). The toxicity order for the treatments in the YAAS is as follows: UV-C and UV-A/B_{aq} > UV-A/B > DC (Fig. S5). Finally, we observed the lowest rate of positive findings in the YAAS (Fig. S5).

3.4. Chemical analysis

The results of the screening via LC-QTOF can be used to get an overview about the chemical complexity of the leachates and for a rough estimation of the released amounts. Due to high differences in detector response for different molecules in LC-MS analysis, a comparison of particular plastic samples is very limited. However,

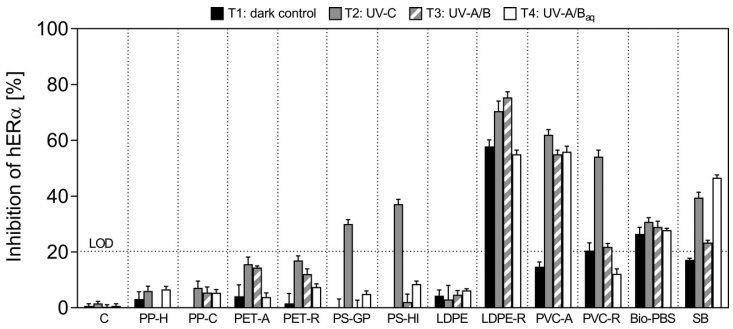

Fig. 3. Yeast anti-estrogen screen. Relative inhibition of the human estrogen receptor hERα (mean ± SEM) of plastic extracts. 111 mg was the maximum tested concentration. Antiestrogenic activities above 20.2% (LOD) were considered as significant. C = control.

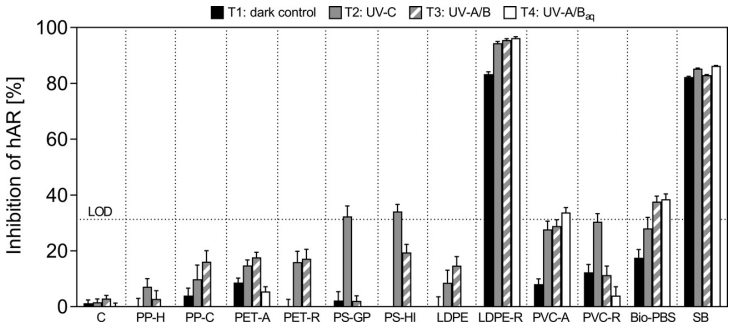

Fig. 4. Yeast anti-androgen screen. Relative inhibition of the human androgen receptor hAR (mean ± SEM) of plastic extracts. 111 mg was the maximum tested concentration. Antiandrogenic activities above 31.3% (LOD) were considered as significant. C = control.

the data revealed obvious differences in leaching from the particular plastic samples and UV induced changes. Fig. 5 illustrates the component counts and intensities of the plastic eluates after subtraction of the procedural blanks. When combining the positively and negatively ionized components, we detected between 42 (T1: PP-H) and 2896 (T4: LDPE-R) compounds (Table S9). LDPE-R, both biodegradable plastics and PVC samples contained the highest counts. PET, PS, PP samples and the LDPE released fewer components (e.g., 855 for T2: PET-R and 410 for T2: PS-HI). In general, we detected a higher count for positively ionized than for negatively ionized components, except for the Bio-PBS and LDPE (Table S9). Moreover, LDPE-R, Bio-PBS and SB showed the highest intensities (Fig. 5, Table S9). A clear influence by UV irradiation could not be observed for these plastics. However, other plastics such as PVC-A and the PET-A and PET-R showed a strong increase in intensities after artificial weathering (Table S9).

4. Discussion

4.1. Toxicity of plastics not subject of irradiation

In the dark control (T1), we identified toxicological activities for five out of 12 plastics (i.e., the LDPE-R, SB, Bio-PBS, PVC-A and PVC-R) (Fig. 5). This highlights that UV light as an initiator of degradation is not an essential prerequisite for plastics to release toxic chemicals. One of the most striking finding is the high toxicity resulting from SB and Bio-PBS, indicating that biodegradable plastics emit active chemicals as well. However, this does not comply with the expectation that degradation products of biodegradable plastics should not exhibit any ecotoxicity (Lambert & Wagner, 2017). In this study, the SB sample induced *in vitro* activities comparable to the most potent non-biodegradable sample (LDPE-R) and thus, was one of the most toxic samples. Both the Bio-PBS and SB are either fully (Bio-PBS) or only partly (SB) derived from renewable feedstock and are both biodegradable. Since such plastics are usually deployed as foils in agriculture, they are usually examined in terms of seed germination. Thus, cell culture studies are rare (Souza et al., 2020). Moreover, 'bio'-associated plastics (i.e., bio-based and/or biodegradable) often comprise blends of several materials, making a toxicological evaluation difficult. The SB sample, for instance, represents a blend of polybutylene adipate terephthalate (PBAT), thermoplastic starch, glycerin and polylactic acid (PLA). The latter can be used as a contact material (e.g., for foodstuff) (Farah et al., 2016) and has been investigated more often than other 'bio'-associated plastics. For instance, Zimmermann et al. (2019) and Yang et al. (2011) used harsh extraction methods and linked PLA products to baseline toxicity and estrogenicity, respectively. Corn-starch based foils were found to stimulate the bioluminescence of *Aliivibrio fischeri*, although the significant stimulant level was not exceeded (Sforzini et al., 2016). Furthermore, Souza et al. (2020) observed decreased cellular viability for HepG2/C3A cells, when exposed to PBAT extracts. Our Bio-PBS sample induced high baseline toxicity as well as medium antiestrogenic and antiandrogenic responses (Fig. 5). In contrast to specific compounds binding to nuclear receptors (e.g., endocrine disruptors), the bacterial and human cell assays are both sensitive to many stressors. The oxidative stress response, however, can be induced selectively as well (Escher et al., 2014). Therefore, it is not surprising that our Bio-PBS mostly affected one unspecific endpoint. Plastics active in the Microtox assay did not necessarily translate to effects in the AREc32 assay and vice versa (Fig. 5). Moreover, Toso et al. (2017) assessed PBS foils with cultured T-lymphocytes as non-toxic. Zimmermann et al. (2020) recently surveyed a broad range of 'bio'-associated plastics and assigned high

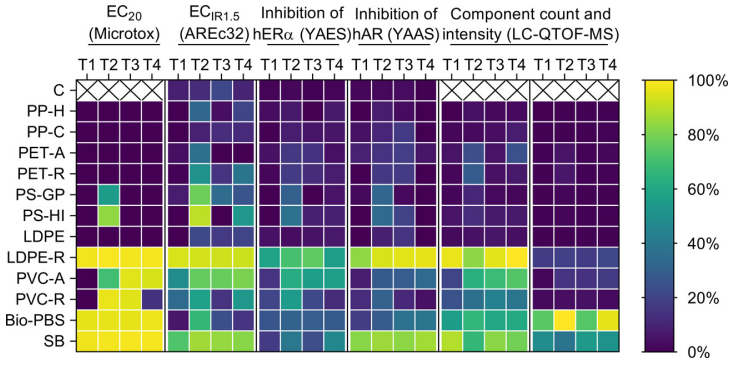

Fig. 5. Relative toxicological activities of all *in vitro* bioassays and detected chemical components (counts and intensities) as heat map. Crossed cells indicate corrected samples. T1 = dark control, T2 = UV-C, T3 = UV-A/B, T4 = UV-A/B_{aq}, C = control.

baseline toxicity as well as antiandrogenicity to materials based on starch, while extracts of PLA, PBS and PBAT resulted in varied toxicities. Overall, these results suggest that the toxicity depends on the entirety of all processed chemicals, i.e., (non-)intentionally added substances, in each plastic material (Groh et al., 2019; Lambert & Wagner, 2017). In view of the biodegradability of Bio-PBS and SB, it could be argued that antagonistic activities were affected by dissolved organic carbon (DOC) as an interfering matrix (Neale et al., 2015). Following this reasoning, both the Bio-PBS and SB should have yielded alike antagonistic activities but this was not the case (e.g., comp. the response of SB in Fig. 3 and Fig. 4). Hence, the DOC is probably but not certainly negligible.

In addition, we identified LDPE-R and the PVC samples (PVC-A and PVC-R) to cause elevated activities. The recycled LDPE resulted in high activities across all bioassays (Fig. 5). Since recyclates can contain significantly higher amounts of hazardous chemicals (comp. Dreolin et al., 2018) and our LDPE-R pellets comprised various colors and forms (Fig. S1), we expected elevated *in vitro* activities due to composite LDPE materials. Along that line, Li et al. (2016) found leachates of recyclable PVC and LDPE consumer products to be toxic for the barnacle larvae *Amphibalanus amphitrite*. PVC leachates were determined as ecotoxicologically relevant as well by Lithner et al. (2012, 2009), Oliviero et al. (2019) and Tetu et al. (2019). In our study, the PVC samples slightly induced oxidative stress (Fig. 2, Table S8), suggesting that PVC contains electrophilic chemicals or chemicals generating reactive oxygen species (Escher et al., 2013). Zimmermann et al. (2019) identified consumer products made from PVC as one of the most potent plastics that induced baseline toxicity, oxidative stress as well as estrogenic and antiandrogenic activities. Our findings further demonstrate that PVC can induce other specific receptor-mediated effects such as antiestrogenicity (Fig. 3). These observations are not surprising because plasticizers and stabilizers are used in high amounts for PVC (Groh et al., 2019; Hahladakis et al., 2018).

4.2. Toxicologically less relevant plastics

Focusing again on the leachables from the dark control (T1), the following samples exhibited no activities almost across all bioassays: PP-H, PP-C, PET-A, PET-R, PS-HI, PS-GP and LDPE (Fig. 5). In the present study, these samples represent plastics of comparably lower chemical content. PP, PET, PS and LDPE are polymers that are commonly used as food packaging materials (PlasticsEurope, 2019) and are thus in contact with human food. The European Union regulates (EU Commission, 2011) certain substances for the production of plastic materials and such in contact with food (Muncke et al., 2020), suggesting that these materials do not contain hazardous chemicals. While Zimmermann et al. (2019) somewhat support this by reporting less but not complete absence of toxicological activities for such plastic products, they also describe that a generalized toxicological statement is not feasible for these materials because their toxicities varied. Yang et al. (2011) provided similar results. PET and PP products exhibited estrogenicity in the E-Screen (MCF7 cells), whereas LDPE and the moiety of PS did not contain estrogen-like compounds. This means in effect that food contact plastics contain concerning chemicals and indicates that regulatory enforcements are not as strict as preferable (comp. Daniel et al., 2018). After all, non-intentionally added substances can only be limitedly assessed and leaching chemical mixtures are currently not taken into account by EU regulations (Muncke et al., 2020). Furthermore, the polymer types discussed in this section can also be used for other applications, e.g., as PP pipes or PS insulation (PlasticsEurope, 2019). Mertl et al. (2014), for instance, determined antagonistic activities for PP granulate intended for use as water pipes. Schiavo et al. (2018) did not detect significant luminescence inhibitions for *A. fischeri* following exposure to virgin PE and PS pellets, whereas minor inductions were observed in the AREc32 and PPARγ assay for milled (<350 µm) PE and PS pellets (Rummel et al., 2019). This highlights once again that the activities depend on the customized chemical composition of each plastic material. Nonetheless, we also demonstrate that seven materials did not release harmful chemicals after the dark control. This could be simply attributed to the mild extraction (i.e., with water and without irradiation) in the reference treatment (T1).

4.3. Complex plastic formulations

We further assumed that the plastic formulation (i.e., additive content) plays an important role when evaluating plastic toxicity. As more and more chemicals are added along the production chain (Real et al., 2015), it could be more likely to detect toxicological activities in final products rather than in pre-production pellets (Rummel et al., 2019; Zimmermann et al., 2020). However, for the present study such a comparison is not advisable because our materials only included two final plastic products and various plastic formulations. We still expected the recyclates to result in higher toxicities than their counterparts since previous studies described unintentional accumulations of hazardous chemicals in plastic recyclates (Fatunsin et al., 2020; Ionas et al., 2014; Pivnenko et al., 2017, 2016; Turner, 2018). As this was true only for the LDPE-R, which represents post-industrial cuttings from manufactured plastic products (Table 1, Fig. S1), this underpins that the recyclates chemical composition depends on the waste

source (Pivnenko et al., 2016). PVC-R, for instance, released bioactive chemicals but displayed mitigated activities compared to PVC-A (Fig. 5). Moreover, we found no baseline toxicity (Fig. 1) and insignificant antagonistic activities for the post-consumer PET-R (Fig. 3, Fig. 4), even though recycled PET bottles have been already determined as a source for the reentrance of endocrine disruptors (Lee et al., 2014). Dreolin et al. (2018) even demonstrated that six out of eight recycled PET pellets contained more than 1 µg of the estrogen-mimicking substance bisphenol A per gram of the examined samples.

4.4. Plastic toxicity after weathering

UV irradiation combined with high ambient temperatures initiates polymer degradation (Andrady et al., 1998), which results in the release of plastic-associated chemicals (Groh et al., 2019) and formation of newly emerging degradation products (Gewert et al., 2018). As already described (see 4.1 and 4.2), UV light as an initiator for degradation is not necessary to detect *in vitro* toxicities (comp. dark control). However, the toxicities were generally enhanced after UV application, e.g., from 57.8% (T1) to 70.5% (T2) of antiandrogenicity for LDPE-R (Fig. 4, Table S8). New activities emerged as well due to UV irradiation (e.g., for PS samples across all bioassays) (Fig. 5), highlighting that even polymeric formulas consisting of low chemical complexity (see Table S9) can release or form toxic chemicals. Our findings are not unexpected but demonstrate the importance of weathering in the environment with regard to the chemical pollution originating from plastics. Yang et al. (2011) determined UV light as a driving force to release estrogen-like compounds. Coffin et al. (2018) support this as they detected higher estrogenic and dioxin-like activities for irradiated virgin plastics than for untreated samples. Rummel et al. (2019) demonstrated as well that most UV-treated plastics exhibited increased oxidative stress compared to a reference treatment. Aside from these *in vitro* studies, weathering also exacerbated the plastics' toxicity in *in vivo* tests (e.g., for virgin and mixed plastics) (Coffin et al., 2018; Xu et al., 2020). Bejgarn et al. (2015), for instance, exposed *Nitocra spinipes* to UV-irradiated plastic leachates and detected increased as well as decreased toxicities after UV irradiation. Sarker et al. (2020) reported as well that two environmentally weathered products, i.e., PVC matting and HDPE grocery bag, affected *Prochlorococcus*, but the bacterial growth was less inhibited compared to the treatment with unweathered plastics. This was observed once in our study. Fig. 2 illustrates a minor reduction of the $EC_{IR1.5}$ for LDPE-R following UV application (from 14.7 mg (T1) to 19.0 mg (T4) plastic-EQs, Table S8). Interestingly, a corn starch and aliphatic polyester based biodegradable bag was significantly more toxic than prior to irradiation (Bejgarn et al., 2015), which is in line with our findings for the SB sample.

Furthermore, we observed elevated inductions after specific weathering types. Most findings stem from UV-C (T2), followed by UV-A/B_{aq} (T4), UV-A/B (T3) and the dark control (T1) (Fig. S5). Based on the literature, it is not really surprising that UV light enhanced the plastics' toxicity. However, it is noteworthy that minor plastic-EQs already induced concerning effects, i.e., either less than 668 mg and 222 mg in the Microtox and AREc32 assay or 111 mg in the antagonistic assays. To give an example, 11.7 mg $well^{-1}$ (T4) of LDPE-R EQs affected 20% of the bacteria in the Microtox assay, while the same effect was achieved with 0.64 µg $well^{-1}$ of 3,5-dichlorophenol as positive control. This corresponds to 4.26 mg of 3,5-dichlorophenol L^{-1}, which is harmful to aquatic life (NORMAN network, 2021). Hence, we believe that this assessment of leachable plastic chemicals is somewhat mirroring the current ongoing process in the environment, considering the million tons of plastics annually polluting the aquatic systems (Lebreton et al., 2017). Since the chemical release can be related to the UV intensity (Andrady et al., 1998), temperature (Yang et al., 2011) and/or surface area of the material (Muncke et al., 2020) in contact with a specific type of solvent (Szczepańska et al., 2016), detectable components and toxicological findings may vary depending on the extraction method used. Our experiments represent mild exposure scenarios due to the UV intensity (Table S2 and Table S3 in SI) and short contact time with a soft medium (i.e., 24 h in water). Because the 'atmospheric' UV irradiation led to elevated temperatures (T2: 45.2 and T3: 40.7°C), after which we noticed yellowed samples and a strong synthetic smell, the toxicities might be closely linked to these temperatures (Table S5). Tsochatzis et al. (2020) depicted that temperature (i.e., 20, 40 and 60°C over a time period of 10 days) had a substantial effect on the release of two compounds typically found in food contact materials. Likewise, Yang et al. (2011) found more detectable estrogenic activities for samples following moist heat treatments (e.g., via autoclave process). In the present study, both the temperature and UV light have to be considered as two influencing factors on the chemical migration. However, Bandow et al. (2017) tested the release rates of (in)organic compounds of recycled plastic pellets or disks and has shown that photo-oxidation has a greater influence on chemical release than thermo-oxidative conditions (i.e., a high temperature). Cai et al. (2018) demonstrated in a similar methodological design that pellets weathered under 'atmospheric' conditions were more prone to degradation than in aqueous medium. The same is true for our weathering scenarios. We observed mitigated toxicological activities for T4 (UV-A/B_{aq}) in comparison to T2 (UV-C) (Fig. S5). This might be attributed to the water diffusing UV light through absorption and reflection. UV-C irradiation, serving as a worst-case scenario, on the other hand probably leads to toxic transformation products due to the high energetic radiation. Since previous studies deliberately increased the plastics' surface area for leaching purposes (Bejgarn et al., 2015; Rummel et al., 2019), we statistically analyzed whether the surface area (Table S1) correlates with the observed toxicities. Our data do not support that a greater surface area leads to a higher toxicity but rather that the effects depend on leachable toxicants present in individual plastic samples. Admittedly, comparing pellets and milled fragments could be more conclusive (comp. Ye et al., 2020).

4.5. Diversity of chemical components

This study aimed to elucidate whether plastic chemicals are increasingly released after UV weathering and whether these chemicals are interlinked with the observed toxicities. Due to weathering, we observed increased component counts for almost every sample. However, the SB leached fewer chemicals after each UV treatment, whereas the LDPE-R released fewer chemicals solely after 'atmospheric' weathering (Fig. 5, Table S9). Most of these chemicals are low-molecular fragments (exemplified in Fig. S6 for PVC-A), which are formed to a higher extent due to degradation. As a result, the number and amount of components generally cluster after the UV treatments (Table S9). This is in line with the toxicological findings (Fig. S5), e.g., UV-C as a worst-case scenario revealed most positive findings. In view of the chemicals, we cannot narrow down the main driver for the toxicities as this would be only feasible via effect-directed analysis. Although we did not majorly focus on chemical identification, future studies should address this in order to determine yet unknown substances responsible for adverse effects. Our chemical data are generally in accordance with other studies, considering analytical differences. Using LC-Orbitrap-MS, Gewert et al. (2018) detected similarly low counts for PP, PS and PE pellets, while PET pellets (Gewert et al., 2018) as well as bottles (Wagner et al., 2013) leached higher chemical counts. Bradley and Coulier (2007) as well as Qian et al. (2018) detected more chem-

icals for PP than in our study (PP-H: 41 (T1), Table S9). However, they deployed either a wide variety of techniques in order to detect (semi-)volatile, polar and/or non-volatile substances (Bradley & Coulier, 2007) or a large selection of plastics (35 PP products in Qian et al., 2018). Interestingly, Bradley & Coulier (2007) ascribed the majority of detected chemicals in the examined high commodity plastics to impurities and breakdown products. As for PVC and 'bio'-associated plastics, Zimmermann et al. (2019) obtained low to high chemical counts for extracts from PVC products analyzed with GC-QTOF-MS. Zimmermann et al. (2020) further employed an analytical system comparable to our study and observed between 880 and 20,965 chemicals for PLA, PBS, PBAT and starch-based materials.

A detailed identification and classification of the plastic-associated chemicals was beyond the scope of our study. We still identified some of the detected components (not displayed) with the attribute tentative, confident or confirmed. These included stabilizers (e.g., benzophenone, benzothiazoles, benzotriazoles), plasticizers (e.g., *n*-butylbenzenesulfonamide, acetyltributylcitrate), organophosphorus compounds (e.g., dibutyl and tributyl phosphates), antioxidants (e.g., Irganox 1098, benzoquinone, fenozan), different benzoic acids and 1,3-diphenylguanidine. Most of these compounds occurred almost after every treatment, while a small fraction was specific to one treatment. We further identified many degradants, especially for the biodegradable plastics. The majority of detected compounds still remained unknown, although this might be attributed to the depth of the chemical analysis. In the main, the chemical analysis demonstrated that plastics comprise low and high chemical contents and that chemicals are increasingly released due to initiating degradation processes for example by UV light and hydrolysis. Overall, this hampers a proper risk assessment for aquatic organisms because new degradation products are continuously formed. Even if degradants would not be an issue, there are many registered chemicals publicly not disclosed (i.e., over 50,000 chemicals as analyzed by Wang et al., 2020); this lack of transparency applies likewise for chemicals used in plastics and needs to be improved urgently (Groh et al., 2019). However, this also puts the application of diverse chemicals into question as toxicologically rather less harmful polymeric formulas already exist.

5. Conclusions

Our results demonstrate that differently formulated plastics include chemicals inducing baseline toxicity, oxidative stress and antagonistic activities. Some plastics affected several *in vitro* endpoints, whereas others caused little to no effects. The toxicities varied even for the same polymer type. As a result, we cannot attribute a toxicological profile to a specific polymer type because activities depended on the customized chemical composition of each plastic. Moreover, we demonstrate that UV irradiation exacerbated the plastics' toxicities, even for plastics initially evaluated as toxicologically harmless. The 'atmospheric' scenario with UV-C irradiation had the strongest impact, followed by 'aquatic' weathering (UV-A/B_{aq}), the 'atmospheric' UV-A/B and the dark control (DC) as reference. A high number of chemical components was detected in the aqueous leachates by non-target analysis. Whether they were toxic or not, every tested plastic sample released chemicals. Such were increasingly observed due to UV irradiation, whereas most chemicals were detected after UV-A/B_{aq}. In general, we exemplified the chemical heterogeneity in polymeric formulations (e.g., for plastic pellets as well as products) and showed that UV light facilitates the chemical release. It is noteworthy that we only assessed some (un)specific mediated effects with bacterial, human and yeast cells, but chemicals in plastics may affect health in ways that are currently not yet fully understood. In order to prevent the exposure to plastic-associated chemicals, mitigation of release of chemicals should be addressed during product development but the application of chemicals could be generally reduced to a minimum. On a regulatory level, the testing of singular compounds is of specific concern. Since our chemical data point to mixture effects, we believe that the evaluation of plastic eluates could be another important focal point.

Declaration of Competing Interest

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

CRediT authorship contribution statement

Kristina Klein: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Delia Hof:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Andrea Dombrowski:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Peter Schweyen:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Georg Dierkes:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Thomas Ternes:** Supervision, Writing - review & editing. **Ulrike Schulte-Oehlmann:** Conceptualization, Supervision, Writing - review & editing. **Jörg Oehlmann:** Conceptualization, Supervision, Writing - review & editing.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117203.

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A2 Chemicals associated with biodegradable microplastic drive the toxicity to the freshwater oligochaete *Lumbriculus variegatus*

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Chemicals associated with biodegradable microplastic drive the toxicity to the freshwater oligochaete *Lumbriculus variegatus*

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ABSTRACT

Microplastics (MPs) as complex synthetic pollutants represent a growing concern for the aquatic environment. Previous studies examined the toxicity of MPs, but infrequently used a natural particle control such as kaolin. The cause of toxicity, either the physical structure of the particles or chemical components originating from the MPs, has rarely been resolved. Moreover, the ecotoxicological assessment of biodegradable plastics has received little attention. To narrow down the main driver for toxicity of irregular biodegradable MPs, we conducted a series of 28-days sediment toxicity tests with the freshwater oligochaete *Lumbriculus variegatus* and recorded the number of worms and dry weight as endpoints. Therefore, MPs containing several biodegradable polymers were either mixed with the sediment or layered on the sediment surface with concentrations from 1 to 8.4% sediment dw^{-1}. Kaolin particles were evaluated in parallel as particle control. Furthermore, aqueous leachates and methanolic extracts as MP equivalents as well as solvent-treated, presumably pure MPs were investigated after mixing them into the sediment. Our results reveal that MP mixed with the sediment induced stronger adverse effects than layered MP. Kaolin particles caused no adverse effects. In contrast, they enhanced dry weight in both applications. The impact of aqueous leachates was comparable to the control without MPs, whereas methanolic extracts affected the worm number at the highest concentration with 100% mortality. Solvent-treated, presumably pure MP resulted in mostly higher worm numbers when compared to untreated MPs mixed into the sediment. This study demonstrates that MPs mixed into the sediment affect *L. variegatus* more than MPs that are layered on the sediment surface. Kaolin as a natural, fine-sized particle control created somewhat favorable conditions for the worm. The main driver for toxicity, however, proved to be chemicals associated with the plastic product and its previous content.

1. Introduction

Because plastic waste is often inadequately disposed, plastic pollution is of increasing environmental concern, especially for freshwater ecosystems (Best, 2019). Annually, up to 10 million tons of plastic debris accumulate in river catchments (Schmidt et al., 2017). Irregularly shaped microplastics (MPs), defined as 1–1000 µm in size by Hartmann et al. (2019), are then formed due to fragmentation (Jahnke et al., 2017). Their spatial distribution in aquatic environments mostly follows their specific density, i.e., particles with a higher density than water (>1 g cm^{-3}) are subject to sedimentation (Rummel et al., 2017). Biofouling can facilitate settlement by increasing the specific density of MPs (Kaiser et al., 2017). Hence, sediments might serve as a potential sink for MPs (Haegerbaeumer et al., 2019). Indeed, various MPs have been detected in riverine shore and bed sediments (Hurley et al., 2018; Käppler et al., 2018; Dierkes et al., 2019). Current concentrations range from 260–11,070 particles kg^{-1} in the Rhine riverbed, which were mostly 11–75 µm in size and had a higher density than freshwater (Mani et al., 2019). As a consequence, endobenthic organisms such as deposit-feeders or sediment-dwellers could encounter diverse compilations of MPs (Besseling et al., 2017; Haegerbaeumer et al., 2019).

Previous MP studies with annelids focused on marine and terrestrial species, e.g., *Arenicola marina* (Besseling et al., 2013; Browne et al., 2013) and *Lumbricus terrestris* (Hodson et al., 2017; Rillig et al., 2017). Even though the literature provides insights into uptake capabilities of MPs for freshwater worms (Imhof et al., 2013; Hurley et al., 2017;

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Scherer et al., 2017), toxicological data is still rare in this context (Haegerbaeumer et al., 2019). For instance, the freshwater oligochaete *Lumbriculus variegatus* has been solely examined by Beckingham & Ghosh (2017) and Redondo-Hasselerharm et al. (2018a,b) despite its ecological role (Chapman, 2001). In its natural environment, *L. variegatus* is exposed to contaminants via two major routes: enteral route by ingestion of sediment particles with pore water and dermal route by uptake from pore and overlying water (Phipps et al., 1993; Leppänen & Kukkonen, 1998b).

Because irregular MPs differ in numerous properties (e.g., size, shape, density, surface and chemical composition) (Lambert et al., 2017; Potthoff et al., 2017), reference particles are used to differentiate between physically and chemically induced effects (Anbumani & Kakkar, 2018; Triebskorn et al., 2019). Thus far, several studies have been carried out with natural (kaolin or silica) (Ogonowski et al., 2016; Straub et al., 2017) or artificial particles (additive-free pellets or glass-beads) (Rummel et al., 2019; Schrank et al., 2019) to elucidate the real cause-and-effect relationship. Research on leached chemicals, originally incorporated in the polymer matrix, has also been conducted (Hahladakis et al., 2018; Franzellitti et al., 2019). However, overarching studies assessing both of these two MP aspects are scarce for freshwater organisms.

The present study aims to address these issues by examining whether physical or chemical properties of a biodegradable plastic (i.e., degradable by microorganisms) affect the survival and dry weight of *L. variegatus*. Bio-based (renewable resources) and/or biodegradable plastics are believed to be a sustainable alternative for conventional plastics. However, an evaluation of potential hazards is important as biodegradability and toxicological impact depend on processed chemicals (Lambert & Wagner, 2017). In addition, biodegradable MPs have been detected in WWTP effluents and rivers (Mintenig et al., 2017; Mani et al., 2019), but only a few studies provide toxicological data on freshwater invertebrates (Sforzini et al., 2016; Straub et al., 2017). On these grounds, we hypothesized that (1) MP influences the survival and dry weight of *L. variegatus*, (2) a reference particle control does not negatively affect the worms, (3) chemicals (e.g., additives) originating from the MP are the main cause for toxicity and thus (4) solvent-treated, presumably pure MP has no impact on *L. variegatus*.

2. Material and methods

2.1. Test organism

Lumbriculus variegatus originated from an in-house culture (Department Aquatic Toxicology at Goethe University) and was kept in 5 L glass aquaria with a quartz sand layer of 2 cm depth as sediment (Baumit, Bad Hindelang, Germany) and aerated reconstituted water (ISO medium containing 294 mg $CaCl_2$ x 2 H_2O L^{-1}, 123 mg $MgSO_4$ x 7 H_2O L^{-1}, 64.8 mg $NaHCO_3$ L^{-1} and 5.75 KCl mg L^{-1} according to OECD guideline 225, OECD 2007). The culture was maintained under constant conditions at 20 ± 2 °C and a light:dark cycle of 16:8 h. Once a week, the overlying water was renewed and worms were fed with finely ground TetraMin® (Tetra GmbH, Melle, Germany).

2.2. Test material

For preparation of MPs, several shampoo bottles labeled as polylactic acid (PLA) with the recycling code 7 were purchased, cleaned with demineralized water and processed by cryogenic milling and sieving ≤150 µm (Mixer Mill MM400 and Vibratory Sieve Shaker AS 200 basic, Retsch GmbH, Haan, Germany). Kaolin (CAS 1332-58-7, Merck KGaA, Darmstadt, Germany; density: 2.6 g cm^{-3}) was used as a naturally occurring reference material to test for physically mediated effects. The MPs' estimated density is 1.2–1.3 g cm^{-3} (Farah et al., 2016). Both particle types were analyzed for size distributions (Coulter Counter, Beckman Coulter, Multisizer 3, Krefeld, Germany) (Fig. S1) and surface properties (Hitachi Scanning Electron Microscope, S-4500) (Fig. S2).

2.3. Experimental design

All experiments were conducted according to the OECD guideline 225: Sediment-Water *Lumbriculus* Toxicity Test Using Spiked Sediment (OECD, 2007). Kaolin is normally used for sediments according to this guideline. Due to our use of kaolin as a reference material, we modified the original sediment composition of the guideline (Table 1). Therefore, quartz sand was washed and annealed at 200 °C overnight and included two size fractions to simulate realistic field conditions, i.e., fine fraction (<0.7 mm, Baumit, Bad Hindelang, Germany) and coarse fraction (2–4 mm, Aquarienkies, Eggert Leuchterhand GmbH, Achim, Germany). The total amount of quartz sand was adjusted downwards with rising particle concentration, except for sediments that were coated with leached and extracted chemicals and thus, were tested as equivalents (EQs) (Table 1). As a result, sediment masses remained identical at 50 g per replicate. As carbon source, a 1:1 mixture of pulverized (<0.5 mm) *Alnus glutinosa* and *Fagus sylvatica* leaves (1.6% dry weight (dw)) was added as described by Nentwig (2007) (Table 1). Negative controls contained neither MPs nor kaolin. Additional controls were prepared following the formulated sediment of OECD guideline 225 (OECD, 2007). These OECD controls deviate from the modified sediment used in our controls without MP and exposure treatments and were only examined to check the vitality of the worm culture. Fourteen days before the tests, worms were artificially fragmented to ensure similar physiological states. Therefore, worms with no signs of recent morphallaxis were cut with a scalpel, leaving 1.5 cm of the posterior end to regenerate new heads. Four replicates of 250 mL test vessels per test concentration (controls: 6 replicates) were filled with 50 g of the modified sediment (Table 1), 200 mL ISO medium and 10 individuals. All glass products were rinsed beforehand with acetone and annealed overnight. Test vessels were aerated via glass pipettes and evaporation losses were compensated with deionized water. Once a week, plus at the beginning and end of each experiment, water parameters (pH, temperature, oxygen and conductivity) were measured with a multimeter (HQ40D, Hach Lange GmbH, Düsseldorf, Germany) (Table S1). Ammonium, ammonia nitrogen and carbonate hardness were determined using MColortest kits (Merck KGaA, Darmstadt, Germany) (Table S1). After 28-days of exposure, all worms were removed from the sediment and counted. Animals were then killed using a drop of ethanol and placed on annealed, pre-weighed weigh pans. After drying at 100 °C overnight and cooling in a desiccator, pans were weighed to record the dry weight per replicate.

Prior to chronic exposure, a miniaturized ingestion study with *L. variegatus* was conducted to confirm the MP uptake. Therefore, four replicates of 100 mL test vessels were prepared per treatment. Test vessels were covered with watch glasses to avoid airborne contaminations. A stock suspension was prepared with 0.5 g MPs L^{-1} and determined by the Coulter Counter to contain 2.2 × 10^6 MPs mL^{-1}. This

Table 1
Modified sedimentary composition [% dw] for chronic experiments with *L. variegatus*. MPs or kaolin mixed into the sediment were examined in Exp. I, MPs or kaolin layered on the sediment surface in Exp. II, leachates or extracts by equivalents (%-EQs) of the MPs in Exp. III and solvent-treated MPs or MPs mixed into the sediment in Exp. IV. The amount of quartz sand was adjusted downwards with rising particle concentrations in Exp. I, II and IV, but not for the quartz sand coated with chemicals from the leachates and extracts of the MPs in Exp. III.

Component [% dw]	Control	Experiments	
		I, II and IV	III
(Solvent-treated) MPs or kaolin	0.00	1.00–8.40	1.00–8.40[a]
Quartz sand	98.4	97.4–90.0	98.4
Food material	1.60	1.60	1.60

[a] Leachate and extract equivalents of the MPs [%-EQs].

suspension was used to obtain diluted MP solutions with the following nominal concentrations: 3, 30, 300 and 3000 MPs mL^{-1}. Thereof, defined volumes were pipetted above the sediment and distributed by slight stirring. A negative control without MPs was examined alongside. Five synchronized worms were added to each test vessel and exposed for 48 h to the MPs. Worms were then transferred into a 2.5% formalin solution. Each worm was cleaned with ultrapure water to rinse off attached particles and lysed at 55 °C and 550 rpm (Eppendorf ThermoMixer® C, Hamburg, Germany) for 24 h using a 30% H_2O_2 and 10% H_2SO_4 solution (1:10). Lysates were then vacuum-filtrated on 25 mm filters with 0.8 µm pore size (Metricel® Membrane, Sartorius AG, Göttingen, Germany). Filters were analyzed for ingested MPs using the Olympus BX50 fluorescence microscope. In order to record the actual MP concentrations inserted in the test vessels, the pipetted volumes were measured with the Coulter Counter.

2.3.1. Sediments with untreated microplastics

MPs were mixed into the dry sediment components of each replicate in the first experiment with a stainless-steel spoon (Exp. I) or layered on the sediment surface without further distribution in Exp. II. ISO medium was then carefully added to avoid MP resuspension. Stirred MPs were still observed but settled on the sediment within one day. Exp. II represents a scenario of synthetic particulates depositing onto sediments, whereas MPs would occur within the sediment only over time (Exp. I). For both tests, kaolin was examined alongside at the same concentrations as MPs to account for physically related effects. Tests were conducted with the following concentrations: 1, 1.7, 2.9, 4.9 and 8.4% in 50 g sediment dw^{-1}. A negative control without MPs and kaolin was examined in parallel for both experiments.

2.3.2. Sediments with microplastic chemicals

In Exp. III, the fine quartz sand was coated with either aqueous leachates (environmentally relevant scenario) or solvent extracts (worst-case scenario) of the MPs. In both scenarios, the amount of quartz sand was consistent for every treatment with 80% dw as fine fraction and 18.4% dw as coarse fraction. To obtain leachates and extracts, 0.5, 0.85, 1.45, 2.46 and 4.2 g of the MPs (corresponding to 1–8.4% MPs sediment dw^{-1}) were processed in bulk for each concentration level in 1 L ultrapure water or in 200 mL methanol, respectively. A leachate and extract control were prepared without MPs and served simultaneously as blanks. Glass bottles were covered with aluminum foil to shield suspensions from light. After 24 h on an orbital shaker (100 rpm) at room temperature, MP suspensions and the controls were vacuum-filtered through 0.2 µm sterile membrane filters (Thermo Scientific Nalgene Rapid-Flow Filter 75 mm, VWR International GmbH, Darmstadt, Germany). Filtrated leachates including the leachate control were enriched by solid-phase extraction (SPE) with Telos C18/ENV cartridges (Kinesis GmbH, Wertheim, Germany) to retain hydrophobic substances (Abbas et al., 2019). Before sample loading, cartridges were conditioned with 2 mL heptane, 2 mL acetone, 6 mL methanol and 8 mL ultrapure water. Columns were dried under a gentle stream of nitrogen and eluted with 5 mL acetone and 5 mL methanol. The eluate was diluted to a total volume of 200 mL with a 1:1 mixture of acetone and methanol, which was added to half of the total used fine quartz fraction for each treatment. Thus, the leachate control without MPs included an acetone-methanol mixture as solvent control and served additionally as a SPE blank. Filtrated methanolic extracts including the extract control, of which 200 µL as retention sample were stored at -22 °C pending analysis, were added directly on half of the total used fine quartz sand for each treatment. The extract control without MPs thus included methanol as solvent control and corresponded as well to a bottle blank. All soaked sediments were evaporated to dryness for 3 days in a fume hood at room temperature and additionally, 24 h in a climate chamber at 30 °C to ensure complete solvent vaporization. The coated sand was transferred into the test vessels, mixed with the remaining quartz sand and food (Table 1) and topped with ISO medium. A negative control without MPs was examined as well.

2.3.3. Sediments with solvent-treated microplastics

Solvent-treated MPs were examined in Exp. IV. Therefore, 0.5–4.2 g of MPs were immersed in methanol for 24 h to remove extractable chemicals, filtrated (same procedure as described in section 2.3.2 up to enrichment) and dried for 3 days in a climate chamber at 30 °C. Solvent-treated, presumably pure MPs were then mixed with the remaining dry sediment components (Table 1). For this experiment, another newly purchased batch of the shampoo bottle was processed due to lack of MP material used in Exp. I to III. Consequently, the chemical load of this particular batch originating from the solvent-treated MPs was tested at the highest extract concentration (8.4%-EQs sediment dw^{-1}). The solvent control was prepared with methanol and evaporated. This procedure enabled a comparison with the highest extract from Exp. III (8.4%-EQs sediment dw^{-1}). Since a new batch of MPs was tested, untreated MPs (similar to Exp. I) were examined in parallel. A negative control without MPs was conducted as well.

2.3.3.1. ISO medium with microplastic migrates. In Exp. IV, MP migrates were additionally examined with four replicates at the highest concentration. Migrates represent MPs leached directly in ISO medium. Therefore, 4 x 4.2 g of MPs were shaken in 4 x 200 mL ISO medium on an orbital shaker at room temperature (same as described in 2.3.2). Solutions were then vacuum-filtrated (0.2 µm) to remove large MPs, but not enriched by SPE. The migrates correspond to 8.4%-EQs of the MP in 200 mL medium.

2.4. Chemical analysis

The polymer type was examined by Fourier transform infrared spectroscopy (FTIR) in attenuated total reflection (ATR) mode, using a Frontier spectrometer (PerkinElmer, Waltham, Massachusetts) equipped with a Universal ATR (UATR) accessory (PerkinElmer, Waltham, Massachusetts) with a Diamond/ZnSe crystal. Spectra were acquired over a wavenumber range of 650 to 4000 cm^{-1} with a spectral resolution of 4 cm^{-1}, a scan speed of 0.2 cm s^{-1} and 4 accumulations.

Furthermore, the polymer was analyzed by pyrolysis–gas chromatography–mass spectrometry (pyr-GC/MS) with an EGA/PY-3030D Multi-Shot pyrolyzer equipped with an AS-1020E autosampler (Frontier Laboratories, Saikon, Japan), coupled to a 7890B gas chromatograph and a 5977B mass spectrometer (Agilent, Santa Clara, California). Chromatographic separation took place on a DB5-MS column (30 m x 250 µm x 0.25 µm; Agilent, Santa Clara, California) with helium as carrier gas (1.2 mL min^{-1}, constant flow mode) and the following temperature program: 40 °C (hold for 2 min), 20 °C min^{-1} to 320 °C (hold for 13 min; overall analysis time: 29 min). The pyrolysis unit was coupled to the GC via a split/splitless injector (split mode, split ratio: 1:100). Approximately 0.5 mg of the sample were pyrolyzed at 600 °C in presence of tetramethylammonium hydroxide (TMAH, 10 µL of 5% w/w solution in methanol, solvent evaporated prior to pyrolysis), causing an in-situ hydrolysis and methylation. Mass spectra were recorded in full scan mode over an m/z range of 40–500. Pyrolysis products were identified by deconvolution of the mass spectra with the software MassHunter Unknown Analysis (Agilent, Santa Clara, California) and comparison with the NIST 14 Mass Spectral library (library match >80) and checked manually. To analyze small molecules incorporated in the polymer matrix, another particle of the sample was analyzed by thermal desorption-GC/MS (TD-GC/MS). The same instrumentation as for pyr-GC/MS was used and similar conditions were applied, but a split ratio of 1:10 was used and no TMAH was added. Thermal desorption took place at 280 °C for 5 min. Two replicates were measured and only compounds identified in both measurements are reported.

The retained sample of the methanolic extract (for details see Experiment III) was analyzed using an Agilent 7890B GC-

chromatograph with electron ionization equipped with an Agilent 7200 QTOF high resolution mass spectrometer (GC-analysis in Supplementary Information (SI)). Chromatograms were evaluated by a non-target approach using the Agilent software Quantitative Analysis Unknown Analysis and compounds were identified by comparison of deconvoluted mass spectra with the NIST 14 library and checked manually. The identification levels (confirmed, confident and tentative) were attributed according to Norwood et al. (2008). Compounds found in both sample and blank were not reported, unless the peak area of the sample is more than tenfold higher.

HPLC analysis of the methanolic extract retention sample and corresponding blank was performed using a TripleTOF 6600 (Sciex) coupled via an ESI (IonDrive) source to a binary HPLC system (1260 Infinity, Agilent) equipped with a reversed phase (C18) column (Zorbax Eclipse Plus, 2.1 mm x 150 mm, 3.5 µm, Agilent) and eluted with a water-acetonitrile gradient acidified with 0.1 vol% formic acid. The HPLC method was further used to quantify two identified compounds (analytical results of the HPLC-analysis in SI).

2.5. Data analysis

Statistical analysis was performed with GraphPad Prism® 8 (GraphPad Software Inc., San Diego, USA). The number of worms and dry weight were tested for Gaussian distribution with Shapiro-Wilks' normality test and for equality of variances with Bartlett's test. If data was normally distributed and in case of variance homogeneity, one-way ANOVA with Dunnett's post-hoc test were conducted. In case of non-normal distribution or variance inhomogeneity, Kruskal-Wallis test with Dunn's post-hoc test was conducted. Negative and solvent controls were checked for normal distribution and equality of variances as well. In case of normal distribution and variance homogeneity, the negative and solvent controls were compared by an unpaired t-test (two-tailed, $\alpha = 0.05$). Otherwise, a Mann-Whitney test ($\alpha = 0.05$) was performed. When no significant differences were detected between these controls, replicates of both groups were pooled. In case of significant difference, exposure treatments were tested against the solvent control. Significant differences between the control without MPs and the exposure treatments were indicated with hollow or black asterisks (* $p < 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3. Results

3.1. Ingestion study with microplastics

The preliminary ingestion study with *L. variegatus* is displayed in Fig. 1. The actual MP concentrations, indicated as exposure treatments, were measured with the Coulter Counter and accounted for 12, 13, 387 and 4240 MPs mL^{-1}. These deviate from the nominal inserted MPs but still show increasing concentrations. This study revealed that the uptake of MPs is concentration-dependent. When exposed to 12 MPs mL^{-1}, worms ingested a mean number of 11.9 ± 4.93 MPs. In contrast, an exposure to 4240 MPs mL^{-1} resulted in 44.2 ± 17.7 MPs individual^{-1}. Worms exposed to more than 12 MPs mL^{-1} resulted in significant differences ($p < 0.001$) to the control. No MPs were added to the control group, but contained on average 4.95 ± 3.17 particles worm^{-1} that resembled features similar to the examined MPs.

3.2. Effects of untreated microplastics

MPs mixed into the sediment caused a significant reduction of survival ($p < 0.05$) in the two highest treatments (4.9 and 8.4% MP sediment dw^{-1}) (Fig. 2a). Dry weight was reduced in a concentration-dependent manner as well, but not affected significantly (Fig. 2b). Although the standard deviations of MP treatments overlap with kaolin treatments, the average number of worms was not affected significantly (from 13.5 ± 2.89 to 10.3 ± 0.50) under kaolin exposure (Fig. 2a). This

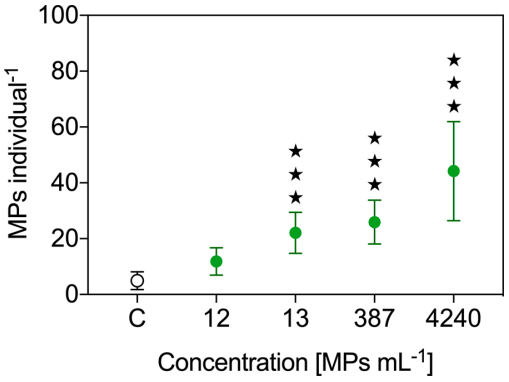

Fig. 1. *Lumbriculus variegatus*. Number of MPs (mean ± SD) found in worm lysates in a preliminary ingestion study. No MPs were added to the control (C), but particles with similar features of the MPs were still found. Significant differences to the control were determined by Kruskal-Wallis test with Dunn's post-hoc test (*** $p < 0.001$). $n = 20$.

is attributed to kaolin having precise means. However, dry weight increased consistently for kaolin from 2.9–8.4% sediment dw^{-1} (Fig. 2b).

Layered MP revealed a significant reduction for survival at 4.9% sediment dw^{-1} ($p < 0.05$) and the highest concentration ($p < 0.01$) in Exp. II (Fig. 2c). Here, worms showed initial avoidance behavior towards the sediment by remaining on the sediment surface instead of burrowing into the sediment. This was not quantitatively measured but merely observed. Dry weight decreased to a minimum of 1.96 ± 1.30 mg at the highest exposure but was not significantly different to the control without MPs (Fig. 2d). Layered kaolin particles did not affect the number of worms (Fig. 2c), though initial sediment avoidance was observed for the two highest concentrations as well. All kaolin treatments had a constant level of approximately 10 individuals per replicate at test end. In contrast, the dry weight increased twofold for treatments 1.7 and 2.9% sediment dw^{-1} in Exp. II and decreased at higher kaolin concentrations, almost reaching the mean control value without any added particles of 5.27 ± 2.88 mg (Fig. 2d). Overall, kaolin did not induce any adverse effect in both experiments.

3.3. Effects of microplastic chemicals and solvent-treated microplastics

In the Exp. III leachates did not affect *L. variegatus*, except for survival in treatment 2.9%-EQs sediment dw^{-1} ($p < 0.05$) (Fig. 2e). In contrast, the number of worms decreased as a function of concentration when exposed to extracts of MP up to a mortality rate of 100% in the highest treatment ($p < 0.01$) (Fig. 2e). Dry weight was already affected significantly at lower concentrations (e.g., 4.9% sediment dw^{-1}, $p < 0.05$) (Fig. 2f). No dry weight could be recorded (i.e., 0.0 mg) for the highest concentration due to 100% mortality ($p < 0.001$).

The solvent-treated particles led to a significant reduction of number of worms at the highest concentration in Exp. IV (8.4% solvent-treated MP sediment dw^{-1}; $p < 0.05$), while the worm number remained at a constant level with 16.3–14.3 for treatments 1–4.9% sediment dw^{-1} (Fig. 2g). Although the number of worms in the control group without MPs (16.2 ± 2.04) was slightly higher than in the respective control groups of Exp. I–III, the negative impact of untreated MPs in Exp. IV was comparable to Exp. I. Survival was significantly reduced under exposure to untreated MP at 4.9–8.4% MP sediment dw^{-1} (Fig. 2g). Regarding dry weight, neither untreated MP nor solvent-treated MP showed any adverse effect (Fig. 2h). Untreated MP in Exp. I, however, reduced the worms' dry weight with increasing concentrations (Fig. 2b). Since a different MP batch of the shampoo bottle was examined in Exp. IV, we tested the highest extract concentration (8.4%-EQs sediment dw^{-1}) from the solvent-treated MPs (Fig. 3) and detected a significant ($p < 0.001$)

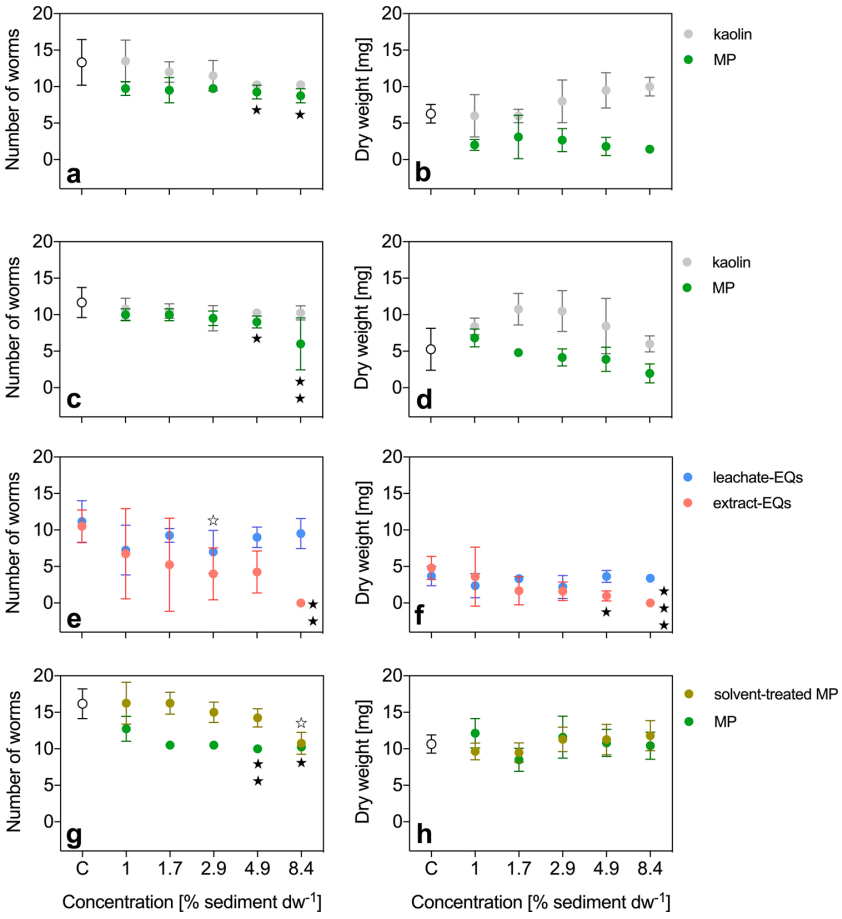

Fig. 2. *Lumbriculus variegatus*. Number of worms and dry weight [mg] (mean ± SD) after 28 days of exposure to MPs or kaolin mixed into the sediment in Exp. I (a, b), MPs or kaolin layered on the sediment surface in Exp. II (c, d), leachates or extracts by equivalents (EQs) of the MPs in Exp. III (e, f) and solvent-treated MPs or MPs mixed into the sediment in Exp. IV (g, h). Significant differences to the control (C) were determined by one-way ANOVA with Dunnett's post-hoc test or Kruskal-Wallis test with Dunn's post-hoc test. Hollow and black asterisks (☆ p <0.05, ★ p <0.05, ★★ p <0.01, ★★★ p <0.001) comply with the first and second listed treatment in the corresponding legend, respectively. n = 4.

lower number of worms (8.00 ± 3.94) (Fig. 3a) and dry weight (p <0.05; Fig. 3b) at test end. This high concentrated extract resulted in similar findings to its counterpart from Exp. III, when focusing on the significant outcome. However, the number of worms was not comparable (Exp. III: no survived worms (Fig. 2e) as opposed to Exp. IV: 8.00 ± 3.94 worms (Fig. 3a)). The same applies for the dry weight (Exp. III: no dry weight due to 100% mortality (Fig. 2f) and in Exp. IV: 4.55 ± 2.28 mg dry weight (Fig. 3b)). The additional treatment at the highest concentration of non-enriched MP migrates did not negatively affect the reproduction or dry weight (Fig. 3a, b). In contrast, under migrate exposure the highest number of worms was detected throughout every conducted experiment (Fig. 3a).

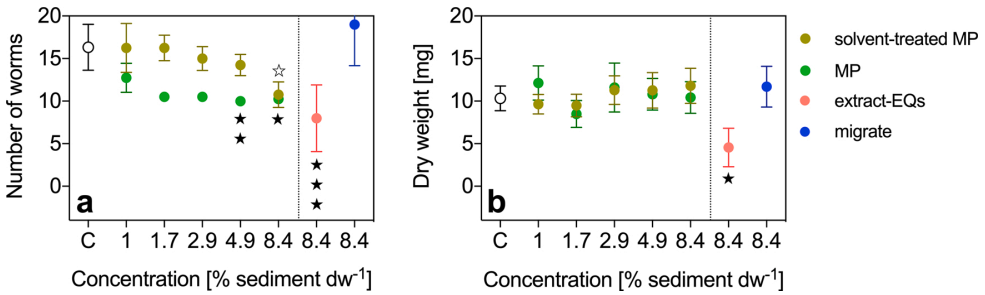

Fig. 3. *Lumbriculus variegatus*. Number of worms and dry weight [mg] (mean ± SD) after 28 days of exposure to solvent-treated MPs or MPs mixed into the sediment in Exp. IV. Since a different batch of MPs was processed for this experiment, the highest extracted concentration of chemicals from the solvent-treated MPs was tested as extract-equivalents (EQs). This treatment is comparable to its counterpart in Exp. III. An additional MP migrate treatment was tested at the highest concentration. Significant differences to the control (C) were determined by Kruskal-Wallis test with Dunn's post-hoc test. The hollow asterisk (☆ p <0.05) corresponds to the first listed treatment in the legend. Black asterisks (★ p <0.05, ★★ p <0.01, ★★★ p <0.001) comply with the second and third listed treatment in the legend. n = 4.

3.4. Chemical analysis

ATR-FTIR analysis displayed that the absorption bands of the shampoo bottle, labeled as PLA, did not show great conformity with those of a PLA reference spectrum (Fig. S3a). The sample spectrum rather resembled that of a starch blend containing 50 w% starch, 46 w% polybutylene adipate terephthalate (PBAT) and merely 4 w% PLA (Fig. S3b). Pyr-GC-MS analysis suggested that the tested material is a blend or copolymer of polybutylene adipate terephthalate (PBAT), polybutylene succinate (PBS), polybutylene succinate adipate (PBSA) and PLA, as indicated by series esters of butandiol and adipic acid (PBAT, PBSA), butandiol and succinic acid (PBS, PBSA), lactic acid (PLA) and butandiol and terephthalic acid, as well as benzoic acid (PBAT) (Table S2). All of these blended materials are considered as biodegradable.

Furthermore, analysis of the methanolic extract showed in both GC-TOF-MS (Table 2) and HPLC-MS (Table S4) many extractable compounds. They could be attributed to two possible sources: i) the polymer matrix and ii) the shampoo formulation (Table 2). HPLC-analysis further detected various series of cyclic and acyclic oligomeric esters composed of butandiol and succinic acid, adipic acid and terephthalic acid and beyond that esters based on lactid acid (Table S4). As listed in Table 2 and S4, declared shampoo ingredients (benzyl alcohol, benzyl benzoate, climbazole, caffeine and the surfactants sodium lauroyl methyl isethionate, sodium lauroyl sarcosinate and 3-(dodecanoylamino)propyl) (dimethyl)ammonio acetate) were detected. The presence of some of these compounds, such as climbazole and benzyl benzoate, was verified by TD-GC-MS analysis of the polymer (Table S3). Moreover, undeclared compounds such as coumarin were found. Some substances like fatty acid related compounds or the biocide iodocarb could not explicitly be assigned to one of the sources.

The identified biocides climbazole and iodocarb were additionally quantified via HPLC-MS using authentic reference substances. The concentrations in the retained methanolic extract sample (for details see Exp. III) were determined with 184 µg mL^{-1} for climbazole and 18 µg mL^{-1} for iodocarb (see quantification of iodocarb and climbazole in SI), corresponding to 7.34 mg of climbazole and 0.72 mg of iodocarb in each test vessel of the highest extract treatment (8.4%-EQs of MP).

Table 2
Identified substances of the methanolic extract retention sample by GC-TOF-MS.

Compound	CAS	Attribute	Possible origin
Dimethyl succinate	106-65-0	confident	PBAT
Benzyl alcohol	100-51-6	confirmed	shampoo
Isophorone	78-59-1	tentative	residual solvent
3,6-Dimethyl-1,4-dioxane-2,5-dione	95-96-5	confirmed	PLA
Chlorophenol	—	tentative	—
4-Methoxybenzaldehyde	123-11-5	tentative	—
Butandiol derivate	—	tentative	PBAT
Anisaldehyde dimethyl acetal	2186-92-7	tentative	—
Coumarin	91-64-5	tentative	shampoo
Dodecanoic acid	143-07-7	tentative	—
Oligomers of adipinic acid and 1,4-butandiol	—	tentative	PBAT
Iodocarb	55406-53-6	confirmed	bottle or shampoo
Butandiol derivate	—	tentative	PBAT
Benzyl benzoate	120-51-4	confident	shampoo
Isopropyl myristate	110-27-0	confident	—
Climbazole	38083-17-9	confirmed	shampoo
Succinic acid ester derivate	—	tentative	PBS
Oleamide	301-02-0	tentative	—
Succinic acid ester derivate	—	tentative	PBS
Dodecanoic acid 3-dodecanoyloxy-propyl ester	26719-54-0	confident	—
Oligomers of adipinic acid and 1,4-butandiol	—	tentative	PBAT
Terephthalate derivate	—	tentative	PBAT
Terephthalate derivate	—	tentative	PBAT
Succinic acid ester derivate	—	tentative	PBS

4. Discussion

4.1. Adverse effects of untreated microplastics

In our study, we demonstrated that *L. variegatus* generally ingests MPs. Thus, we tested the MP effects. Significant effects became evident when MP was mixed into the sediment (Fig. 2a–b) as well as layered on the sediment surface. So far, *L. variegatus* has only rarely been used in microplastic effect studies (Triebskorn et al., 2019; Haegerbaeumer et al., 2019). Redondo-Hasselerharm et al. (2018b) examined *L. variegatus* to analyze potential effects of car tire particles in mass partitions of 0–10% of sediment dw^{-1} on reproduction and dry weight of the oligochaete. Natural and synthetic rubbers as well as chemical processing aids are usually used for tire production, which was confirmed by chemical analysis in their study. They even detected zinc as inorganic filler in the material, but did not observe any effect. In contrast, our results display a clear concentration-response relationship for MPs, whereas kaolin as particle control caused no negative effect (Fig. 2a–b). This indicates chemically mediated effects of MPs. Besides, food quality and quantity in sediments can influence the response of oligochaetes to a pollutant. Due to the high encounter rate to synthetic particles with increasing concentration of MPs, the food material could have been diluted (Lwanga et al., 2016) or diminished in quality (Green et al., 2016), resulting in decreased nutritional value. This explanation has been used often in previous MP studies, of which several ascribed an effect to this mechanism (reviewed by de Ruijter et al., 2020). The reproduction of worms can be in fact positively influenced under organic-rich test conditions (Phipps et al., 1993; Camusso et al., 2012). Worm number is indeed higher (comp. vitality of worm culture in SI) when sediments were prepared following the optimized conditions of the OECD guideline 225 (OECD, 2007). However, we changed test conditions to better detect chemically mediated effects of MPs, which might be masked by oversupply of organic matter as in OECD formulated sediments (OECD, 2007) or in the study of Redondo-Hasselerharm et al. (2018b). Compared to the latter, the observed response in our experiment might be the result of lower organic content (Leppänen & Kukkonen, 1998a) combined with the high bioaccumulation potential for MP as pollutant (Mäenpää et al., 2003).

Reference particles should preferably display comparable physico-chemical properties as the examined MP without adding nutritional value (O'Connor et al., 2019). In our study, MPs and kaolin as particle control had specific densities >1 g cm^{-3} and thus, both are bioavailable for worms. Surface structures of both particle types seem comparable at first (Fig. S2), but really are not. The MPs were irregularly shaped, to which many small particles attached, whereas kaolin appears to form stacked plate-like agglomerates. This observation is in accordance with its actual shape, therewith connected is the high adsorption potential for organic molecules (Awad et al., 2017). Moreover, the size fractions of both particles were not identical despite the congruent particle distribution for the size-range 8–18 µm (Fig. S1). We further observed that kaolin had a higher particle count than the MPs despite same weighed-in masses, which is linked to its high amount of fine-sized matter combined with a high density. Since uptake capabilities depend mostly on particle size (Scherer et al., 2017), this property could have contributed to adverse effects of the MP. Because the size fractions of both particulates were within an ingestible range for *L. variegatus* (Beckingham & Ghosh, 2017), we reject the size as a major influence and assume that MP chemicals induced adverse effects (see first section of 4.1). This was not confirmed by kaolin findings as it proved to be a biologically active control due to enhanced dry weights in Exp. I and II (Fig. 2b, d). However, the first indication on chemical toxicity of MP was supported by the result (Fig. 2e, f) from similarly distributed solvent-treated MPs (Fig. S1) combined with the high mortality from enriched, extracted

chemicals (Fig. 2e).

4.2. Subsurface pollutants as the main exposure route

Layered MPs significantly decreased worm number at the two highest concentrations (4.9 and 8.4% MP sediment dw^{-1}) (Fig. 2c), which represents an extreme type of particle deposition on the sediment surface. Again, this effect could be attributed to the MP, since no negative impacts were detected in the layered kaolin exposure (Fig. 2c–d). Initially worms avoided burrowing into the high layer of 8.4% MP sediment dw^{-1}, what can be regarded as normal behavior for contaminated sites (Dermott & Munawar, 1992). After 24 h we noticed that the worms' anterior parts were enclosed by the thick layer of MP (8.4% MP sediment dw^{-1}) and thus, particles with no nutritional value. Even though oligochaetes are active burrowers and thereby provide bioturbation of the sediment (US EPA, 2000), *L. variegatus* was not capable of reworking the thick layered MPs after 28-days of exposure.

As an upward conveyor belt-feeder the worm ingests particles in the sediment and egests feces to the sediment surface (Tevesz et al., 1980; Reible et al., 1996). When MPs are mixed into the sediment as in Exp. I, individuals are likely to encounter these particles. In Exp. II, however, MPs were applied on the upper layer of the sediment. For 1–2.9% of layered MPs sediment dw^{-1}, oligochaetes were observed to burrow their anterior (feeding) part under the MP layer. Therefore, the worms' feeding part was surrounded with natural materials such as quartz sand and food, while the posterior part passed through the MP layer and surfaced at the water-sediment interface (Tevesz et al., 1980). In this case, MPs were not even bioavailable as synthetic particles were out of reach after burrowing. Therefore, it is not surprising that MPs mixed into the sediment caused higher impacts on dry weight (Fig. 2b) than layered MPs (Fig. 2d). However, when worms were exposed to a thick layer of MPs (4.9–8.4% sediment dw^{-1}), they initially avoided to burrow into the sediment. In this context, the toxicity of MP depends greatly on the application type or spiking method, as described by Scherer et al. (2019).

4.3. Beneficial effects of kaolin as natural mineral

Exposure to kaolin not only demonstrated that MPs were more toxic than the (biologically active) particle control, it highlighted that subsurface kaolin particles (Fig. 2b) influenced worms more than layered kaolin (Fig. 2d). This indicates that the scenario in Exp. I is an effective exposure route for *L. variegatus* as individuals are likely to ingest these particles. Moreover, kaolin increased the worms' dry weight (Fig. 2b, d). Scherer et al. (2019) observed increased weight of emerged *Chironomus riparius* midges when exposed to 2% of deposited kaolin. Further findings showed that kaolin mixed into the sediment positively affected the success and time of emergence for chironomids. Despite our observation of increasing dry weight due to layered kaolin, dry weight decreased again at the highest concentration, probably as a result of initial sediment avoidance interlinked with a reduced ability to reach the food material (Fig. 2d). However, endobenthic organisms seem to benefit from natural fine-sized matter such as kaolin (Scherer et al., 2019), which is recommended as sediment constituent in OECD guideline 225 (OECD, 2007). It is evident that some oligochaetes feed selectively on fine organic-rich particles due to nutritional value (Rodriguez et al., 2001). *Lumbriculus variegatus* is considered a generalist feeder and ingests fine-grained particles regardless of their organic content (Kukkonen & Landrum, 1995). Although the worm tolerates a wide range of physico-chemical sediment properties (Ankley et al., 1993, 1994; Ingersoll et al., 1995), it reproduces better in fine-sized sediments (Leppänen & Kukkonen, 1998a; Sardo et al., 2007). We assume that kaolin provides more favorable conditions as it has a high capacity to bind organic molecules (Awad et al., 2017) and thereby, provides nutritional value.

4.4. Chemical toxicity of microplastics

Aqueous leachates of the MP had almost no adverse effects on *L. variegatus*, despite the enrichment by SPE via C18-cartridges and thus, enrichment of medium polar compounds. A single minor effect ($p < 0.05$) on worm number was observed at 2.9%-EQs sediment dw^{-1}. However, hydrophobic substances migrate slowly into water (Teuten et al., 2009), which is probably the main reason that we could not detect severe impacts from leachates. Due to our enrichment, it could be argued that inorganic substances were not considered in this study, even though *L. variegatus* is sensitive to metals (e.g., Cu, Cd, Pb) (Phipps et al., 1995). Hydrophilic compounds could be underrepresented as well. Therefore, we examined the MP migrate, i.e., MPs directly leached in ISO medium, filtered but not enriched. Again, no effects were observed at test end, suggesting that possible migrating compounds were not relevant (Fig. 3a, b). Bejgarn et al. (2015) showed as well that PLA (3D printer plastic) leachates did not induce toxicity on *Nitocra spinipes*, whereas the leachate of a UV radiated biodegradable bag (corn starch and aliphatic polyester) did. This emphasizes that ecotoxicological effects of plastics strongly depend on the chemical composition and degradation products (Lambert & Wagner, 2017).

Methanolic extracts of MP were most potent with 100% mortality in the highest treatment (8.4%-EQs sediment dw^{-1}) (Fig. 2e, f). These inhibitory effects are not surprising since extracts represent worst-case conditions. It is noteworthy that the organic extraction was only an experimental construct to elucidate if chemical compounds from the MPs contribute to the toxicity. Under the exposure with a potential semblance of environmental relevance (e.g., 1%-EQs sediment dw^{-1}), very little biological impact is actually seen. Zimmermann et al. (2019) demonstrated that methanolic extracted chemicals from labeled PLA consumer products induced strong *in vitro* toxicity. One of the four investigated PLA samples ('PLA 3'; Zimmermann et al., 2019) represents the same product as in our study and yielded high baseline toxicity. Because we added hydrophobic compounds to the sediment, the observed mortality of *L. variegatus* might be induced by non-specific narcosis, complying with intercalation of hydrophobic pollutants into membranes (Escher & Hermens, 2002). Chemicals may desorb from ingested sediment particles during gut passage (Browne et al., 2013; Beckingham & Ghosh, 2017) or can be taken up percutaneously from pore water (Leppänen & Kukkonen, 1998b). In the present study, the ingestion of pore water and contaminated sediment particles with food are likely both operating routes for exposure.

The shampoo bottle was labeled with the recycling code 7 and amendment PLA. However, based on analytical results obtained by IR-spectroscopy (Fig. S3), pyrolysis-GC-MS (Table S2) and chromatographic analysis of extracted compounds (Table 2, S3 and S4), the plastic bottle was presumably composed of a blend or copolymer at least of PBAT, PBS and PLA as biodegradable materials. PBAT, PBS and PBSA are thermoplastic polyesters synthesized through condensation polymerization of a diol and a dicarbonic acid. The synthesis can be incomplete, leaving monomers and series of oligomers in the polymer. Identified chemicals from the MP indeed included several series of aliphatic and aromatic ester oligomers related to the polymers PLA, PBAT, PBS and PBSA (Table 2). These low-molecular weight substances can migrate from the polymer matrix in contact with medium. In addition, we identified declared shampoo ingredients (e.g., benzyl benzoate and climbazole) from the plastic bottle (Table S3) and the methanolic extract (Table 2 and S4). Consequently, a migration of these compounds from the shampoo into the polymer matrix or adsorption on the particle surface must have occurred and thus, MPs served as a vector for contaminants (Teuten et al., 2009). Undeclared compounds such as coumarin could be part of the plant extracts, while iodocarb might originate from the plastic bottle or shampoo formulation (Table 2). Both biocides were quantified in high concentrations, corresponding to 7.34 mg climbazole and 0.72 mg iodocarb in each test vessel of the highest extract treatment. We assume that they contributed to the extracts'

toxicity since both are ecotoxicologically relevant (Richter et al., 2013). However, it is evident that a mixture toxicity is the main cause for the observed effects since this specific biodegradable plastic represents a complex mixture of various compounds. This underlines the challenging work with sparsely declared plastic products, especially for environmentally friendly marketed products that comprise several polymeric blends.

4.5. Toxicity of solvent-treated microplastics

By extracting chemicals from MPs before exposure, we evaluated physically mediated effects in Exp. IV (Fig. 2g, h). To account for the chemical load of this newly processed MP batch, untreated MPs were examined again as well as the highest extract concentration originating from the solvent-treated MPs (Fig. 3). The result illustrates that solvent-treated MPs induced mitigated impacts on worm number than untreated MPs (Fig. 2g). Moreover, the highest extract resulted in a significant reduction of both worm number ($p < 0.001$) and dry weight ($p < 0.05$). The outcome of the extract exposure compared to its counterpart in Exp. III was quite distinct, although the same but only newly purchased product was tested. Previous studies demonstrated that solvent-treated MPs have no impact on reproduction of *L. variegatus* (Redondo-Hasselerharm et al., 2018a) and *L. terrestris* (Lwanga et al., 2016). However, the latter study observed significant reductions on growth rate. Straub et al. (2017) described weight loss in G*ammarus fossarum* when exposed to biodegradable polyhydroxybutyrate MPs. Since these particles were processed several times (i.e., with heat, sonication and washing) prior to their experiment, we assume that they removed most chemicals from the tested material. These studies found either no effects or impacts likely caused by reduced food uptake. We therefore believe that the reduction in the highest treatment of solvent-treated MP (Fig. 2g) is related to the high exposure to non-nutritious MPs and not an effect of remaining harmful chemicals. This observation could highlight that worms are not selective regarding food source and therefore, affected by food shortage when exposed to high non-nutritious fractions of either untreated or solvent-treated MP. However, we do not know whether the solvent-treated MPs underwent exhaustive extraction. Thus, we cannot ascribe a specific mechanism to this effect. Since the kaolin used in our study proved to be somewhat beneficial for the worms, we recommend using chemically purified plastic particles (Pikuda et al., 2019; de Ruijter et al., 2020) or glass beads as controls for MP experiments with worms. Although the latter particle type lacks an irregular shape (Schrank et al., 2019), it provides chemically inert and well-known physical properties as well as sufficient interstitial space for burrowing worms (Taghon, 1982; West & Ankley, 1998).

5. Conclusions

Our study highlights that MPs mixed into sediments affected *L. variegatus* more than MPs layered on the sediment surface. MPs generally caused toxic effects, whereas kaolin as particle control did not. Even though the choice of appropriate reference material depends on the study objectives, the use of kaolin displayed the importance of natural fine-sized matter for the endobenthic worm. Kaolin proved to be a biologically active control. It is therefore crucial to assess the mere chemical load of MPs to elucidate the source of toxicity. Severe adverse effects were observed in the experiment with an exposure of worms to extracted chemicals from the MPs. Here, industrially processed chemicals and their degradation products were decisive for the survival of worms. Solvent-treated, presumably pure MPs mostly resulted in higher reproduction than for untreated MPs. This indicates that chemicals originating from the polymer or even from the shampoo formulation that migrated into or adsorbed on the polymer are the main driver for toxicity. It is noteworthy that MP concentrations in the experiment exceeded environmental concentrations and that we used the organic extraction to investigate chemical-specific MP properties. In any case, the results indicate that biodegradable plastic is not necessarily a better alternative for conventional plastics since the toxicological impact depends on processed chemicals and their degradation products.

CRediT authorship contribution statement

Kristina Klein: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Visualization, Writing - original draft, Writing - review & editing. **Theresa Piana:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Tim Lauschke:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Peter Schweyen:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Georg Dierkes:** Investigation, Data curation, Formal analysis, Writing - review & editing. **Thomas Ternes:** Supervision, Writing - review & editing. **Ulrike Schulte-Oehlmann:** Conceptualization, Supervision, Writing - review & editing. **Jörg Oehlmann:** Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105723.

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A3 Particle shape does not affect ingestion and egestion of microplastics by the freshwater shrimp *Neocaridina palmata*

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RESEARCH ARTICLE

Particle shape does not affect ingestion and egestion of microplastics by the freshwater shrimp *Neocaridina palmata*

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Abstract
The ingestion of microplastics (MPs) is well documented for various animals and spherical MPs (beads) in many studies. However, the retention time and egestion of MPs have been examined less, especially for irregular MPs (fragments) which are predominantly found in the environment. Furthermore, the accumulation of such particles in the gastrointestinal tract is likely to determine whether adverse effects are induced. To address this, we investigated if the ingestion and egestion of beads are different to those of fragments in the freshwater shrimp *Neocaridina palmata*. Therefore, organisms were exposed to 20–20,000 particles L^{-1} of either polyethylene (PE) beads (41 μm and 87 μm) or polyvinyl chloride (PVC) fragments (<63 μm). Moreover, shrimps were exposed to 20,000 particles L^{-1} of either 41 μm PE and 11 μm polystyrene (PS) beads or the PVC fragments for 24 h, followed by a post-exposure period of 4 h to analyze the excretion of particles. To simulate natural conditions, an additional fragment ingestion study was performed in the presence of food. After each treatment, the shrimps were analyzed for retained or excreted particles. Our results demonstrate that the ingestion of beads and fragments were concentration-dependent. Shrimps egested 59% of beads and 18% of fragments within 4 h. Particle shape did not significantly affect MP ingestion or egestion, but size was a relevant factor. Medium- and small-sized beads were frequently ingested. Furthermore, fragment uptake decreased slightly when co-exposed to food, but was not significantly different to the treatments without food. Finally, the investigations highlight that the assessment of ingestion and egestion rates can help to clarify whether MPs remain in specific organisms and, thereby, become a potential health threat.

Keywords Polymer · Microplastic · Uptake · Excretion · Freshwater invertebrate · Crustacea · *Neocaridina palmata*

Introduction

The ingestion of microplastics (MPs) has been previously described for more than 70 freshwater organisms (summarized by Scherer et al. 2018). With regard to egestion, a comparatively small number of publications are available (Burns and Boxall 2018), focusing on the investigation of either spherical MPs (beads), irregularly shaped MPs (fragments), and fibers or a combination thereof (Au et al. 2015; Blarer and Burkhardt-Holm 2016; Frydkjær et al. 2017; Scherer et al. 2017; Straub et al. 2017; Canniff and Hoang 2018; Weber et al. 2018; Hoang and Felix-Kim 2020). However, the ingestion and egestion capabilities of animals are both important aspects that contribute to potential adverse effects (Fueser et al. 2020), because the residence time of MPs in the digestive system probably determines the level of toxicity (Anbumani and Kakkar 2018). Particle shape could be a relevant factor on handling and passaging time (Frydkjær et al. 2017; Gray and Weinstein 2017) as well as on the relative toxicity. Therefore, it is of particular interest whether rounded beads or sharp-edged fragments need more time to pass the gastrointestinal tract (de Ruijter et al. 2020). After all, comprehensive data on the consumption and elimination of MPs are still lacking for freshwater organisms (Hoang and Felix-Kim 2020).

To address these aspects, we used the freshwater invertebrate *Neocaridina palmata* (var. White Pearl). This shrimp is characterized by a transparent exoskeleton and, therefore, eggs in breeding females, and food uptake is easy to detect.

Kristina Klein and Sebastian Heß contributed equally to this work.

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The genus is native to Asia (Karge and Klotz 2013) and typically used there as a model organism in ecotoxicology (EPA/ROC 2013) due to its wide distribution in lakes, streams and ponds (de Grave et al. 2008; Karge and Klotz 2013; Kohal et al. 2018), adaptation to diverse water parameters, relatively short reproduction period and sensitivity to endocrine disrupting chemicals (Huang et al. 2006; Mykles et al. 2016; Huang et al. 2020). Besides, the freshwater organism is increasingly used to address questions relating to decapod physiology (Sonakowska et al. 2015, 2016; Włodarczyk et al. 2017) and genomics (Mykles and Hui 2015; Mykles et al. 2016). Today, it has been found in European rivers (Klotz et al. 2013; Jabłońska et al. 2018), most likely as a result of global trade as an exotic species for hobby aquarists and the unintentional release into the aquatic environment (Schoolmann and Arndt 2018; Jaskuła et al. 2019). We deployed *Neocaridina* as a surrogate organism for decapods in order to approach approximate values for the ingestion of MPs by higher crustaceans such as the endangered noble crayfish *Astacus astacus* (Hilber et al. 2020). We expected that the epibenthic shrimp ingests settled MPs (Haegerbaeumer et al. 2019) and thus incorporated concentrations that cover recently presented data on MPs in the sediment phase (i.e., converted to volumetric units for comparative purposes: 0.51 to 64,900 MPs L^{-1}) of global rivers (Scherer et al. 2020). In detail, we investigated the ingestion rate for two differently shaped MPs (i.e., beads and fragments). We further analyzed the retained number of particles in the gut and the egested particles 4 h after the stop of exposure. Finally, we examined whether food interferes with the uptake of fragments, since animals could encounter such particles along with food under environmental conditions.

Material and methods

Test organism

Neocaridina palmata (var. White Pearl) was purchased and cultured in 20 L glass aquaria at Goethe University (Department Aquatic Ecotoxicology). Individuals were acclimatized at least for 1 week and were kept under constant conditions at 23 ± 2 °C and a 16:8 h light/dark cycle (460 lux). Reconstituted water based on the *OECD guideline 242*: *Potamopyrgus antipodarum Reproduction Test* (OECD 2016) was used in diluted form (i.e., 60%) to obtain a pH of 7.5 ± 1.0 and conductivity of 400 ± 100 µS cm^{-1}. Therefore, 1.8 g Tropic Marin® sea salt and 1.08 g $NaHCO_3$ were dissolved per 10 L of deionized water. The aquaria were provided with nano corner filters (Dennerle GmbH, Münchweiler an der Rodalb, Germany) and continuous aeration. Twice a week, the medium was partially renewed, and the shrimps were fed *ad libitum* with CrustaGran and Shrimp King Mineral (Dennerle GmbH). The number of individuals in the culturing aquaria varied greatly, depending on the reproduction rate of the individuals at the time.

Test materials

Spherical MPs and fluorescent polyethylene (PE) beads (excitation maximum: 414 nm, emission maximum: 515 nm) of two different size ranges (UVPMS-BG-1.035g/cc 38–45 µm and UVPMS-BG-1.025g/cc 75–90 µm) were purchased from Cospheric LLC© (Santa Barbara, USA) and Fluoresbrite® YG 10 µm polystyrene (PS) beads in a 2.5% aqueous suspension (article no. 18140, excitation: 441 nm, emission: 486 nm) from Polysciences Europe GmbH (Hirschberg an der Bergstrasse, Germany). Irregular MPs (fragments) were prepared from a fluorescent (excitation: 400–410 nm, emission: 455 nm) polyvinyl chloride (PVC) cord (Modulor GmbH, Berlin, Germany); the PVC cord was cut into small pieces (<1 cm) and milled cryogenically for 1–2 min at 30 Hz (Mixer Mill MM400, Retsch GmbH, Haan, Germany). The grinding steps were repeated until a fine powder was formed, which was sieved (<63 µm) using the Vibratory Sieve Shaker AS 200 basic (Retsch GmbH, Haan, Germany). Since there were no data available for the specific density of the PVC cord, the density was determined based on the weight and volume of one PVC cord piece (Table 1). The average size of each MP was determined by measuring 100 beads and 150 fragments with the Olympus BX50 fluorescence microscope and a connected digital camera (JVC KY-F75U and Olympus UC90). PVC fragments ≤5 µm were generally not considered for analysis due to optical limitations (Table 1, Fig. S1 and

Table 1 Properties of beads and fragments used in the ingestion and egestion studies

Experiment	Ingestion study			Egestion study		
	Beads[a]		Fragments	Beads[a]		Fragments
Polymer type	PE	PE	PVC	PE	PS	PVC
Density [g cm^{-3}]	1.03	1.04	1.26	1.04	1.05	1.26
Mean size ± SD [µm]	87.0 ± 4.83	41.1 ± 3.42	22.0 ± 16.8	41.1 ± 3.42	11.5 ± 0.87	22.0 ± 16.8

[a] Exposed as mixtures (1:1)

Fig. S2). Since the PVC fragments comprised irregular forms, the surface structure of these particles was analyzed with the S-4500 Hitachi Scanning Electron Microscope (Fig. S3).

Each bead type was suspended with ultrapure water and the surfactant Tween®20 (CAS 9005-64-5, Sigma-Aldrich) to avoid the agglomeration of beads (Frydkjær et al. 2017), not exceeding a final solvent concentration of 0.01% (v/v). Stock suspensions of fragments were prepared directly with medium (Table S1). Stock suspensions with beads were shaken for 24 h at 120 rpm, while 300 rpm were necessary to disperse the fragments (GFL 3017, Burgwedel, Germany). In order to determine the particle concentration of each suspension, aliquots were taken and vacuum-filtered onto cellulose nitrate membrane filters of 0.8 μm pore size (Sartorius AG, Göttingen, Germany). Retained particles were optically counted using the fluorescence microscope; particles ≤5 μm were not considered. Based on the derived concentrations, volumes from the stock suspensions, corresponding to the test concentrations, were rechecked to ensure that nominal and actual particle concentrations matched (see Table S1). Subsequently, the appropriate volumes were added to the test vessels. All vessels were prepared at least 20 h before the addition of the shrimps and remained without aeration to allow MP settlement. As the physical properties of the examined MPs differed (Table 1), we analyzed the agglomeration behavior of the particles. Due to their bright coloring, we could observe that beads accumulated on the bottom of the test vessels. Since fragments were not fully visible to the eye, the fragment settlement was investigated further (Fig. S4). Settlement of the fragments was confirmed after 20 h and remained at a similar level when the test vessels were aerated for an additional 24-h period (Fig. S4). The latter resembled the actual exposure conditions for 24 h.

Ingestion and egestion studies

Prior to the experiments, adult organisms were selected by size and allocated to other tanks that included the minimum number of adults needed for each experiment. The individuals were then held for 24 h in vessels with particle-free medium to allow gut clearance; all tested individuals had a mean body length of 12.7 ± 1.48 mm (Table S3). All treatments had eight replicates, with one individual per vessel and 500 mL medium, respectively, and were conducted once. In order to prevent the resuspension of particles, the test vessels were aerated a few centimeters below the surface of the medium for the test period. At the beginning and end of all tests, water parameters (pH, conductivity, oxygen, and temperature) were measured (Table S2).

For the ingestion study, individuals were exposed to four concentrations of beads and fragments (20, 200, 2000, and 20,000 particles L^{-1}) for 24 h (Table 1), respectively. These concentrations mirror global concentrations of MPs in sediments of rivers (Scherer et al. 2020). We expected the shrimps to encounter such MPs since the epibenthic organism feeds on biofilm material on the substrate (Pantaleão et al. 2017). We chose an exposure period of 24 h in order to reach a steady MP buildup (Rist et al. 2017). Negative controls without MPs were conducted in parallel. Since Pikuda et al. (2019) demonstrated that surfactants can negatively impact *Daphnia magna*, we included a solvent control with 0.01% (v/v) of Tween®20 as we dispersed the beads with this solution. To elucidate whether the shrimps feed preferentially within a specific size range, the experiments with beads were conducted with mixtures (1:1) of 75–90 μm and 38–45 μm PE beads in the ingestion study and 38–45 μm PE and 10 μm PS beads in the egestion study, respectively (Table 1). In addition to the ingestion study with MP fragments <63 μm, the effect of available food on the ingestion of fragments was investigated. Thus, *N. palmata* was exposed to similar treatments (20, 200, 2000, and 20,000 fragments L^{-1}) for 24 h but with added 4–5 mg of CrustaGran per test vessel; this food quantity corresponds approximately to 10% of the shrimps' wet weight (Vazquez et al. 2017). In addition, food was added to the negative control in order to detect potential synthetic particles introduced by the food source itself (Table S4). The food was added once and settled to the bottom of the test vessels.

To determine the number of ingested particles from the aforementioned experiments, individuals were rinsed with ultrapure water at the end of the exposure period to ensure the complete runoff of attached particles, snap-frozen in liquid nitrogen, and stored at −20 °C until further analysis. The body length (defined as the distance from the rostrum to the posterior margin of the last abdominal segment) and the sex (by means of the *appendix masculina*) were determined for each individual using an Olympus SZ40 stereo microscope (Table S3). Animals were again rinsed with ultrapure water and lysed in a 1:10 solution of 10% H_2SO_4 and 30% H_2O_2 for 72 h (40 °C, 300 rpm) (Heidolph Titramax 1000 with Inkubator 1000, Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). Lysates were then vacuum-filtered onto cellulose nitrate membrane filters and analyzed for ingested particles using the fluorescence microscope. The data were corrected for the negative control of the beads that served as a blank and for the airborne control that was necessary during the microscopical fragment analysis (Table S4).

For the egestion study, 16 shrimps were exposed for 24 h to the highest concentration (20,000 particles L^{-1}) of a PE-PS beads mixture and PVC fragments, respectively. Half of the individuals were then transferred into particle-free vessels with food (10 mg CrustaGran), which was added once to the vessels. A higher food amount than in the fragment ingestion study with food was chosen to increase the encounter rate for natural particles and, thereby, enhance the excretion. A post-exposure period of 4 h (t = 4 h) for the egestion of particles was chosen since preliminary tests revealed that <4 h is

sufficient for the shrimps to egest more than 50% of beads. The other half of the individuals not intended for excretion analysis were removed from the test after particle exposure to serve as a reference for particle uptake (t = 0 h). After the egestion period, the shrimps were cleaned and lysed under the same conditions as previously described. Lysates and excretions were vacuum-filtered and analyzed microscopically next to the shrimps that had no egestion period (t = 0 h). Here, the negative control of the beads study served as a blank, while another filter accounted for the introduction of airborne fragment-like particles during microscopy. A further blank accounted for potential fragment-like particles introduced by the food source during post-exposure (Table S4).

Data analysis

Data were analyzed with GraphPad Prism® (5.00 and 9.00) (GraphPad Software Inc., San Diego, USA). The data were tested for normal distribution. If the data were not normally distributed or in cases of variance inhomogeneity, the Kruskal-Wallis test followed by Dunn's post hoc test was conducted; otherwise a one-way ANOVA with Dunnett's post hoc test was performed. Statistical comparisons were made between the control group without MPs and the exposure treatments. Relationships between the body length and ingested or egested particles were analyzed using the Pearson or Spearman correlations, depending on whether the data met the parametric criteria. In order to test if the particle type, sex, and added food influenced the ingestion or egestion, a two-way ANOVA with Bonferroni's post hoc test was performed. The significance level was defined with $\alpha = 0.05$ ($p < 0.05$, $p < 0.01$, $p < 0.001$, and $p < 0.0001$).

Results

Ingestion study

Neocaridina palmata ingested both beads and fragments in a concentration-dependent manner (Fig. 1a). The respective negative controls, including the solvent control for the bead testing, contained neither beads nor PVC-like particles. It was necessary to correct the fragment data since one PVC-like fragment was detected in the airborne blank (Table S4). In general, the number of PE beads found in the lysates increased (0.63–64.6 beads individual^{-1}) with rising exposure concentrations (20–20,000 beads L^{-1}) (Table S4), whereas mostly beads of the smaller size class (38–45 μm) were detected compared to the 75–90 μm beads. Regarding the 20,000 beads L^{-1} exposure treatment, for instance, shrimps ingested 60.8 beads of the 38–45 μm size class and 3.80 beads of the 75–90 μm size class. Compared to the control without MPs, significant increases were observed for the exposure to 2000 ($p < 0.001$) and 20,000 ($p < 0.0001$) beads L^{-1}. During exposure to 2000 beads L^{-1}, one individual out of eight individuals died. Regarding the PVC particles, the mean number of

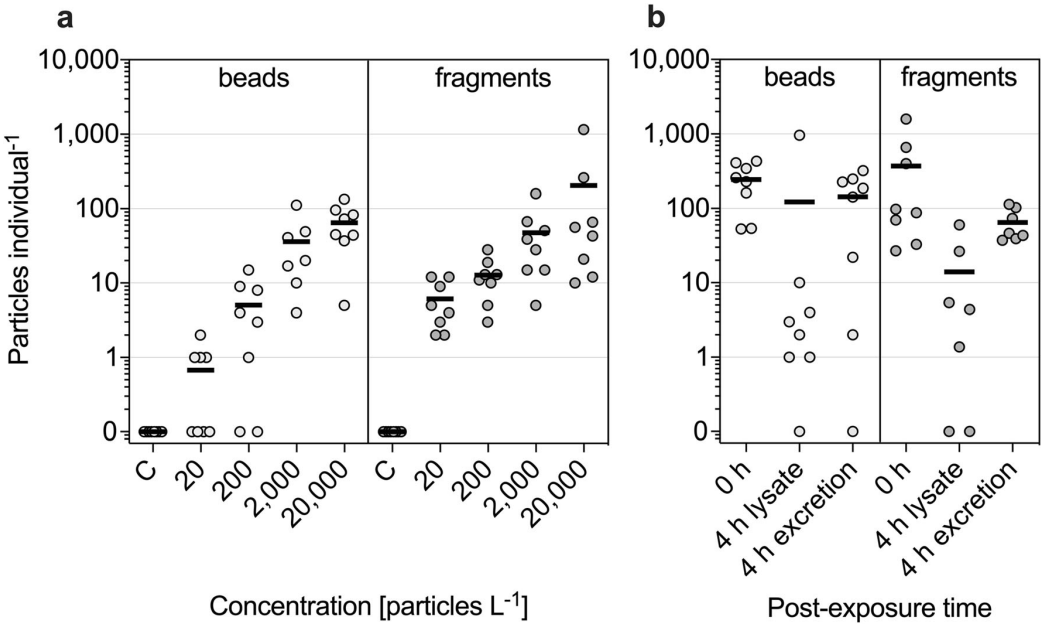

Fig. 1 *Neocaridina palmata*. **a** Mean number (lines) of detected beads and fragments individual^{-1} in shrimp lysates for the ingestion study. **b** Mean number (lines) of detected beads and fragments in shrimp lysates exposed to 20,000 particles L^{-1} (t = 0 h), as well as in the shrimp lysates and their corresponding excretions after an additional egestion period of 4 h (t = 4 h). No beads or fragments were added to the controls (C) in the ingestion study. One independent experiment with $n = 7–8$ replicates for each treatment

ingested particles ranged from 6.13 to 204 fragments individual^{-1} after exposure to 20–20,000 fragments L^{-1}. Once again, significant increases were observed for 200 fragments L^{-1} (p <0.01) as well as for 2000 and 20,000 fragments L^{-1} (p <0.0001) when compared to the control group. A significant influence of the differently shaped MPs in relation to the uptake was not detected. Moreover, neither significant differences for the MP ingestion between males and females nor a correlation between MP ingestion and body length were observed (Table S3).

Egestion study

Based on the results of the ingestion study (i.e., shrimps ingested a higher number of the smaller sized PE beads (38–45 μm) compared to the 75–90 μm PE beads), the organisms were further exposed to a PE-PS beads mixture of an even smaller size range in the egestion experiment (Table 1). For the egestion experiment (Fig. 1b), a reference (t = 0 h) was carried out for the ingestion of 38–45 μm PE and 10 μm PS beads and the <63 μm PVC fragments for shrimps exposed to 20,000 particles L^{-1}. In another treatment, individuals had an additional post-exposure time (t = 4 h) in particle-free medium to allow the measurement of egested particles. Here, the shrimps' excretions as well as lysates were examined to elucidate whether particles remained in the digestive system. On average, the shrimps contained 243 beads individual^{-1} (t = 0 h), i.e., 146 of 11 μm beads and 96.4 of 41 μm beads, and egested 143 beads individual^{-1} after 4 h (i.e., 59% of the previously ingested beads); the latter was corrected for two beads found in the corresponding negative control (Table S4). After 4 h of post-exposure time, 123 beads individual^{-1} remained in the shrimp lysates but were still significantly different to the reference treatment (p <0.05) (Table S4, Fig. 1b). *Neocaridina palmata* was further observed to excrete irregularly shaped MPs. In the excretions, 65.1 PVC fragments individual^{-1} were detected, while the food itself introduced 1.63 PVC-like particles (Table S4). The mean number of fragments individual^{-1} decreased significantly (p <0.01) from 371 in the reference (t = 0 h) to 14.0 in the lysate within 4 h of post-exposure. One individual died in the egestion treatment (t = 4 h) (Table S4, Fig. 1b). No significant difference between the egestion of beads and fragments was observed. Furthermore, no correlations between the body length and egestion or sex-specific differences could be detected. Due to the high variability that could potentially mask effects, the ingestion and egestion data were corrected for statistical outliers (Grubb's test) and evaluated again. This data resulted in similar findings as already described.

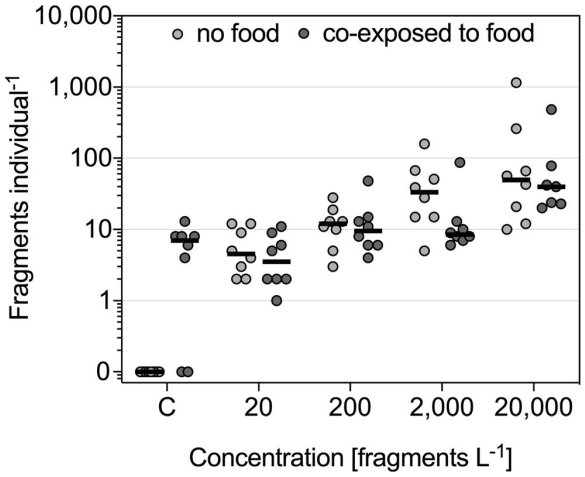

Fig. 2 *Neocaridina palmata*. Mean number (lines) of detected fragments individual^{-1} in shrimp lysates exposed to PVC fragments in the absence and presence of food. No fragments were added to the controls (C), but the food source introduced PVC-like fragments. One independent experiment with n = 7–8 replicates for each treatment

Food availability

Finally, we investigated whether food availability influenced the ingestion of fragments (Fig. 2). The ingested fragments without food resemble the same data as illustrated in Fig. 1a. The negative control with food contained 5.88 PVC-like particles individual^{-1} and, therefore, included more particles than the exposure treatment with 20 fragments L^{-1}. Here, an average of 4.75 PVC particles was detected per shrimp (Fig. 2, Table S4). The setup demonstrated similar ingestion rates as in the experiment without food, but with slightly lower mean ingested particles individual^{-1} for the two highest treatments. However, no significant difference was found between the treatments in the presence and absence of food. During the exposure to 20,000 fragments L^{-1}, one individual died. Overall, mortality occurred for one individual each in the ingestion experiment exposed to 2000 beads L^{-1}, co-exposed to 20,000 fragments L^{-1} and food as well as in the fragment egestion experiment following the 4 h excretion period (t = 4 h).

Discussion

Ingestion rates of beads and fragments are comparable

The current study aimed to examine differences in the gut passaging for microplastic beads and fragments by the atyid shrimp *Neocaridina palmata*. In addition, we used MP concentrations measured in the sediment of global freshwaters

(Scherer et al. 2020). The ingestion for both MP shapes was concentration-dependent (Fig. 1a). Based on the ingestion and egestion (t = 0 h) study, shrimps frequently ingested medium- and small-sized beads (i.e., 41 μm PE and 11 μm PS beads, respectively) compared to the large-sized beads of the respective exposure scenario (Fig. 1a, b). Thus, we detected size-related uptake preferences. However, the ingestion of both MP shapes did not differ significantly. In contrast, the estuarine shrimp *Palaemonetes pugio* was observed to ingest significantly higher numbers of 34 μm and 93 μm polypropylene (PP) fragments than of 30–165 μm PE and PS beads (Gray and Weinstein 2017); this could indicate shape-related influences. Lehtiniemi et al. (2018) somewhat support this as the mysid shrimp *Mysis relicta* ingested high rates of acrylonitrile butadiene styrene (ABS) fragments, but not polyethylene terephthalate (PET) fragments, when compared to PS beads. This could be attributed to the individual's ingestible size range since ABS fragments were smaller than the PET. Moreover, diverse MP properties (e.g., size, density, and surface chemistry) could contribute to bioavailability issues (Lambert et al. 2017). For instance, Frydkjær et al. (2017) showed that fragment uptake can decrease in daphnids, despite rising concentrations, when MPs agglomerate and are out of reach. Although the physical properties of our MPs differed (see Table 1), we detected that beads and fragments sedimented (Fig. S4) and so both were similarly available to the shrimps; this is in line with Setälä et al. (2016). They examined PS beads as used in the present study and observed them to settle, thereby becoming available for ingestion by the mysid shrimps *Neomysis integer* and *Praunus flexuosus*. Therefore, we do not assume that the sedimentation had a major impact on the study results. Considering the preferential uptake of the lower sized beads, it could be argued that the PVC fragments with the broad dimension range have been ingested disproportionally compared to the spheres with tight size specifications (Table 1). However, we generally excluded the lowest size range of the fragments (i.e., ≤5 μm) and, thereby, disregarded at least the smallest MPs. In general, our results indicate a rather unselective ingestion of MPs by *N. palmata*, which is likely connected to its opportunistic omnivorous feeding strategy (Yam and Dudgeon 2005; Weber and Traunspurger 2016). Thus, it is not surprising that the ingestion of beads and fragments were not significantly different.

Fragment uptake tends to be lower in the presence of food

We investigated the ingestion of fragments while food was available to the shrimps. We could not detect significant differences between the fragment ingestion in the absence and presence of food but observed a tendency towards a slightly reduced fragment uptake for individuals co-exposed to food (Fig. 2). Along this line, other freshwater invertebrates such as *D. magna* and *Gammarus pulex* have been shown to have reduced uptake rates for MPs in the presence of algae or leaf material (Scherer et al. 2017; Aljaibachi and Callaghan 2018). Bour et al. (2020) reported that the brine shrimp *Artemia* ingested less PE beads when co-exposed to food. However, the feeding type of these animals is not the same as for *Neocaridina*. Recent studies have described different outcomes when focusing on caridean shrimps as used in the present study. For instance, Saborowski et al. (2019) examined the uptake of polyacrylic wool fibers and different food concentrations with the Atlantic ditch shrimp *Palaemon varians*. In the cases where commercial food was present compared to when exposure took place without food, they demonstrated that the number of ingested microfibers was higher. This was explained by fibers attaching to the food source and, thereby, facilitating ingestion. They observed regurgitation of large microfibers via the esophagus of *P. varians*, highlighting the ability to remove indigestible particles. Korez et al. (2020) found PS beads in the stomach and midgut gland of the brown shrimp *Crangon crangon*, but, due to this organism being a predator, they generally included food to increase particle interaction. Therefore, it cannot be distinguished whether the beads would be ingested to a higher or lower extent in the absence of food by the brown shrimp. However, they examined the ingestion of inorganic particles (e.g., quartz grains and fragments from the remains of bivalve shells) and detected high loads of natural particles. This indicated active particle uptake enabling food to be mechanically fragmented. Based on this observation, they concluded that shrimps may be less selective in their search for food and therefore less susceptible towards MP contamination in their environment. Our findings are plausible in that food probably reduces the animals' encounter rate for MPs due to dilution effects (summarized by de Ruijter et al. 2020). However, it does not seem necessarily relevant for the epibenthic shrimp whether food is present or not because they likely feed on various sediment constituents. We argue that *N. palmata* does not appear to selectively feed on certain particles; this agrees with its omnivorous feeding behavior.

Comparably fast excretions of beads and fragments

Our egestion experiments demonstrated that *N. palmata* can excrete previously ingested MPs (i.e., t = 0 h) within 4 h of post-exposure. The shrimps only partially egested the particles within this specific excretion period as 123 beads and 14 fragments remained in the gastrointestinal tract (Fig. 1b). Interestingly, we did not observe a statistical difference between the egestion of beads and fragments. Similarly, Gray and Weinstein (2017) tested the egestion of 11 different MPs with the estuarine shrimp *P. pugio* and observed no apparent trend towards a prolonged residence time of differently sized as well as shaped MPs. Likewise, the same species egested the

majority of ingested PE spheres and PP fragments within 2 days (Leads et al. 2019). Korez et al. (2020) demonstrated that *C. crangon* egested the majority of PS beads after 24 to 48 h. However, they could not exclude the reentrance of MPs from feces due to coprophagy. The same may be relevant for our study since we found some MPs in the shrimps' lysates (Fig. 2b) during the post-exposure time. In order to conclude about incomplete excretion, the excretion time should be longer and the experimental design must monitor the excretion over time without allowing the organisms to re-ingest excreted particles. After all, the egestion of MPs is crucial in terms of limited gut space for the consumption of real nutritious food, which could result in energy depletion and developmental delays (Hoang and Felix-Kim 2020). We selected 4 h as the post-exposure period based on a preliminary conducted egestion study (data not shown) for beads at different times (4, 8, 16, and 32 h). Here, we could not detect significant differences between the excretion groups. Our data indicate that 59% of beads were excreted after 4 h (Fig. 2b). However, when we combined the groups of different excretion times from the preliminary test to obtain a large dataset (n = 32 replicates), we observed a comparably higher excretion rate for beads (85%), while only a small fraction was found in the digestive systems, and the rest could not be detected due to methodological reasons. Saborowski et al. (2019) demonstrated that the stomachs of *P. varians* were emptied from beads and fibers after 16–24 h. Bour et al. (2020) support this observation since they demonstrated major and complete bead depuration in *Artemia* after 24 and 48 h, respectively. Leads et al. (2019) showed that the egestion of different MP shapes is not affected in shrimps, which were previously injected with the bacterium *Vibrio campbellii* to increase their susceptibility to MPs. Taken together, our results are mostly in line with other publications and highlight that beads as well as fragments pass the shrimp's gut. Due to the numerous aspects that can influence the ingestion and egestion of MPs, a transfer of our results to other species (e.g., crayfish as higher crustaceans) is very limited, and solely the analysis of sampled animals would elucidate true accumulation rates of MPs (comp. Zhang et al. 2020).

It is noteworthy that three individuals died, which was however not exclusive to one MP shape. Canniff and Hoang (2018), for instance, used high concentrations of up to 100 mg L^{-1} of similar PE beads and did not detect adverse effects on the survival of *D. magna*. Cytotoxic effects could not either be detected in *in vitro* models with human cell lines (Çobanoğlu et al. 2021; Stock et al. 2021), except at really high concentrations (i.e., >75 mg mL^{-1}) for PE beads and powdered PVC particles by Stock et al. (2021). Given the comparably low MP concentrations examined in the present study, we cannot ascribe a specific toxicity mechanism to the low mortality of *Neocaridina*. In order to elucidate the real cause for the mortality, further research has to be performed with specific regard to internal injuries due to sharp-edged fragments or migrating chemicals from MPs.

Conclusions

We exposed *Neocaridina palmata* to realistic MP concentrations measured in the sediment of freshwaters and showed that shrimps generally ingest MPs. We further demonstrated that both the ingestion and egestion of beads and fragments do not differ in the freshwater organism. The particle size but not the shape affected the uptake. Moreover, we did not detect any significant differences between the fragment ingestion in the presence and absence of food, but we observed a slight tendency towards lower fragment uptake with the availability of food. This could reflect environmental conditions. Taken together, we could not detect any influencing factors on the ingestion other than the individuals' mouth opening probably limiting the ingestible particle size. Our results indicate that *Neocaridina* is not very selective regarding food properties, which might be linked to its omnivorous feeding behavior. We further observed that shrimps rapidly but only partially egested beads and fragments within 4 h. As the depuration was incomplete within this time frame, long-term effects cannot be fully excluded based on our study. Moreover, it is not reasonable to ascribe the low observed mortality rate to a specific toxicity mechanism, considering the low MP concentrations used. However, since we mostly observed few remaining particles in the digestive tract and shrimps are known to ingest high natural particle loads, we assume that the physical impact of MPs would be minor for freshwater shrimps. Overall, we are convinced that the assessment of ingestion and egestion rates is an important preliminary step for chronic studies. This could generally help to clarify whether MPs accumulate in organisms and, thereby, become a potential health problem at the individual level or even for higher animals via trophic transfer.

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Availability of data and materials The data used in the present study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate The German Animal Welfare Act does not apply for the present study because only invertebrates were used. The shrimps were still handled with the utmost care.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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A4 Locomotor behavior of *Neocaridina palmata*: a study with leachates from UV-weathered microplastics

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Co-authors USO, JO:	10% (concept)
(2) Conducting tests and experiments	
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Doctoral candidate KK:	50% (compilation of final data and preparation of figures and tables)
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(4) Analysis and interpretation of data	
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(5) Drafting of manuscript	
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Locomotor behavior of *Neocaridina palmata*: a study with leachates from UV-weathered microplastics

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ABSTRACT

Weathering of plastics leads to the formation of increasingly smaller particles with the release of chemical compounds. The latter occurs with currently unknown environmental impacts. Leachate-induced effects of weathered microplastics (MPs) are therefore of increasing concern. To investigate the toxicity of the chemical mixtures from such plastics, we exposed the freshwater shrimp *Neocaridina palmata* to enriched leachates from unweathered and artificially weathered (UV-A/B light) MPs (≤1 mm) from recycled low-density polyethylene (LDPE-R) pellets and from a biodegradable, not fully bio-based starch blend (SB) foil. We analyzed the individual locomotor activity (moved distance and frozen events) on day 1, 3, 7 and 14 of exposure to five leachate concentrations equivalent to 0.40–15.6 g MPs L^{-1}, representing the upper scale of MPs that have been found in the environment. The median moved distance did not change as a function of concentration, except for the unweathered SB treatment on day 14 that indicated hyperactivity with increasing concentrations. Significant impacts were solely detected for few concentrations and exposure days. Generally, no consistent trend was observed across the experiments. We further assessed the baseline toxicity of the samples in the Microtox assay and detected high bioluminescence inhibitions of the bacterium *Aliivibrio fischeri*. This study demonstrates that neither the recycled nor the biodegradable material are without impacts on test parameters and therefore cannot be seen as safe alternative for conventional plastics regarding the toxicity. However, the observed *in vitro* toxicity did not result in substantial effects on the behavior of shrimps. Overall, we assume that the two endpoints examined in the atyid shrimp *N. palmata* were not sensitive to chemicals leaching from plastics or that effects on the *in vivo* level affect other toxic endpoints which were not considered in this study.

Subjects Animal Behavior, Ecology, Ecotoxicology, Environmental Impacts
Keywords Microplastic, Weathering, Freshwater shrimp, Locomotion, Toxicity, Behavioral assay, *Neocaridina*, Polymer, Crustacea, Invertebrate

INTRODUCTION

Plastics contain chemicals that fulfill certain functions, *e.g.*, plasticizers are used to increase the materials' plasticity. Stabilizers, antioxidants, colorants, flame retardants and biocides are other functional compounds that can be admixed to the base polymer (*Hartmann*

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et al., 2019; *Bridson et al., 2021*). The European Chemicals Agency (ECHA) has compiled more than 400 substances of such additives that represent high production volume chemicals used in plastics (*European Chemicals Agency (ECHA), 2021*). In addition, processing aids, impurities and reaction by-products can be introduced unintentionally during production and are usually unknown to the public (*Groh et al., 2019*, *2021*; *Bridson et al., 2021*). These chemical mixtures can leach into an aqueous phase under laboratory conditions (*Gewert et al., 2018*; *Capolupo et al., 2020*). Plastic chemicals have also been detected in rivers (*e.g.*, organophosphates, phthalates and bisphenols) and some of them even exceeded the Environmental Quality Standards (EQS) as the maximum allowable concentration of defined substances in surface waters (*Schmidt et al., 2019*, *2020*; *Bolívar-Subirats et al., 2021*). Besides, degradation products formed due to weathering processes (*e.g.*, UV irradiation and hydrolysis) are often overlooked, but display an emerging chemical fraction once plastics enter the aquatic environment (*Arp et al., 2021*).

Moreover, the mortality, reproduction and development of freshwater invertebrates have been frequently examined in the field of ecotoxicology, whereas the behavior has been less often evaluated (*Oehlmann, Oetken & Schulte-Oehlmann, 2008*; *Oehlmann et al., 2009*; *Triebskorn et al., 2019*; *Thomas et al., 2021*). However, behavioral alterations are considered as early warning signs since behavioral responses to environmental factors (*e.g.*, anthropogenic pollutants) are reported to be 10–1,000 times more sensitive than commonly used endpoints like mortality, reproduction and development (*Hellou, 2011*; *Melvin & Wilson, 2013*). In recent years, the swimming behavior or locomotion has been increasingly tested for freshwater species exposed to plastic particles of the nano (*Pikuda et al., 2019*; *Lin et al., 2019*; *Xu et al., 2020*) and micro range (*Gorokhova et al., 2017*; *De Felice et al., 2019*; *Pannetier et al., 2020*; *Bartonitz et al., 2020*; *Xu et al., 2020*; *Chen et al., 2020*; *De Oliveira et al., 2020*). To our knowledge, leachable chemicals derived from such particles have been rarely addressed in behavioral studies with freshwater invertebrates as by *Xu et al. (2020)*. Related studies have worked though with marine (*Langlet et al., 2020*) and intertidal animals (*Seuront, 2018*; *Seuront et al., 2021*). *Sun et al. (2021)* analyzed both physically- and chemically-mediated effects of microplastics (MPs) on the locomotor activity of aquatic organisms and reported a significant decrease in movements at environmentally measured MP (≤1 mg L^{-1}) concentrations.

In this paper we aimed to extend the current database for leachate-induced effects of weathered plastics on the locomotor performance of a benthic freshwater invertebrate. We used the atyid shrimp *Neocaridina palmata* and recorded the moved distance and frozen events during the exposure to enriched leachable chemicals from MPs ≤1 mm. *Neocaridina* is easy to culture and known to be sensitive to environmental pollutants (*Siregar et al., 2021*) and therefore is frequently used as a model organism (*Mykles et al., 2016*). The sublethal endpoints were analyzed with the open-source software ToxTrac (*Rodriguez et al., 2018*). For the materials, we selected post-industrial recycled low-density polyethylene (LDPE-R) pellets and a biodegradable, partly bio-based starch blend (SB) foil that we previously identified to induce high *in vitro* toxicities including baseline toxicity, oxidative stress and endocrine activities (*Klein et al., 2021a* and K. Klein et al., 2020, unpublished data). Comparable to *Klein et al. (2021a)*, we further weathered the MPs

with artificial UV-A/B light and leached them afterwards in ultrapure water, taking into consideration the formed degradation products. Finally, we conducted the Microtox assay in order to assess the baseline toxicity of the leachable chemicals from the unweathered and UV-weathered MP samples.

MATERIALS & METHODS

Test organism

Individuals of *N. palmata* (var. White Pearl) were purchased from Garnelenhaus GmbH (Barsbüttel, Germany) and acclimatized at least one weak in 20 L reconstituted water at 23 ± 2 °C and a 16:8 h light:dark cycle prior to testing (Goethe University, Department Aquatic Ecotoxicology). For the experiments, only acclimatized adult individuals or adult offspring of the culture were used. The culturing medium was adapted from the OECD guideline 242: *Potamopyrgus antipodarum* Reproduction Test (*OECD, 2016*) as described in *Klein et al. (2021b)*. Nano corner filters (Dennerle GmbH, Münchweiler an der Rodalb, Germany) and aeration were provided in the culturing aquaria. Rocks and java moss (*Taxiphyllum barbieri*) from an *in vitro* culture were included to offer hiding spots. Shrimps were fed *ad libitum* with CrustaGran and ShrimpKing Mineral (Dennerle GmbH, Münchweiler an der Rodalb, Germany). One third of the medium was renewed twice a week.

Test materials

We obtained post-industrial recycled low-density polyethylene (LDPE-R) pellets and a biodegradable, partly bio-based starch blend foil (SB). The latter contains 50 w% starch, 46 w% polybutylene adipate terephthalate (PBAT) and 4 w% polylactic acid (PLA) and, therefore, is not fully derived from renewable resources because it contains petroleum-based PBAT. The materials were selected because recycled and biodegradable plastics are increasingly used as alternative materials for conventional plastics (*De Schoenmakere et al., 2019*; *Shen et al., 2020*) and because of their known high *in vitro* toxicities (comp. Table S3, *Klein et al., 2021a*). The foil was cut into 1 × 1 cm pieces with stainless steel scissors. Both test materials were then cryogenically milled and sieved (≤1 mm) using the Mixer Mill MM400 and Vibratory Sieve Shaker AS 200 basic (Retsch GmbH, Haan, Germany). Stereo microscopy (Olympus SZ40) as well as scanning electron microscopy (Hitachi Scanning Electron Microscope, S-4500) were used to characterize the MPs (Fig. S1).

Artificial weathering

Each MP (100 g) was weathered with UV-A/B irradiation (280–400 nm) for 24 h in a climate chamber (ThermoTEC 1501; ThermoTEC Weilburg GmbH & Co. KG, Weilburg, Germany), similarly to the procedure in *Klein et al. (2021a)* for plastic pellets where the irradiation time and intensity led to accelerated weathering of the plastics in terms of the enhancement of chemical leaching. The present study, however, includes MPs with a comparably higher surface area due to the milling process. Moreover, a closed climate chamber was used. Two data loggers (HOBO Pendant®; Onset Computer Corporation,

Bourne, USA) recorded the temperature (20.1 ± 0.26 °C). The UV-A intensity was measured using a Radiometer RM-12 (Opsytec Dr. Göbel GmbH, Ettlingen, Germany) and accounted for 2.30 ± 0.30 W m^{-2} with an approximate sun exposure equivalent of 8.16 h following the calculation provided by *Gewert et al. (2018)*. In comparison, the amount of UV-B irradiation was nine times lower. Following the one-stage batch test EN 12457-1:2002 (*EN 12457-1:2002, 2003*), the 100 g of unweathered and UV-treated MPs were leached in 1 L of ultrapure water for 24 h using an overhead shaker at 10 turns min^{-1} (Heidolph Reax 20; Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) and were prepared as duplicates. Hence, each MP sample was available twice. A procedural blank included 1 L of ultrapure water and was conducted in parallel. In order to protect the MP suspensions from light, aluminum foil was used to cover the glass bottles. Prior to all experiments, glass ware was rinsed with acetone and annealed to 200 °C for a minimum of 3 h. The MP suspensions and the blank were vacuum-filtered onto sterile membrane filters of 0.2 µm pore size (Thermo Scientific Nalgene Rapid-Flow Filter 75 mm; VWR International GmbH, Darmstadt, Germany).

Solid-phase extraction

The MP leachates and the procedural blank were acidified to pH 2.5 (3.5 mol H_2SO_4 L^{-1}) and enriched *via* solid-phase extraction (SPE) using the Telos C18/ENV cartridges (Kinesis GmbH, Wertheim, Germany). The elution and enrichment were performed as described in *Klein et al. (2021a)*. Afterwards, the duplicates of the respective MP extracts (2 × 200 µL) were combined and resulted in 400 µL extracts with dimethyl sulfoxide (DMSO) as solvent. These 5,000-fold concentrated extracts included a mass equivalent (EQ) of 198 g of MPs. This unit describes the leachable chemicals stemming from the MPs, *e.g.*, 1 mg of MP-EQs includes the components that were leached from 1 mg of MPs and enriched *via* the cartridges. The samples were stored at −20 °C until analysis.

Microtox assay

In order to examine whether the MP extracts contain chemicals that induce baseline toxicity, we tested the extracts in the Microtox assay using the luminescent bacterium *Aliivibrio fischeri* according to *ISO 11348-3 (2007)*. Negative (growth medium) and solvent (DMSO) controls as well as the blank, a reference compound as positive control (3,5-dichlorophenol (CAS: 591-35-5): from 1.73 × 10^{-6} to 2.21 × 10^{-4} mol L^{-1}, corresponding to 0.04 to 5.40 µg $well^{-1}$) and the MP extracts (5.22–668 mg EQs $well^{-1}$ (equals 0.03–4.45 kg EQs L^{-1}) of unweathered and UV-treated LDPE-R or SB foil, respectively) were tested. One well in the test plates contained 150 µl as final test volume and included the test medium, sample extracts and the bacteria. The used mass concentrations represent high environmental concentrations, considering findings with similar sized MPs (*Lasee et al., 2017*; *Schmidt, Krauth & Wagner, 2017*). The final solvent concentration did not exceed 1% (v/v) per well. The effects of the MP extracts were compared to the findings of the previously published study *Klein et al. (2021a)*. Moreover, the *in vitro* response of the MP extracts was compared to that of the positive control in order to evaluate the

environmental relevance. Three independent experiments were conducted with two technical replicates for each sample.

Sub-chronic *in vivo* experiments

One day prior to the toxicity testing of the MP extracts, adult shrimps were pre-sorted based on total body length. Adult individuals were chosen and separated from the culture. The mean body length (from the rostrum to the beginning of the telson) of the tested animals was 12.6 ± 1.16 mm, while 49% were identified as males and 51% as females (Table S2). Eight replicates of 600 mL test vessels (glass beakers, approximately Ø 9 cm, 63.6 cm^2 as bottom surface) per test concentration were filled with 200 mL test medium (same as in culture) and prepared with the following concentrations: 0.40, 1.00, 2.50, 6.25 and 15.6 g MP-EQs L^{-1} (0.0004–0.0156 kg MP-EQs L^{-1}). These are lower equivalent concentrations of plastics compared to a previous study using up to 100 g of plastic material L^{-1} (*Lithner et al., 2009*), but display MP concentrations of the upper scale that have been found in the environment (*Schmidt, Krauth & Wagner, 2017*). In addition to these five treatments, we conducted a negative (medium), solvent (0.003% of DMSO) and positive (4.5 g NaCl L^{-1}) control. The solvent control corresponds to the highest solvent concentration of the analyzed MP extracts. Afterwards, one individual and 2 mg of ground CrustaGran as food (Dennerle GmbH) was included into each vessel. The test vessels were aerated with glass pipettes and covered with watch glasses. The food was provided daily. On day 1, 3, 7 and 14 of exposure, the behavioral tracking was conducted. In order to minimize a potential ammonium increase, the test solutions were renewed on day 4, 8 and 12 of the exposure. Moreover, we daily recorded the mortality and molting (Table S2) as additional endpoints. After the water renewal on day 8 of exposure and at the end of the experiment, the pH, temperature, oxygen saturation and conductivity were measured (HQ40D multimeter; Hach Lange GmbH, Düsseldorf, Germany). MColortest kits were used to determine the ammonium and calcium carbonate concentration (Merck KGaA, Darmstadt, Germany) (Table S1). After 14-days of exposure, all shrimps were removed from the test vessels, shock-frozen with liquid nitrogen and stored at −20 °C until further analysis. We determined the body length (*i.e.*, from the tip of the rostrum to the telson) and the sex by means of the *appendix masculina* with a stereo microscope (Olympus SZ61) and a digital camera (JVC KY-F75U, Bad Vilbel, Germany) (Table S2).

Prior to the sub-chronic exposure, we conducted acute toxicity tests (72 h) with *N. palmata* and exposed them to sodium chloride (NaCl, CAS: 7647-14-5) in the following concentration range: 1.47–3.59 g L^{-1}. A negative control was conducted in parallel. Every treatment included ten replicates with one individual per vessel. In order to determine the concentration that is lethal to 50% of the organisms (LC_{50}), we recorded the mortality after 24, 48 and 72 h. This enabled us to assess the sensitivity of different individuals towards the same substance throughout every behavioral experiment. The derived LC_{50} was 2.17 g L^{-1} (95%-CI [1.73–2.72] g L^{-1}) after 72 h. As already mentioned, we included 4.5 g NaCl L^{-1} as a positive control in order to achieve a high behavioral response to a known toxicant.

Locomotor tracking

The locomotor activity of four replicates was recorded simultaneously. The vessels in which the animals were recorded correspond to the exposure glass beakers containing the concentrated leaching chemicals and the individual shrimps. Replicates were placed on a bright background and were surrounded by white styrofoam plates to minimize external interferences and light variations. A Sony DSC-RX100 digital camera was mounted on a tripod at a distance of 62 cm. Each replicate was filmed for 10 min after the placement; the last 5 min of the recordings were analyzed as we observed varied locomotor activity at the beginning. Therefore, the initial 5 min of the videos were solely used for acclimatization purposes. Five minutes for analysis was sufficient since preliminary testing revealed insignificant differences between different recording lengths (5, 10, 15 and 20 min) (Fig. S2). The record settings (file format: 50i 24M (FX), ISO: 800, aperture: 3.2, shutter speed: 1/30 s, focus mode: automatic focus) were adjusted to high contrast for efficient tracking of the objects. The use of 200 mL as test medium prevented for the most part vertical movements of the individuals. All recordings were converted into MPEG-4 format using the highest possible resolution. We finally obtained a sample rate of 25 frames per second and a video resolution of 1,920 × 1,080 pixels. For the behavioral analysis, we used the open-source software ToxTrac (ver. 2.91) (*Rodriguez et al., 2018*). Every time the camera was positioned (*e.g.*, at the beginning of the experiments or following battery change), a calibration pattern was recorded (Fig. S3). This was implemented into ToxTrac. The arena definition had to be adjusted for every replicate. Background noise was subtracted. Initially, a threshold was defined, *i.e.*, animals with a visibility rate ≥95% were analyzed. Following this approach, some replicates had to be disregarded from the statistical analysis. We thus decided to also include individuals that had a comparably lower visibility rate. The averaged visibility rate throughout every experiment was still high with 96.5%. A minimum of 83.8% visibility rate was detected for the exposure treatment containing the leachable chemicals from the UV-treated SB foil MPs. False object detections were checked as well (*e.g.*, due to low visibility rate) and corrected when necessary. ToxTrac generated several locomotor parameters (average speed, acceleration, mobility rate, distance traveled and frozen events). We chose the two latter ones as endpoints because the moved distance is commonly used as a parameter (*Faimali et al., 2017*) and the number of frozen events could indicate anxiety as in zebrafish (*De Oliveira et al., 2020*). The frozen events were recorded when objects did not move more than 3 sec and/or 5 mm. In total, we analyzed 896 trajectories, excluding the sodium chloride treatment because of the high mortality observed. A detailed step-by-step instruction for ToxTrac can be made available upon request.

Data analysis

Nonlinear regressions with a four-parameter logistic function were performed in order to derive concentration-response curves and effect concentrations (ECs) for samples of the Microtox assays (GraphPad Prism® 9; GraphPad Software Inc., CA, USA). We derived EC_{20} values, which are provided in mg of MP-EQs $well^{-1}$ and also in g of MP-EQs L^{-1} that

induced 20% of luminescence inhibition. To calculate this, the minimum and maximum effect were constrained to 0 and 100% inhibition, respectively.

Negative and solvent controls were tested for normality using the D'Agostino & Pearson test ($n \geq 8$) or Shapiro-Wilk test ($n = 7$) and for homogeneity of variances with F test. If the data were normally distributed and in case of equality of variances, an unpaired t-test (two-tailed, $\alpha = 0.05$) was performed. Otherwise, a Mann-Whitney test was performed. Both control groups were pooled when no significant differences were detected. Otherwise, we used the solvent control to test against the exposure treatments.

The behavioral data were tested for normal distribution using the D'Agostino & Pearson test and homogeneity of variances with Bartlett's test. In case of normally distributed data and equality of variances, one-way ANOVA with Dunnett's post-hoc test was performed. For non-normally distributed data or in case of variance inhomogeneity, Kruskal-Wallis test with Dunn's post-hoc test was performed. This analysis was conducted to identify significant differences to the control for each individual exposure day. However, since the experiments really represent paired observations, we further performed repeated-measures two-way ANOVA using Geisser-Greenhouse's correction and Tukey's post-hoc test to discern the impact of the exposure day and concentration. In case of significance, we performed repeated-measures ANOVA in order to determine which matched treatments changed over time. If matching was not effective, ordinary one-way ANOVA with Tukey's post-hoc test was conducted. Friedman's test with Dunn's post-hoc was used for non-normally distributed data. The significance level was set to $\alpha = 0.05$ ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$).

RESULTS

Baseline toxicity

The SPE blank and solvent control did not inhibit the bioluminescence of *Aliivibrio fischeri*. In contrast, the enriched MP chemicals, stemming from either the unweathered or UV-treated LDPE-R or SB foil, affected the metabolism of the bacteria (Fig. 1). Both the recycled and biodegradable material released chemicals that were toxic, resulting in low EC_{20} values. The efficacies ranged from 3.48 ± 0.08 mg to 2.97 ± 0.43 mg EQs well^{-1} (= 0.02–0.01 kg EQs L^{-1}) for the unweathered and UV-treated LDPE-R MPs, respectively. In contrast, the unweathered and UV-treated SB foil MPs had comparably lower effects with 12.2 ± 2.06 mg (0.08 kg EQs L^{-1}) and 14.0 ± 1.13 mg EQs well^{-1} (0.09 kg EQs L^{-1}), respectively. While the UV-treated LDPE-R was the most potent MP sample in this assay, the UV treatment did not provoke an elevated toxicity for the SB foil MPs. The toxicities of the tested MPs can be ranked as follows: UV-weathered LDPE-R > LDPE-R > SB > UV-weathered SB. The highest tested concentration of every MP extract (*i.e.*, 668 mg EQs well^{-1} or 4.45 kg EQs L^{-1}) had a comparable impact as 1.40 µg well^{-1} (corresponding to 5.50 × 10^{-5} mol L^{-1}) of 3,5-dichlorophenol applied as the positive control in the Microtox assay (Fig. 1). The derived EC_{20} value of the sigmoidal-shaped curve of the positive control was 3.04 × 10^{-5} mol L^{-1} (*i.e.*, 0.74 µg well^{-1}).

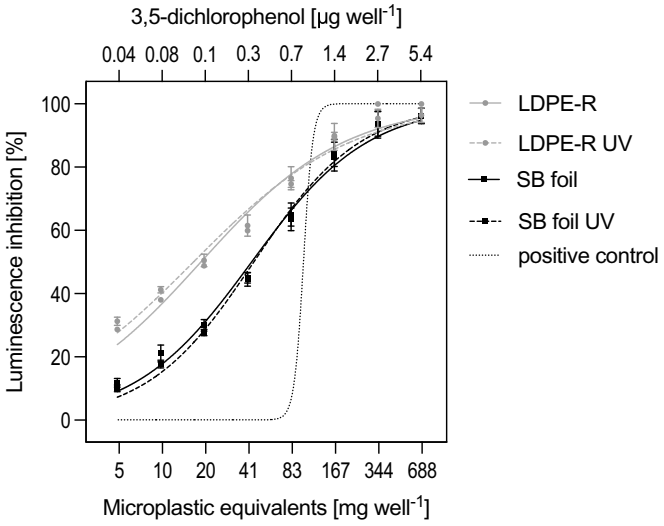

Figure 1 Activity in the Mikrotox assay. Relative luminescence inhibition (mean ± SEM) by unweathered and UV-treated MP samples as well as by the positive control (3,5-dichlorophenol). The LDPE-R and SB foil were tested in mg of MP-equivalents (EQs) well^{-1}; the tested concentration ranged between 5.22 and 688 mg MP-EQs well^{-1}. The positive control was tested in the range of 1.73×10^{-6} to 2.21×10^{-4} mol L^{-1}, which corresponds to 0.04 to 5.40 µg well^{-1}. n = 3 independent experiments. Full-size DOI: 10.7717/peerj.12442/fig-1

LOCOMOTOR ACTIVITY

Unweathered LDPE-R MPs

The moved distance of *N. palmata* individuals was not affected by the chemicals leaching from unweathered LDPE-R MPs (Fig. 2). The statistical analysis with a two-way repeated-measures ANOVA showed that neither the concentration nor the day of the examination (= duration of the experiment) or the combination of both factors had a significant influence ($p > 0.05$). In contrast, the analysis showed that the moved distance of the individuals was the only significant source of variation ($p < 0.0001$). In general, the median moved distances in the MP-EQs exposure groups were either comparable to or lower than the respective control group. Over time, the median distance of the control group decreased from 5.03 m on day 1 (min: 0.10 m, max: 15.3 m, mean: 4.63 m, SD: 4.08 m) to 3.94 m on day 14 (min: 0.18 m, max: 9.68 m, mean: 3.83 m, SD: 3.23 m). This decrease is not statistically significant. Moreover, no obvious trend can be seen, apart from the fact that the NaCl treatment (positive control) led to a fast reduction in locomotor activity on day 3 and to an overall high mortality. The main purpose of the positive control was to assess the sensitivity of the individuals and the analyzed endpoints during the experiments. It turned out that the response of the shrimps was similar between the experiments. Like the moved distance, also the number of frozen events was not affected by the chemicals from LDPE-R MPs (Fig. S4). Again, the two-way repeated-measures ANOVA excluded the concentration and exposure day or the combination thereof as

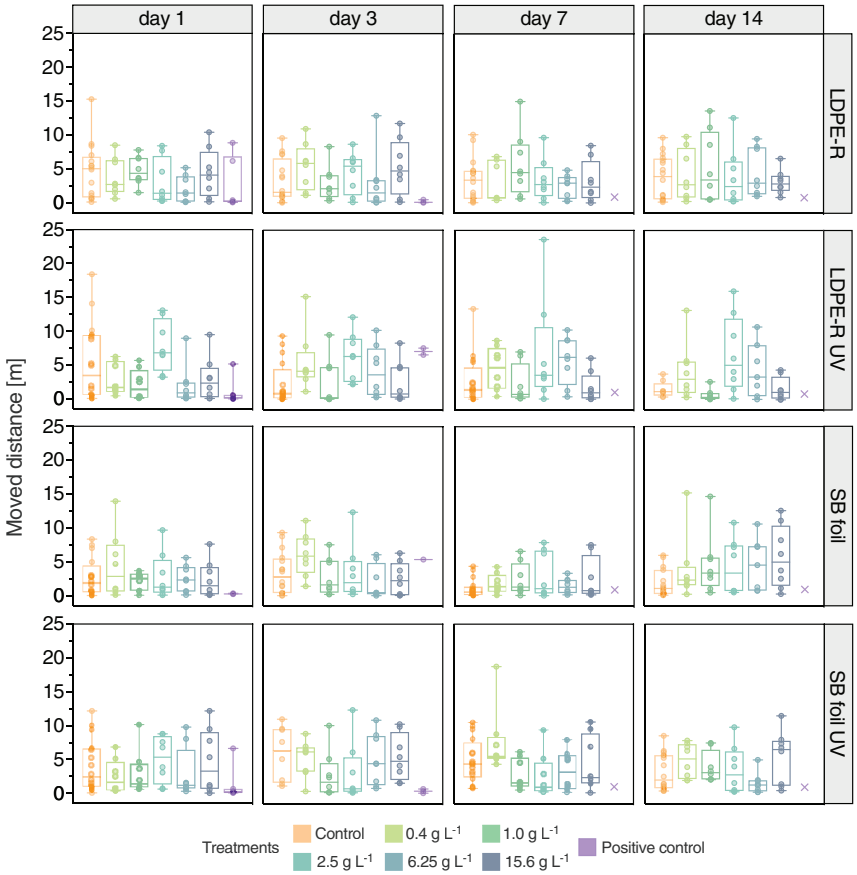

Figure 2 Locomotor behavior of *Neocaridina palmata* in the experiments. Moved distance (m) (median with min–max) of *N. palmata* exposed to the leachable chemicals from unweathered and UV-treated MP samples as well as to 4.5 g L^{-1} of sodium chloride (positive control) on day 1, 3, 7 and 14. Tested concentrations ranged from 0.4 to 15.6 g L^{-1} of MP-equivalents (EQs) for both the LDPE-R and SB foil. Treatments with extinct (crosses) individuals are displayed on day 7 and 14 in the positive control treatments. n = 7–8 for MP-EQs treatments, n = 1–8 for positive control.

Full-size DOI: 10.7717/peerj.12442/fig-2

influencing factors, but confirmed the individuals as the only significant source of variation ($p < 0.01$). The exposure could have resulted either in hypoactivity or hyperactivity (*i.e.*, decreased or increased locomotion) depending on the toxicity mechanism of the tested chemicals. We initially expected a decline in locomotor activity with increasing concentration and exposure duration, especially since 20% of the bacteria in the Microtox assay were inhibited (EC_{20}) by 3.48 ± 0.08 mg LDPE-R MP-EQs $well^{-1}$ (= 0.02 kg MP-EQs L^{-1}, Fig. 1). Furthermore, we observed that the medium in the vessels of the highest concentration had a distinct coloration due to the enriched leachable chemicals (Fig. S5). Hence, *N. palmata* was exposed to a concentrated mixture of LDPE-R chemicals.

No individuals died during the 14-days exposure period, except in the positive control treatment.

UV-treated LDPE-R MPs

In the second experiment, we detected a similar variation of the data when shrimps were exposed to the chemicals leaching from the UV-treated LDPE-R MPs. The median moved distances in the pooled control group decreased from 3.49 m on day 1 (min: 0.07 m, max: 18.4 m, mean: 5.40 m, SD: 5.68 m) to a comparably more clustered locomotor activity on day 14 (median: 1.22 m, min: 0.44 m, max: 3.81 m, mean: 1.55 m, SD: 1.18 m). Significant differences among the MP treatments were detected between 2.5 and 6.25 g L^{-1} for the first day of exposure and between 1 and 2.5 g L^{-1} for the last day of exposure ($p < 0.05$). In the positive control the moved distance was reduced significantly on day 1 ($p < 0.05$), when compared to the control treatment. The median moved distance then increased insignificantly in comparison to the control treatment ($p > 0.05$) on the following recording day. We further could not observe any significant differences within a given exposure group over time. While the concentration of leached chemicals affected the moved distances in some exposure groups ($p < 0.01$), the wide range of individual locomotion was observed to be the dominating source of variation ($p < 0.001$). Generally, there was no concentration-dependent trend towards reduced or increased movements. For frozen events (Fig. S4), the individual moved distances were determined as the source for the variation ($p < 0.001$). After 10 days of exposure, one test organism died in the 1 g of UV-treated LDPE-R MPs L^{-1} exposure group.

Unweathered SB foil MPs

When shrimps were exposed to the chemicals leaching from SB foil MPs, median moved distances were similar for day 1 and 3 (Fig. 2). There were no significant differences among the treatments for a given exposure day or within a given exposure group over time. However, repeated-measures two-way ANOVA revealed that the exposure day was a significant factor ($p < 0.01$). Because a trend of increasing moved distances with increasing concentrations can be seen on day 7 and 14, the locomotor data on day 7 differ significantly to day 3 ($p < 0.05$) and day 14 ($p < 0.01$). This outcome is in contrast to our assumption. Frozen events were not affected by the concentration of leached chemicals, the exposure duration and the combination thereof, but were generally observed to slightly increase up to day 7 and for some treatments on day 14. After nine and 12 days of exposure, one individual died in the 6.25 g L^{-1} and solvent treatment, respectively.

UV-treated SB foil MPs

Regarding the UV-treated SB foil MPs, the leachable chemicals did not affect the moved distance of *N. palmata*. Neither the differences among the treatments nor between the exposure groups and the pooled control group were statistically significant. The comparison of the repeated measurements within a given exposure group over time revealed a similar picture, except that the 0.4 g L^{-1} treatment differed on day 1 and 7 ($p < 0.05$) and the 6.25 g L^{-1} treatment differed on day 3 and 14 ($p < 0.01$). The variation in

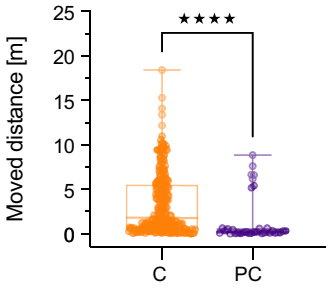

Figure 3 Cumulated data of the moved distance (m) (median with min–max) for *Neocaridina palmata*. Cumulated replicates (n = 239) of the pooled negative and solvent control (C) were compared to all replicates (n = 33) of the positive control (PC) containing 4.5 g L^{-1} of sodium chloride. Significant differences between these groups were determined by Mann-Whitney test (****$p < 0.0001$).
Full-size DOI: 10.7717/peerj.12442/fig-3

locomotor data was the main reason for this statistical outcome ($p < 0.001$). The latter is true as well for the number of frozen events ($p < 0.01$). The positive control was significantly different to the corresponding control treatment ($p < 0.05$ on day 1 and 3). In this last experiment, no mortality occurred except for the positive control.

Sodium chloride as positive control

In the positive control (4.5 g NaCl L^{-1}) all organisms died either by the sixth or seventh day of exposure, revealing a comparative sensitivity throughout the experiments. As displayed in Fig. 2, the locomotion declined rapidly followed by a high mortality. In sum, significant behavioral differences between the positive control and the control group were observed on days 1 and 3 in the experiments with UV-treated MPs ($p < 0.05$). On day 3, the remaining three shrimps responded with high (LDPE-R UV) as well as low locomotion (SB foil UV) in the positive control ($p < 0.05$). In the two other experiments (LDPE-R and SB foil), the opposite locomotor behavior was observed. However, when we compare the cumulative data for all time points of the pooled control (negative and solvent control) to the replicates of the positive control, the difference for the moved distance between these two groups was highly significant ($p < 0.0001$, Fig. 3). Median values of the pooled and positive control were 1.79 m (min: 0.03 m, max: 18.4 m, mean: 3.40 m, SD: 3.45 m) and 0.24 m (min: 0.01 m, max: 8.86 m, mean: 1.60 m, SD: 2.72 m), respectively. Despite the high variation of moving activity, this analysis underpins the negative impact of the positive control on the condition of the test organisms after a short period of time, which is subsequently reflected in hypoactivity and mortality.

DISCUSSION

Chemicals of toxicological concern in recycled and biodegradable plastic

We examined the locomotor activity of *N. palmata* during the exposure to chemicals leaching from unweathered and UV-weathered MPs that were originally recycled LDPE

pellets and a biodegradable starch blend foil. For a first indication of whether the swimming behavior of the aquatic invertebrate could be affected or not, we evaluated the *in vitro* impact potential of the leachable chemicals from the MPs in the Microtox assay. Independent of the UV irradiation, both plastics leached chemicals that induced high baseline toxicities in the Microtox assay. The enriched leachate from LDPE-R MPs resulted in a higher effect than from SB MPs. The findings are in line with the previous analysis (comp. Table S3 and *Klein et al., 2021a*) where both materials highly inhibited the bioluminescence of *A. fischeri*. This is not surprising because plastics have been demonstrated to release chemicals that are toxic in *in vitro* bioassays (*Yang et al., 2011*; *Coffin et al., 2018*; *Rummel et al., 2019*; *Zimmermann et al., 2019*). This toxicity is *inter alia* attributed to (non-)intentionally added substances, transformation products and yet unknown chemicals (*Groh et al., 2019*). Because plastics consist of different chemical formulations (*Rummel et al., 2019*; *Klein et al., 2021a*), even plastics with the same base polymer cause different *in vitro* effects. This impedes a toxicological ranking based on the polymer type (*Zimmermann et al., 2019*). Recognizing this, neither recycled plastics as such nor starch-based materials in general can be expected to induce adverse effects in different test scenarios. Depending on the application area of plastics different regulatory requirements come into force, *e.g.*, as for food-contact materials (*Daniel et al., 2019*; *Ong, Samsudin & Soto-Valdez, 2020*) and construction products (*Bandow et al., 2018*). Finally, the diversity of substances applied contributes to the complexity of the leached chemical mixtures that can be thoroughly analyzed by different screening methods (*Bradley & Coulier, 2007*; *Capolupo et al., 2020*).

In contrast to an extraction procedure, the leaching method represents more realistic conditions with regard to the exposure condition in the environment. Hence, we focused on chemicals migrating from plastics that could harm aquatic life. To contextualize, the concentration of the leachable chemicals inhibiting the bacteria by 20% (EC_{20}) can be compared to that of 3,5-dichlorophenol as the positive control in the Microtox assay (Fig. 1). Thereby, we can draw conclusions on the environmental relevance of the leached chemicals from our MP samples as it is otherwise difficult to assess. Based on the EC_{20} of 3,5-dichlorophenol (0.74 µg $well^{-1}$, same as 4.95 mg L^{-1}) and data of the *NORMAN network (2021)*, the derived concentration of the positive control would negatively affect *Daphnia magna*. This means that the leachable chemicals from the recycled and biodegradable plastic can be harmful at least to some freshwater invertebrates. This finding is relevant with regard to the circular economy because recycled and bio-based materials as more sustainable alternatives for newly produced petroleum-based plastics are current issues of concern (*De Schoenmakere et al., 2019*). We have shown that these plastics emit chemicals classified as toxicologically harmful. This is in line with the results of *Li et al. (2016a)*, *Li et al. (2016b)*, *Dreolin et al. (2019)* and *Zimmermann et al. (2020)*, who have shown that recyclates and environmentally friendly marketed biodegradable and/or bio-based materials (often called 'bioplastics') can both contain bioactive compounds. Taken together, the data point to toxic plastic-associated chemicals affecting aquatic invertebrates. However, neither recyclates nor 'bioplastics' should be overhauled or stabilized with harmful chemicals (*Lambert & Wagner, 2017*; *Shen et al., 2020*;

Muncke et al., 2020). To counter this problem, materials have to be deliberately developed in view of their sustainability and (eco)toxicological safety (*Mitrano & Wohlleben, 2020*).

UV-weathering causes changes in the toxicological profile of plastics

We further aimed at investigating the impact of UV-weathering on the toxicity of the two materials. Compared to their unweathered counterparts, we observed an increased as well as decreased baseline toxicity of the UV-irradiated MPs. Together with the findings of the baseline toxicities of the original states of the materials as pellets and foil, we mainly found elevated *in vitro* toxicities after the treatment with the artificial UV-A/B light (Table S3). This is consistent with the previously conducted work (*Klein et al., 2021a*) and other findings (*Coffin et al., 2018*; *Rummel et al., 2019*). However, there was one exception: The UV-weathered SB MPs induced a slightly lower toxicity than its unweathered form (Fig. 1). Generally, the findings suggest that the UV irradiation leads to a different toxicological profile of the plastics as a consequence of chemical changes. Moreover, this indicates that the toxicity of the biodegradable microscopic fragments decreases over the course of degradation. This should be further examined in future studies. Based on the adverse effects of the MPs measured *in vitro*, the evaluation of the locomotion of shrimps helps to assess the altered chemical composition, which is important as plastics are subject to degradation processes in the environment (*Sarker et al., 2020*). Earlier, *Bejgarn et al. (2015)* exposed different plastics at several time points to artificial UV light and determined the toxicity of the leachates towards the copepod *Nitocra spinipes*. They observed increased, decreased and constant toxicities and thus could not detect any consistent trend with the increasing irradiation time. Particularly, a LDPE cotton-swab box induced no toxicity, whereas a biodegradable bag (corn starch and aliphatic polyester) was more toxic after the weathering. *Sarker et al. (2020)* observed that long-term environmental weathering reduced the toxicity of a high-density PE bag and PVC matting towards a marine microbe. A comparatively lower hazard can result from aged plastics taken from the environment because the release of chemicals already occurs during the aquatic lifetime (*Gardon et al., 2020*; *Arp et al., 2021*). In contrast, *Luo et al. (2020)* has shown that aged MPs (<500 µm PE that includes chrome yellow) caused an inhibition in algal cells because a comparably higher amount of chromium and lead was released. We studied the compounds that leached within a 24 h time frame. Our mitigated activity of the UV-weathered SB MPs (see EC_{20} values in Table S3) represents only a minor reduction that could be simply attributed to the processing of the foil into MPs, linked to the low temperature of the used liquid nitrogen or the altered surface area (Fig. S1) that was in contact with the leaching medium. Considering the latter, the toxicity should theoretically increase due to the higher surface-area-to-volume ratio (*Muncke et al., 2020*), which we only observed for the LDPE-R MPs compared to its pellets. However, the toxicity is not necessarily related to the concentration of released chemicals but more so to individual toxicants and complex mixtures present in and leaching out from plastic materials (*Klein et al., 2021a*). *Capolupo et al. (2020)* did not evaluate the environmental impact on the chemical release and toxicity of plastics, but they assessed the leachables as well as extractables of post-consumer recyclates (<1 mm) of polypropylene (PP), polyethylene

terephthalate (PET) and polystyrene (PS). Their (non-)target approach led to the detection of various compounds, some of which were specific to the leachate and/or extract and masked otherwise (similarly performed by *Bradley & Coulier, 2007*). To some extent, the leachates (except for PET) were found to elicit effects on freshwater and marine algae as well as a Mediterranean mussel species, which was linked to the detected chemicals. In this section, we outlined that the toxicity of plastic leachates is related to the chemical composition of the plastic, often being unique to the material. As already described by *Bridson et al. (2021)* and discussed here, the adverse effects of plastic leachates are not under debate. However, the extent to which hazardous chemicals are released due to weathering and affect aquatic organisms is of specific concern (*Arp et al., 2021*).

Although we did not chemically analyze the MP leachates in this study, we are convinced that *N. palmata* was exposed to a high number of compounds as measured in our previous work after a similar treatment with and without UV-A/B irradiation (*Klein et al., 2021a*). In the following, we address the identified chemicals labeled as 'confirmed' or 'confident' and excluded the tentatively identified chemicals in order to provide a brief overview. The compounds were compared to the information provided by the Chemicals associated with Plastic Packaging database (CPPdb, *Groh et al., 2019*), *NORMAN network (2021)* and *Aurisano et al. (2021)* and are as follows: di-and tributyl phosphate, acetyltributylcitrate, octabenzone, 5-chlorobenzotriazole and 2,4-dihydroxybenzophenone; these chemicals were released from the LDPE-R pellets. The biodegradable SB foil released 4-acetylbenzoic acid, dibutyl phosphate and many degradants, *e.g.*, related to PBAT and PLA. While the LDPE-R released plenty degradants as well, these were tentatively identified. According to *Groh et al. (2019)*, the substances are used as lubricant, filler, plasticizer, UV stabilizer or absorber, antioxidant, adhesive, antifoaming agent, colorant, solvent and processing aid. Our observation highlights the variety of substances associated with only two synthetic materials. We assume that the chemical mixtures drove the *in vitro* toxicity since high counts of components were detected (*e.g.*, 2,984 for the SB foil and 2,804 for the LDPE-R leachate without UV light). Obviously, the leachates might contain single concerning substances. For some of the plastic chemicals identified in this study, ecotoxicological reports are available (*Capolupo et al., 2020*; *Bolívar-Subirats et al., 2021*; *Sakuragi et al., 2021*). However, it is noteworthy that in this work we did not elucidate whether singular compounds or the mixture caused the *in vitro* toxicity. This should be one research focus of future studies performing effect-directed analysis in order to substitute hazardous chemicals with (eco)toxicological safe alternatives (*Muncke et al., 2020*). Moreover, some of the chemicals appeared solely after the UV treatment, while others leached without the additional UV stressor. Most of them were found after both treatments. *Klein et al. (2021a)* also detected that the enriched leachates of the unprocessed LDPE-R pellets and SB foil triggered other *in vitro* endpoints, *e.g.*, oxidative stress and antagonistic activities. On these grounds, we expected to find behavioral alterations of the atyid shrimp during the exposure to the plastic-associated chemicals.

Plastic chemicals did not substantially alter the locomotor behavior of *Neocaridina*

The moved distance and frozen events as sublethal endpoints were recorded on day 1, 3, 7 and 14 of exposure of the freshwater shrimp to the leachates (Figs. 2 and S4). Despite the *in vitro* findings, the chemical mixtures did not substantially alter the locomotor behavior of the test organism. We detected few statistically significant impacts for the concentration or exposure day and observed mostly varied movements of the shrimps. We further could not observe a common trend in the experiments, except for the SB treatment on days 7 and 14. Here, the median moved distance increased as a function of concentration, suggesting hyperactivity. *Sun et al. (2021)* recently published the results of a meta-analysis on locomotor data of aquatic biota exposed to environmentally measured MP concentrations and included physically- and chemically-mediated (*i.e.*, based on the particulate matter of the pollutant or its intrinsic properties) MP impacts. They outlined a decrease in the average speed and moved distance by 5% and 8%, respectively. Moreover, they have found that a longer exposure duration significantly increased the locomotion. This was explained by an adaptive response to external stressors. Based on this, we additionally analyzed the activity of all individuals, except of the control groups, and observed a slight but insignificant ($p = 0.27$) increase with the exposure duration (Fig. S6). Likewise, a minor but insignificant ($p = 0.64$) reduction in activity can be observed with increasing concentrations. Our locomotor data are not conclusive regarding stimulating or inhibiting effects of leachable chemicals. We still cannot conclude that the shrimps were not affected at all because of the few detected significant impacts. To really assess whether these are negligible or not, it could be beneficial to use our study as a baseline and adjust the test variables. We applied similar test variables in terms of acclimatization period (*Chen et al., 2020*; *Wang et al., 2020*), recording time (Wang et al., 2020*)* and replicate number per treatment as other studies (*Gerhardt, 2020*; *Xu et al., 2020*). It is noteworthy that the test variables for the assessment of the swimming activity can vary greatly (*Faimali et al., 2017*; *Siregar et al., 2021*; *Sun et al., 2021*). Since we have analyzed several recording lengths in a preliminary study and detected no differences between the groups (Fig. S2), we assume that our recording time was sufficient. In any case, we exclude experimental biases for video processing based on the ToxTrac analytics performed by *Henry, Rodriguez & Wlodkowic (2019)*.

On a different note, part of our results indicate that the swimming activity does not change as a function of concentration, except for the unweathered SB treatment on day 14 that suggests hyperactivity because median moved distances increased with the concentration, although this effect was not statistically significant. *Chen et al. (2020)* as well monitored excessive movements of adult zebrafish exposed to 5 µm PS particles and attributed this to the upregulation of estrogen, caused by the exposure to the particulate matter itself. Because behavioral changes can appear at low doses (*Hellou, 2011*; *Melvin & Wilson, 2013*) and accelerated movements can be induced by endocrine disrupting chemicals (*Clotfelter, Bell & Levering, 2004*), hormone-mimicking chemicals in plastics (*Groh et al., 2019*) could theoretically result in nonmonotonic responses (*i.e.*, a biphasic

response or hormesis effect) (*Zala & Penn, 2004*; *Gerhardt, 2007*). The median moved distances of *e.g.*, the LDPE-R UV (day 1) and SB foil UV (day 3) suggest (inverted) U-shaped response curves. Overall, we could not detect any substantial early warning sign indicated by behavioral changes. This is in accordance with *Langlet et al. (2020)* who demonstrated unaffected foraminiferal activity during the exposure to PP leachates. We can add to these findings that the molting behavior of the shrimps occurred regularly (Table S2). *Gerhardt (2020)* used new and aged PET and PLA foils as substrate for *Gammarus fossarum* and similarly detected high variation in the swimming data; the ventilation increased probably as a result of migrating compounds from the PLA that the organism has to cope with (*Amiard-Triquet, 2009*). Moreover, *Xu et al. (2020)* determined that the chemical mixtures from weathered plastic debris influenced the curling rate of Daphnids but not the swimming activity. The debris were weathered under realistic exposure conditions for 20 days and the leachates contained (sub)micro-sized particles, metals, bisphenol A and other compounds. Behavior was also examined in fish (*Jacob et al., 2020*). Here, exploration and locomotor activity were influenced in more than half of the cases by virgin plastic particles. It could be argued that the obtained shrimps were not sensitive to the specific plastic chemicals, thereby eliminating potential effects. To somewhat characterize this, we used a positive control that inhibited the swimming behavior (Fig. 3) and eventually caused death (Fig. 2). In this study, it remains elusive whether the few observed effects stem from plastic-associated chemicals since we detected the high variation of the data as a major influencing factor. Based on the *in vitro* analysis, we showed that the plastics leach toxicants that did not really affect *N. palmata* under the chosen experimental conditions. However, they might affect other responses not assessed in this work. Therefore, harmful compounds associated with plastics must be identified and removed entirely from the production process. To begin with, manufacturers could disclose and reduce the chemicals used in plastics (*Gardon et al., 2020*), especially because weathered plastics release chemicals into the aquatic environment of yet unknown impacts on aquatic biota (*Arp et al., 2021*).

CONCLUSIONS

Weathered and unweathered samples elicited high bioluminescence inhibitions in the bacterium *Aliivibrio fischeri*. While we only detected few significant influences on the shrimps, most of the data were explained by the high varied movements of the individuals. Therefore, no substantial effects could be found. We ascribe the negative results to the low sensitivity of the two examined endpoints of our test organism towards the particular leached compounds. This does not generally rule out hazards of leached plastic chemicals for animals. *Neocaridina* could be more robust to the toxicants than other freshwater organisms. Despite this, we suggest that manufacturers should properly disclose the chemicals used in plastic production as the plastic chemicals triggered some test parameters. While the identification of hazardous chemicals and substitution with (eco)toxicologically safe substances is a promising strategy, it is very time consuming. The materials have to be designed in view of their sustainability and toxicological safety

right from the beginning. At the same time, the diverse chemicals in application have to be reduced and simplified to avoid mixture toxicities.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

Jörg Oehlmann is an Academic Editor for PeerJ. The other authors declare that they have no competing interests.

Author Contributions

- Kristina Klein conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
- Sebastian Heß conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
- Ulrike Schulte-Oehlmann conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
- Jörg Oehlmann conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:
The raw measurements are available in the Supplementary Files.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.12442#supplemental-information.

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A5 Further results

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This annex provides two tabular summaries of the analytical results conducted for the study A1. The chemical analysis was performed by the cooperation partner BfG (Federal Institute of Hydrology), whereas the data was analyzed by the BfG and Goethe University. Some of the data was included in the publication A4 (SB foil (which was not included in A1) and LDPE-R). The compounds were identified with the attribute tentative, confident or confirmed and are annotated according to their detection following the different weathering treatments (DC, UV-C, UV-A/B and UV-A/B_{aq}) and their potential function based on the Chemicals associated with Plastic Packaging database (CPPdb). The identified substances have not been published in A1 in such detail. In publication A1, only a small portion of the data below was summarized. The many detected degradation products are not reported in the tables below.

Table A1: Chemical identification of plastic leachates after different weathering treatments

Substance	CAS	Formula	Attribute	Mass	Retention time	Sample	Treatment
5-Methyl-1H-benzotriazole	136-85-6	$C_7H_7N_3$	confident	134.0701	6.95	LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
Salicylic acid	69-72-7	$C_7H_6O_3$[a]	tentative	137.0236	7.76	SB	DC, UV-A/B, UV-A/B_{aq}
4-Hydroxybenzoic acid	99-96-7	$C_7H_6O_3$	tentative	137.024	5.28	Bio-PBS	UV-C
						SB foil	DC, UV-A/B_{aq}
3-Methylbenzoic acid	99-04-7	$C_8H_8O_2$	confirmed	137.0582	8.66	PET-A	UV-C
4-Hydroxybenzoic acid	99-96-7	$C_7H_6O_3$	confirmed	139.0379	5.4	PET-A	UV-C
						PET-R	UV-C
				139.0381	5.38	SB foil	UV-A/B_{aq}
			tentative	139.0588	5.43	SB	UV-C
Adipic acid	124-04-9	$C_6H_{10}O_4$	tentative	145.0499	6.14	SB	UV-C, UV-A/B_{aq}
				145.05	4.9	Bio-PBS	DC, UV-C, UV-A/B, UV-A/B_{aq}
						SB foil	UV-A/B
				145.0501	4.93	SB	UV-C, UV-A/B
5-Chlorobenzotriazole	94-97-3	$C_6H_4ClN_3$	confirmed	152.0011	7.67	LDPE-R	DC, UV-A/B, UV-A/B_{aq}
2-Hydroxybenzothiazole	934-34-9	C_7H_5NOS	confirmed	152.0146	7.87	PET-A	UV-C
						PET-R	UV-C
4-Acetylbenzoic acid	586-89-0	$C_9H_8O_3$	confirmed	163.0391	6.89	SB foil	UV-C, UV-A/B
			tentative	163.0392	6.51	SB	UV-C, UV-A/B_{aq}
			confirmed	163.0393	6.92	PET-A	UV-C, UV-A/B
						PET-R	UV-C, UV-A/B, UV-A/B_{aq}
			tentative	163.0399	6.95	SB	UV-C
Terephthalic acid	100-21-0[a]	$C_8H_6O_4$	confident	165.0191	5.62	PET-A	DC, UV-C, UV-A/B, UV-A/B_{aq}
						PET-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
4-Acetylbenzoic acid	586-89-0	$C_9H_8O_3$	confirmed	165.0533	6.9	PET-A	UV-C
						PET-R	UV-C, UV-A/B_{aq}

Terephthalic acid related compound		$C_9H_{10}O_3$	tentative	165.0547	6.56	SB	DC, UV-C, UV-A/B, UV-A/B_{aq}
				165.0552	6.61	SB foil	DC, UV-C, UV-A/B, UV-A/B_{aq}
4-Toluenesulfonic acid	104-15-4	$C_7H_8O_3S$	confirmed	171.012	4.71	LDPE-R	UV-C
2-Hydroxyterephthalic acid	636-94-2[a]	$C_8H_6O_5$	tentative	181.0131	5.85	PET-R	UV-C
				181.0135	5.55	PET-A	DC, UV-C, UV-A/B_{aq}
						PET-R	DC, UV-C, UV-A/B
5-Methyl-1H-benzotriazole	136-85-6	$C_7H_7N_3$	confident	181.0713	4.71	PVC-A	UV-C
						PVC-R	UV-A/B
Dibutyl phosphate	107-66-4	$C_8H_{19}O_4P$	confirmed	209.0935	8.16	SB foil	DC, UV-A/B_{aq}
			confident	209.095	7.04	LDPE-R	UV-C
				209.0951	7.12	LDPE-R	DC, UV-A/B, UV-A/B_{aq}
				211.1082	7.04	LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
N-Butylbenzenesulfonamide	3622-84-2[a]	$C_{10}H_{15}NO_2S$	tentative	212.0736	10.79	LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
1,3-Diphenylguanidine[a]	102-06-7	$C_{13}H_{13}N_3$	tentative	212.118	6.01	PVC-A	UV-A/B, UV-A/B_{aq}
						PVC-R	DC, UV-C, UV-A/B
2,4-Dihydroxybenzophenone[a]	131-56-6	$C_{13}H_{10}O_3$	confirmed	213.0546	10.71	LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
Benzothiazole-2-sulfonic acid	941-57-1	$C_7H_5NO_3S_2$	confirmed	213.9626	5.5	PET-A	UV-A/B_{aq}
						PET-R	UV-A/B
				213.9633	5.64	LDPE-R	UV-A/B_{aq}
N-Butylbenzenesulfonamide[a]	3622-84-2	$C_{10}H_{15}NO_2S$	confident	214.0882	10.8	PVC-A	UV-C, UV-A/B, UV-A/B_{aq}
						PVC-R	DC, UV-A/B
N-Butylbenzenesulfonamide	3622-84-2[a]	$C_{10}H_{15}NO_2S$	tentative	214.0886	10.8	PP-H	DC, UV-A/B, UV-A/B_{aq}
						PS-GP	DC, UV-C, UV-A/B, UV-A/B_{aq}
				214.0891	10.79	LDPE	DC, UV-A/B_{aq}
						LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
2,6-Di-tert-butyl-P-benzoquinone	719-22-2	$C_{14}H_{20}O_2$	tentative	221.1521	11.64	LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
Dicyclohexylurea	2387-23-7[a]	$C_{13}H_{24}N_2O$	confident	225.1955	10.85	PVC-A	DC, UV-C, UV-A/B, UV-A/B_{aq}
						PVC-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
Drometrizole	2440-22-4	$C_{13}H_{11}N_3O$	confident	226.0971	15.18	PVC-A	DC, UV-C, UV-A/B, UV-A/B_{aq}
						PVC-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
3,5-Di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	$C_{15}H_{22}O_2$	tentative	233.1534	14.44	LDPE	DC, UV-C, UV-A/B, UV-A/B_{aq}
						LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
				235.1689	14.44	LDPE	DC, UV-C, UV-A/B, UV-A/B_{aq}
						LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
PEG 5 (Polyethylene glycol[a])	4792-15-8[a]	$C_{10}H_{22}O_6$	tentative	239.1482	4.85	PVC-R	DC, UV-C, UV-A/B
N-(2-Hydroxyethyl)-dodecanamide	142-78-9[a]	$C_{14}H_{29}NO_2$	tentative	244.2261	12.79	LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
Tributyl phosphate (TNBP[b])	126-73-8	$C_{12}H_{27}O_4P$	confirmed	267.1714	14.16	LDPE	UV-C
						LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
2-Phenylbenzimidazole-5-sulfonic acid (Ensulizole[a])	27503-81-7	$C_{13}H_{10}N_2O_3S$	confirmed	275.0477	4.85	PET-R	UV-A/B_{aq}
Fenozan	20170-32-5	$C_{17}H_{26}O_3$	tentative	277.1797	13.27	LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione[a]	82304-66-3	$C_{17}H_{24}O_3$	tentative	277.1798	14.91	LDPE	DC, UV-C, UV-A/B, UV-A/B_{aq}
						LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
PEG 6		$C_{12}H_{26}O_7$	tentative	283.1746	5.03	PVC-R	UV-C, UV-A/B, UV-A/B_{aq}
Lauric acid diethanolamide	120-40-1	$C_{16}H_{33}NO_3$	tentative	288.2527	12.45	LDPE	DC, UV-C, UV-A/B, UV-A/B_{aq}
						LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
PPG 5		$C_{15}H_{32}O_6$	tentative	309.2256	7.47	PVC-R	UV-A/B, UV-A/B_{aq}
Octabenzone	1843-05-6	$C_{21}H_{26}O_3$	confident	327.1948	19.12	LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
PEG 7		$C_{14}H_{30}O_8$	tentative	327.2009	5.16	PVC-R	UV-C, UV-A/B, UV-A/B_{aq}
	59599-57-4[a]	$C_{19}H_{32}O_3S$	tentative	341.2157	5.72	PVC-A	UV-C, UV-A/B_{aq}

Benzenesulfonic acid, tridecyl-[a]						PVC-R	UV-A/B, UV-A/B_{aq}
2-hydroxy-123-propanetricarboxylic acid tributyl ester (Tributyl citrate[a] or TBC[b])	77-94-1	$C_{18}H_{32}O_7$	tentative	361.2215	16.55	PVC-A	UV-C, UV-A/B, UV-A/B_{aq}
						PVC-R	DC, UV-A/B_{aq}
				361.2219	15.44	LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
PPG 6 (Polypropyenlglycol[a])		$C_{18}H_{38}O_7$	tentative	367.2687	10.32	PVC-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
Isopropylphenyl diphenyl phosphate[a]	64532-94-1	$C_{21}H_{21}O_4P$	confirmed	369.1225	16.05	PET-R	UV-C
PEG 8		$C_{16}H_{34}O_9$	tentative	371.2273	5.35	PVC-R	UV-C, UV-A/B, UV-A/B_{aq}
2-hydroxy-123-propanetricarboxylic acid tributyl ester +Na	77-94-1	$C_{18}H_{32}O_7$	tentative	383.2036	15.4	LDPE	DC, UV-A/B
						LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
Tris(2-butoxyethyl) phosphate (TBOEP[b])	78-51-3	$C_{18}H_{39}O_7P$	confident	399.2506	14.98	PVC-A	DC, UV-C, UV-A/B, UV-A/B_{aq}
						PVC-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
Acetyltributylcitrate[a] (ATBC[b])	77-90-7	$C_{20}H_{34}O_8$	confident	403.2316	16.57	LDPE	DC, UV-A/B, UV-A/B_{aq}
						LDPE-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
			tentative	403.2316	16.55	PVC-A	DC, UV-C, UV-A/B, UV-A/B_{aq}
						PVC-R	DC, UV-C, UV-A/B, UV-A/B_{aq}
PEG 9		$C_{18}H_{38}O_{10}$	tentative	415.2536	5.52	PVC-R	UV-C, UV-A/B
PPG 7		$C_{21}H_{44}O_8$	tentative	425.3089	9.3	PVC-R	DC, UV-C, UV-A/B_{aq}
PEG 10		$C_{20}H_{42}O_{11}$	tentative	459.279	5.68	PVC-R	UV-A/B
PPG 8		$C_{24}H_{50}O_9$	tentative	483.3506	10.2	PVC-R	DC, UV-C, UV-A/B
PEG 11		$C_{22}H_{46}O_{12}$	tentative	503.3058	5.85	PVC-R	DC, UV-A/B_{aq}
PPG 9		$C_{27}H_{56}O_{10}$	tentative	541.3923	11.08	PVC-R	UV-C, UV-A/B
PEG 12		$C_{24}H_{50}O_{13}$	tentative	547.3318	5.98	PVC-R	DC, UV-A/B
PEG 13		$C_{26}H_{54}O_{14}$	tentative	591.3601	6.2	PVC-R	UV-A/B, UV-A/B_{aq}
PPG 10		$C_{30}H_{62}O_{11}$	tentative	599.4334	11.89	PVC-R	UV-A/B
Irganox 1098	23128-74-7	$C_{40}H_{64}N_2O_4$	tentative	637.4894	16.8	LDPE	UV-A/B, UV-A/B_{aq}
PPG 11		$C_{33}H_{68}O_{12}$	tentative	657.4756	12.72	PVC-R	DC, UV-A/B
PPG 12		$C_{36}H_{74}O_{13}$	tentative	715.5181	13.51	PVC-R	UV-C, UV-A/B_{aq}
PPG 13		$C_{39}H_{80}O_{14}$	tentative	773.5588	14.3	PVC-R	UV-C

[a] added or adapted and verified according to PubChem classification (accessed April 10, 2022; URL: https://pubchem.ncbi.nlm.nih.gov)

[b] Aurisano et al. (2021) Chemicals of concern in plastic toys. Environment International 146, 106194. https://doi.org/10.1016/j.envint.2020.106194

Table A2: Identified chemicals of plastic leachates present in several samples

Substance	CAS	Sample
2-Hydroxy-123-propanetricarboxylic acid tributyl ester	77-94-1	PVC-A, PVC-R, LDPE-R
2-Hydroxy-123-propanetricarboxylic acid tributyl ester +Na	77-94-1	LDPE, LDPE-R
2-Hydroxybenzothiazole	934-34-9	PET-A, PET-R
2-Hydroxyterephthalic acid	636-94-2[a]	PET-A, PET-R
2-Phenylbenzimidazole-5-sulfonic acid	27503-81-7	PET-R, LDPE-R
3,5-Di-tert-butyl-4-hydroxybenzaldehyde	1620-98-0	LDPE, LDPE-R
4-Acetylbenzoic acid	586-89-0	SB foil, SB, PET-A, PET-R
4-Hydroxybenzoic acid	99-96-7	PET-A, PET-R, Bio-PBS, SB, SB foil
5-Methyl-1H-benzotriazole	136-85-6	LDPE-R, PVC-A, PVC-R
7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	82304-66-3	LDPE, LDPE-R
Adipic acid[a]	124-04-9	Bio-PBS, SB, SB foil
Benzothiazole-2-sulfonic acid	941-57-1	PET-A, PET-R, LDPE-R
Dibutyl phosphate	107-66-4	SB foil, LDPE-R
Dicyclohexylurea	2387-23-7[a]	PVC-A, PVC-R
Drometrizole	2440-22-4	PVC-A, PVC-R
Lauric acid diethanolamide	120-40-1	LDPE, LDPE-R

N-Butylbenzenesulfonamide	3622-84-2[a]	PP-H, PS-GP, LDPE, LDPE-R, PVC-A, PVC-R
1,3-Diphenylguanidine[a]	102-06-7	PVC-A, PVC-R
Terephthalic acid	100-21-0[a]	PET-A, PET-R
Terephthalic acid related compound		SB, SB foil
Tributyl phosphate	126-73-8	LDPE, LDPE-R
Acetyltributylcitrate[a]	77-90-7	LDPE, LDPE-R, PVC-A, PVC-R
Benzenesulfonic acid, tridecyl-[a]	59599-57-4[a]	PVC-A, PVC-R
Tris(2-butoxyethyl) phosphate	78-51-3	PVC-A, PVC-R

[a] added or adapted and verified according to PubChem classification (accessed April 10, 2022; URL: https://pubchem.ncbi.nlm.nih.gov)

Table A3: Functions of identified chemicals known to be associated with plastic packaging or toys

Substance	CAS	Sample	Function according to CPPdb and *Aurisano et al. (2021)*
Salicylic acid	69-72-7	SB	Food contact; antistatic; viscosity adjustor; hardener; process regulator; filler; colorant dye pigment; adhesive; monomer; additive
4-Hydroxybenzoic acid	99-96-7	PET-A, PET-R, Bio-PBS, SB, SB foil	Manufacturing plastics; process regulator; colorant; monomer
Adipic acid	124-04-9	Bio-PBS, SB, SB foil	Food contact; process regulator; plasticizer; oxidant; softener; solvent; surface treatment finishing agent; hardener; lubricant; filler; adhesive; co-monomer; monomer; additive
Terephthalic acid	100-21-0[a]	PET-A, PET-R	Food contact, drinking water contact; adhesive; co-monomer; monomer
4-Toluenesulfonic acid	104-15-4	LDPE-R	Manufacturing plastics; manufacturing rubber; food contact; catalyst; antifoaming agent; dispersion agent; processing aid; solvent; raw material for plastics production; surface treatment finishing agent; hardener; filler; adhesive; catalyst
Dibutyl phosphate	107-66-4	SB foil, LDPE-R	Process regulator; lubricant; filler; adhesive
N-Butylbenzenesulfonamide	3622-84-2[a]	PP-H, PS-GP, LDPE, LDPE-R, PVC-A, PVC-R	Plasticizer; adhesive
1,3-Diphenylguanidine[a]	102-06-7	PVC-A, PVC-R	Food contact; drinking water content; process regulator; accelerator; hardener; filler; adhesive; secondary accelerator; foam stabilizer
2,4-Dihydroxybenzophenone[a]	131-56-6	LDPE-R	Food contact; drinking water contact; stabilizer; UV stabilizer; colorant; adhesive consumer use; UV absorber; stabilizer; antioxidant; additive
2,6-Di-tert-butyl-P-benzoquinone	719-22-2	LDPE-R	NIAS, break-down product
Drometrizole	2440-22-4	PVC-A, PVC-R	Food contact; drinking water contact; stabilizer; UV stabilizer; raw material for plastics production; solvent; adhesive; UV absorber; additive
Tributyl phosphate (TNBP[b])	126-73-8	LDPE, LDPE-R	Food contact; antifoaming; softener; process regulator; solvent; lubricant; flame retardant; filler; colorant; adhesive; *plasticizer*
Fenozan	20170-32-5	LDPE-R	Seal material; antioxidant; stabilizer; lubricant; NIAS, break-down product of additives
7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione[a]	82304-66-3	LDPE, LDPE-R	NIAS, break-down product of additives
Lauric acid diethanolamide	120-40-1	LDPE, LDPE-R	Food contact; antistatic; additive
Octabenzone	1843-05-6	LDPE-R	Rubber; food contact; drinking water contact; process regulator; raw material for plastics production; softener; stabilizer; UV stabilizer; colorant; UV absorber; antioxidant; additive; light stabilizers / UV absorbers
2-hydroxy-123-propanetricarboxylic acid tributyl ester (Tributyl citrate[b] or TBC[b])	77-94-1	PVC-A, PVC-R, LDPE-R	Food contact; process regulator; *plasticizer*; colorant; processing aid; solvents; other additives for liquid systems
2-hydroxy-123-propanetricarboxylic acid tributyl ester +Na	77-94-1	LDPE, LDPE-R	Food contact; process regulator; *plasticizer*; colorant; processing aid; solvents; other additives for liquid systems

Tris(2-butoxyethyl) phosphate (TBOEP[b])	78-51-3	PVC-A, PVC-R	Food contact; softener; solvent; surface treatment film forming; surface treatment; lubricant; *flame retardant*; colorant; adhesive
Acetyltributylcitrate[a] (ATBC[b])	77-90-7	LDPE, LDPE-R, PVC-A, PVC-R	Food contact; food packaging; viscosity adjustor; *plasticizer*; colorant; adhesive; additive; processing aids
Irganox 1098	23128-74-7	LDPE	Food contact; antioxidant; process regulator; oxidant; UV stabilizer; additive

[a] added or adapted and verified according to PubChem classification (accessed April 10, 2022; URL: https://pubchem.ncbi.nlm.nih.gov)

[b] Aurisano et al. (2021) Chemicals of concern in plastic toys. Environment International 146, 106194. https://doi.org/10.1016/j.envint.2020.106194;

CPPdb = Chemicals associated with Plastic Packaging database (accessed April 10, 2022; URL: https://zenodo.org/record/1287773#.YlK4ey-21QL) according to Groh et al. (2019) Overview of known plastic packaging-associated chemicals and their hazards. Science of the Total Environment 651, 3253-3268. https://doi.org/10.1016/j.scitotenv.2018.10.015

A6 Publications and conference contributions

Publications

Klein K, Heß S, Schulte-Oehlmann U, Oehlmann J (2021) Locomotor behavior of *Neocaridina palmata*: a study with leachates from UV-weathered microplastics. PeerJ 9:e12442. https://doi.org/10.7717/peerj.12442

Klein K, Heß S, Nungeß S, Schulte-Oehlmann U, Oehlmann J (2021) Particle shape does not affect ingestion and egestion of microplastics by the freshwater shrimp *Neocaridina palmata*. Environmental Science and Pollution Research 28, 62246–62254. https://doi.org/10.1007/s11356-021-15068-x

Klein K, Hof D, Dombrowski A, Schweyen P, Dierkes G, Ternes T, Schulte-Oehlmann U, Oehlmann J (2021) Enhanced *in vitro* toxicity of plastic leachates after UV irradiation. Water Research 199, 117203. https://doi.org/10.1016/j.watres.2021.117203

Wick N, **Klein K**, Schulte-Oehlmann U, Dombrowski A, Oehlmann J, Krause S, Schaum C (2021) Mikroplastik in limnischen Systemen: Einträge aus der Siedlungswasserwirtschaft und ökotoxikologische Effekte. DWA Korrespondenz Wasserwirtschaft (KW) 14, Nr. 3

Klein K, Piana T, Lauschke T, Schweyen T, Dierkes G, Ternes T, Schulte-Oehlmann U, Oehlmann J (2021) Chemicals associated with biodegradable microplastic drive the toxicity to the freshwater oligochaete *Lumbriculus variegatus*. Aquatic Toxicology 231, 105723. https://doi.org/10.1016/j.aquatox.2020.105723

Conference contributions

Klein K, Dombrowski A, Hof D, Schweyen P, Dierkes G, Ternes T, Schulte-Oehlmann U, Oehlmann J (2020) Induction of *in vitro* toxicity by UV-weathered microplastic leachates. International online conference MICRO2020 (Lanzarote) (presentation)

Klein K, Piana T, Schulte-Oehlmann U, Oehlmann J (2019) Effekte von irregulären PLA-Mikropartikeln auf den benthischen Süßwasseroligochaeten *Lumbriculus variegatus*. 24th annual meeting of the SETAC GLB in Landau (presentation)

Heß S, **Klein K**, Schulte-Oehlmann U, Oehlmann J (2019) Aufnahme und Ausscheidung von sekundärem Mikroplastik bei der Süßwassergarnele *Neocaridina* sp. 24th annual meeting of the SETAC GLB in Landau (presentation)

Piana T, **Klein K**, Schulte-Oehlmann U, Oehlmann J (2019) Ingestion of irregularly shaped microplastics by the freshwater oligochaete *Lumbriculus variegatus*. SETAC Young Environmental Scientists Meeting in Ghent (Belgium) (presentation)

Klein K, Nungeß S, Schulte-Oehlmann U, Oehlmann J (2018) Aufnahme und Ausscheidung von Mikroplastik bei der Süßwassergarnele *Neocaridina palmata*. 23rd annual meeting of the SETAC GLB in Münster (Westfalen) (poster)

Piana T, **Klein K**, Schulte-Oehlmann U, Oehlmann J (2018) Aufnahme irregulärer Mikroplastikpartikel durch den benthischen Süßwasserorganismus *Lumbriculus variegatus*. 23rd annual meeting of the SETAC GLB in Münster (Westfalen) (poster)

Klein K, Scherer C, Wagner M (2016) Aufnahme von irregulärem Mikroplastik durch *Daphnia magna*, 21st annual meeting of the SETAC GLB in Tübingen (presentation)

Further presentations

Klein K, Schulte-Oehlmann U, Oehlmann J (2020) Mikroplastik in der Umwelt: Eintrag, Verbleib und Auswirkungen. Hydrobiological seminar series of the Institute Hydrobiology at the Technical University Dresden

Klein K, Schulte-Oehlmann U, Oehlmann J (2019) Alles klar? mikro – Schadstoffe und Plastik in Wasser und Umwelt. Information event in the city hall of Walldorf, Amt für Umwelt Mörfelden-Walldorf

Further work

Klein K, von Randow M, Schulte-Oehlmann U, Oehlmann J (2020) *Chaoborus* und Mikroplastik – Büschelmückenlarven als Räuber von mit Plastikpartikeln beladenen Wässerflöhen