Effects of synthetic polymers on freshwater invertebrates

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Summary

Synthetic polymers (plastics) are essential for our everyday lives. Since the mid-20th century, their production, usage, and consequently their waste, have increased exponentially, introducing a new suite of environmental contaminants. Hence, plastic pollution has received increasing attention from researchers, the media, and the public.

Among the major sources of plastic contamination are mismanaged waste, materials from packaging, construction, and transportation sectors. In the environment, large plastic items undergo chemical and physical degradation, leading to their fragmentation. When they reach a size between 1 and 1000 µm (ISO 24187:2023), these particles are defined as microplastics (MP). MP formed from fragmentation are defined as secondary MP, while those intentionally produced as such (e.g., pellets for manufacture or for personal care products) are called primary. Their persistence causes accumulation in virtually every habitat, leading to ubiquitous contamination with potential ecological risks. Moreover, plastics are often associated with intentionally and unintentionally added chemicals that can leach out during their life cycle, contributing to potential MP toxicity. As a more sustainable alternative, new bio-based and/or biodegradable plastics are promoted. While bio-based plastics are produced from renewable resources, biodegradable ones are not necessarily bio-based but should degrade under appropriate conditions. The use of these so-called bioplastics (bio-based and biodegradable) should reduce the carbon footprint connected to plastic production and its environmental impact, thanks to biodegradability. However, while the use and so the emissions of bioplastics are expected to increase with their market demand, proof of their environmental safety is missing. Early investigations provide first indications that bioplastics might exert similar adverse effects as conventional petroleum-based polymers. Nevertheless, ecotoxicological data are still limited.

Furthermore, synthetic polymer contamination is not exclusively represented by solid plastic. There is, in fact, a class of widely neglected compounds that can dissolve in water. Such water-soluble polymers (WSP) are highly employed in industries, medicine, water-treatment plants, dietary and biomedical products. Once dissolved, WSP are not easily discernible from their matrix, but they are present in the environment. WSP potential effects are just now starting to be explored. Just like MP from bioplastics, WSP interact with the biota, but, due to terminological ambiguity and the current lack of regulations, they have been widely under-researched. In fact, while recent regulations like the EU amendment to REACH (25.9.2023) limit the use of certain MP, biodegradable plastics, and WSP are exempted because they are assumed, given the supposed lack of ecotoxicological data, not to pose an environmental risk (as per article 44).

Given the current lack of knowledge about the potential effects of both biodegradable and bio-based plastics and WSP, in this thesis, I aimed to 1) assess the potential effects of biodegradable bio-based plastics on different freshwater organisms, including unicellular eukaryotes and invertebrates, and 2) provide new knowledge on the effects of five commonly employed WSP on the freshwater cladoceran *Daphnia magna.*

In detail, I pursued the following research objectives:

I. Assess MP uptake and potential trophic transfer in the lower trophic levels, employing unicellular eukaryotes as models;
II. Compare the effects of MP from bio-based and biodegradable as well as petroleum-based plastics and their extracts on *D. magna,* including analysis of clonal sensitivity;
III. Investigate the potential threat represented by five WSP on *D. magna*.

In my thesis, I show the potential trophic transfer of MP in freshwater food webs. I could show the uptake of different MP particles (PS, PET, PP, PVC, PA, LDPE, PHB, PDLLA, PLLA, PBS) in Ciliophora from low trophic levels (Article I) and that trophic transfer occurs

from two Ciliophora *(P. Caudatum* and *Tetrahymena pyriformis)* to an Amoebozoa *(Amoeba proteus*) (Article II). Moving up along the food web to primary consumers, I employed *Daphnia magna* as model organism. *Daphnia* is a keystone species in freshwater ecosystems, linking primary production with higher trophic levels. The use of *Daphnia* as model organisms is widely accepted also because of their amictic parthenogenetic life-cycle, which allows the use of clonal lineages for experimental designs. Using *Daphnia,* I show that adverse effects are not only elicited by conventional plastics (PET) but also by bio-based and biodegradable ones (PBS and PLA) (Article III). Moreover, I show that genetic clonal differences have a significant impact on the outcome of MP exposure studies comparing the effects of one conventional one (PET) to two bio-based and biodegradable (PBS and PLA) (Article IV). Lastly, I investigated for the first time the potential hazard of five common WSP (PVOH, PVP, PAA, PEO, PEG) on *D. magna*. In this study, I showed that WSP can impair *D. magna*'s life history, which over time could cascade over higher trophic levels and impair ecosystem dynamics (Article V).

My results will help to add new knowledge on the potential ecological implications associated with the contamination of MP from biodegradable and bio-based polymers and set the foundation for a better understanding of WSP potential threat.

Zusammenfassung

Synthetische Polymere (Kunststoffe) sind für unser tägliches Leben von wesentlicher Bedeutung. Seit Mitte des 20. Jahrhunderts haben ihre Produktion und Verwendung, und damit auch die entstehenden Abfallmengen, exponentiell zugenommen und eine neue Klasse von Umweltkontaminanten hervorgebracht. Daher hat die Verschmutzung durch Kunststoffe zunehmend Aufmerksamkeit von Forschung, Medien und Öffentlichkeit erhalten.

Zu den wichtigsten Quellen der Kunststoffkontamination zählen unsachgemäß entsorgte Abfälle sowie Materialien aus Verpackung, Bauwesen und Transport. In der Umwelt unterliegen große Kunststoffgegenstände chemischen und physikalischen Abbauprozessen, die zu ihrer Fragmentierung führen. Erreichen diese Partikel eine Größe zwischen 1 und 1000 µm (ISO 24187:2023), werden sie als Mikroplastik (MP) definiert. Durch Fragmentierung entstandenes MP wird als sekundäres MP bezeichnet, während Partikel, die absichtlich in dieser Form hergestellt werden (z. B. Pellets für die Kunststoffproduktion oder Körperpflegeprodukte), als primäres MP gelten.

Aufgrund ihrer Persistenz reichern sich Kunststoffe in nahezu allen Lebensräumen an und führen zu einer allgegenwärtigen Kontamination mit potenziellen ökologischen Risiken. Zudem sind Kunststoffe häufig mit absichtlich oder unbeabsichtigt zugesetzten Chemikalien verbunden, die während ihres Lebenszyklus auslaugen und so zur potenziellen Toxizität von MP beitragen können.

Als nachhaltigere Alternative werden biobasierte und/oder biologisch abbaubare Kunststoffe gefördert. Biobasierte Kunststoffe werden aus nachwachsenden Rohstoffen hergestellt, während biologisch abbaubare Kunststoffe nicht zwangsläufig biobasiert sind, sich jedoch unter geeigneten Bedingungen zersetzen sollen. Die Nutzung dieser sogenannten Biokunststoffe (biobasiert und biologisch abbaubar) soll dank ihrer biologischen

Abbaubarkeit den mit der Kunststoffproduktion verbundenen CO_2-Fußabdruck und Umweltwirkungen verringern.

Obwohl mit steigender Marktnachfrage auch Verwendung und Emissionen von Biokunststoffen voraussichtlich zunehmen werden, fehlen bislang belastbare Nachweise für ihre Umweltsicherheit. Erste Studien deuten darauf hin, dass Biokunststoffe ähnliche schädliche Auswirkungen haben könnten wie konventionelle erdölbasierte Polymere. Dennoch sind verfügbare ökotoxikologische Daten weiterhin begrenzt.

Darüber hinaus beschränkt sich die Kontamination durch synthetische Polymere nicht ausschließlich auf feste Kunststoffe. Es existiert eine bislang weitgehend vernachlässigte Klasse von Verbindungen, die sich in Wasser lösen können. Solche wasserlöslichen Polymere (WSP) werden in Industrie, Medizin, Wasseraufbereitungsanlagen sowie Lebensmittel- und biomedizinischen Produkten eingesetzt. Nach ihrer Auflösung sind WSP schwer von ihrer Matrix zu unterscheiden, verbleiben jedoch in der Umwelt.

Die potenziellen Auswirkungen von WSP werden erst kürzlich untersucht. Ähnlich wie MP aus Biokunststoffen können WSP mit Organismen interagieren, sind jedoch aufgrund terminologischer Unklarheiten und fehlender regulatorischer Vorgaben bislang weitgehend unerforscht. Jüngste Regelungen wie die EU-Änderung der REACH-Verordnung (25.9.2023) beschränken die Verwendung bestimmter Mikroplastikpartikel, doch biologisch abbaubare Kunststoffe und WSP sind davon ausgenommen, da aufgrund des Mangels an ökotoxikologischen Daten angenommen wird, dass sie kein Umweltrisiko darstellen (Artikel 44).

Angesichts des Wissensmangels über die potenziellen Auswirkungen biologisch abbaubarer und biobasierter Kunststoffe sowie von WSP verfolge ich in dieser Dissertation zwei Hauptziele:

1. die potenziellen Auswirkungen biologisch abbaubarer biobasierter Kunststoffe auf verschiedene Süßwasserorganismen, darunter einzellige Eukaryoten und Wirbellose, zu untersuchen, und
2. neue Erkenntnisse über die Auswirkungen von fünf häufig verwendeten WSP auf den Süßwasser-Cladoceran *Daphnia magna* zu gewinnen.

Im Einzelnen verfolgte ich folgende Forschungsziele:

I. Untersuchung der Aufnahme von MP und des potenziellen trophischen Transfers in unteren trophischen Ebenen unter Verwendung einzelliger Eukaryoten als Modellorganismen;

II. Vergleich der Auswirkungen von MP aus biobasierten und biologisch abbaubaren sowie erdölbasierten Kunststoffen und deren Extrakten auf *D. magna*, einschließlich einer Analyse der klonalen Empfindlichkeit;

III. Untersuchung der potenziellen Gefährdung von *D. magna* durch fünf WSP.

In dieser Dissertation zeige ich den potenziellen trophischen Transfer von MP in Süßwasser-Nahrungsnetzen. Ich konnte die Aufnahme verschiedener MP-Partikel (PS, PET, PP, PVC, PA, LDPE, PHB, PDLLA, PLLA, PBS) durch Ciliophora aus niedrigen trophischen Ebenen nachweisen (Artikel I) und zeigen, dass ein trophischer Transfer von zwei Ciliophora (*Paramecium caudatum* und *Tetrahymena pyriformis*) zu einem Amoebozoa (*Amoeba proteus*) stattfindet (Artikel II).

Um entlang des Nahrungsnetzes zu den Primärkonsumenten vorzudringen, verwendete ich *Daphnia magna* als Modellorganismus. Daphnia ist eine Schlüsselart in Süßwasserökosystemen, da sie die Primärproduktion mit höheren trophischen Ebenen verbindet. Ihre Verwendung als Modellorganismus ist auch aufgrund ihres amiktischen parthenogenetischen Lebenszyklus weithin anerkannt, der die Nutzung klonaler Linien in experimentellen Designs ermöglicht.

Anhand von Daphnia zeige ich, dass schädliche Effekte nicht nur durch konventionelle Kunststoffe (PET), sondern auch durch biobasierte und biologisch abbaubare Kunststoffe (PBS und PLA) ausgelöst werden können (Artikel III). Darüber hinaus zeige ich, dass genetische klonale Unterschiede einen signifikanten Einfluss auf die Ergebnisse von MP-Expositionsstudien haben, indem ich die Auswirkungen eines konventionellen Kunststoffs (PET) mit denen von zwei biobasierten und biologisch abbaubaren Kunststoffen (PBS und PLA) vergleiche (Artikel IV).

Abschließend untersuchte ich erstmals die potenzielle Gefährdung von *D. magna* durch fünf häufig verwendete WSP (PVOH, PVP, PAA, PEO, PEG). Dabei zeigte sich, dass WSP die Lebensgeschichte von *D. magna* beeinträchtigen können, was sich im Laufe der Zeit als trophische Kaskade auf höhere trophische Ebenen auswirken und die Dynamik von Ökosystemen beeinflussen könnte (Artikel V).

Meine Ergebnisse tragen dazu bei, neue Erkenntnisse über die potenziellen ökologischen Auswirkungen einer Kontamination durch Mikroplastik aus biologisch abbaubaren und biobasierten Polymeren zu gewinnen und eine Grundlage für ein besseres Verständnis der potenziellen Gefährdung durch WSP zu schaffen.

Figures

Figure 1: plastic classification based on their composition and degradability. The ellipses surround the bioplastics, in yellow are marked the biodegradable ones and in blue those that are biobased. (Adapted from Plastics Europe 2022).

Figure 2: individual of *Aldabrachelys gigantea* ingesting a red string of plastic (Prison Island, Zanzibar; © Simona Mondellini).

Abbreviations

DMSO – Dimethyl sulfoxide

EDS – energy dispersive X-ray spectroscopy

FT-IR – Fourier Transformed – Infra Red spectroscopy

GPC – gel permeation chromatography

LOD – limit of detection

LOQ – limit of quantification

MALDI-TOF MS – matrix-assisted laser desorption/ionization time of flight mass spectrometry

MP – microplastic

MS – mussel shells

MW – molecular weight

NALDI-TOF MS – nanostructured-assisted laser desorption/ionization time of flight mass

PA – poly amide

PAA – poly acrilic acid

PBAT – poly butylene adipate terephtalate

PBS – poly butlylene succinate

PCL – poly capro lactone

PDLLA – poly D,L-lactic acid

PE – poly ethylene

PEG – poly ethylene glycol

PEO – poly ethylene oxide

PET – poly ethylene therephtalate

PLA – poly lactic acid

PLLA – poly L-lactic acid

PMMA – poly methyl metacrilate

PP – poly propylene

PPCP – pharmaceutical and personal care products

PS – poly styrene

PVC – poly vinyl chloride

PVOH – poly vinyl alcohol

PVP – poly vinyl pyrrolidone

Py GC/MS – pyrolysis gas chromatography/mass spectroscopy

QA/QC – quality assurance/quality control

REACH – regulation, evaluation, authorization, and restriction of chemicals; Regulation EC No 1907/2006

SEC-ESI-HRMS – size exclusion chromatography – electrospray ionization – high resolution mass spectrometry

SEM – scanning electron microscopy

WSP – water soluble polymers

WWTP – waste water treatment plants

1. Introduction

1.1 Synthetic polymers – a brief introduction

The world polymer is of Greek origin, where *polys* means "many" and *meros* stands for "parts". In chemistry a polymer is a compound composed by molecules, called monomers, chained repeatedly together. These substances can be both of natural origin (e.g., cellulose, nucleic acids) or man-made. The advent of man-made polymers, that signed the beginning of the "Plastic Age", came with the invention of "Bakelite" in 1909 by Leo Hendrik Baekeland. This event initiated a prolific period, characterized by the invention of several synthetic polymers (e.g., PVC in 1913, PMMA in 1924, PE in 1933, PA in 1938, PET in 1941, PS in 1944, PP in 1954), which revolutionized our way of living. Light weight, low production costs, malleability and durability are just few of the properties that marked the success of the materials we call plastics. The general global excitement about the new possibilities and innumerable applications of these compounds, changed in the beginning of the 21st century, when the consequences of the mass production of plastics began to be apparent (Thompson et al. 2004). The same properties that made plastics valuable are those that make them a persistent environmental contaminant. Raising concern of the environmental implications of the use and abuse of conventional petroleum-based plastics triggered the demand of a more sustainable alternative, which increased the production of bio-based (produced totally or partially from renewable resources) and biodegradable (petroleum- or bio-based plastics that can be degraded in appropriate environmental conditions) plastics. These materials are supposed to reduce the environmental footprint connected to plastic production and contamination, as bio-based ones are produced from biomass, while biodegradable ones are meant to degrade under a specific set of conditions. Biodegradable plastics can be either bio- or petroleum- based.

When we think about synthetic polymers, we often neglect those that are water soluble. These compounds, at certain molecular weight and temperature, can totally dissolve in water and alter its physicochemical properties. Due to their characteristics together with misleading terminology, water soluble polymers (WSP) do not fit in the conventional definition of plastics. Despite they are not as discussed as traditional plastics, these polymers account for approximately 6% of the global polymer market (Wang et al. 2021), which translates in millions of tons of yearly production. Given the variety of applications they are employed to, their presence in the environment and so the interaction with the biota is likely.

1.2 Plastics: from miracle product to environmental problem

Plastic properties have made these materials irreplaceable in our everyday lives. Plastic packaging has increased food shelf life and decreased the carbon footprint caused by transportation thanks to its light weight. In medicine, plastic ensures sterility. Due to its insulating properties, plastic reduces energy consumption, and became essential for the construction sector as part of cables, pipes, seals, and adhesive. Even the most recent technology (e.g. smartphones and computers) rely on the use of plastics (Laforsch et al. 2021).

Since the 1950s the use and production of plastic has always increased. Global data collected by OECD (2022) showed that plastic production reached 460 Mt in 2019, the same report predicts that the amount will triplicate by 2060, reaching 1230 Mt of produced plastic. To date, all sectors employ plastics, nevertheless the packaging one dominates the global plastic market, having reached 36% of the total production in 2017, followed by the building and construction sector (16%) and textiles (14%) (Geyer 2020). The packaging sector is not only the one that produces most primary plastics, but it is also the one that generates the highest

amount of post-consumer waste, as most retail packaging products are single use (Geyer 2020).

The generation of plastic waste increases together with its production, and reached 353 Mt in 2019 alone (OECD, 2022). With plastic waste comes the problem of its disposal. Currently only 9% of the used plastic gets recycled, while 12 % is incinerated and 79% is disposed in managed landfills, open dumps or the environment (Geyer et al. 2017). So far, the efforts spent on plastic recycling have been limited, as this process typically produces products of lower technical and economic value which only delays the final disposal. Therefore, as the vast majority of the plastics produced today have very low degradability, they either get incinerated or they eventually enter the environment (Geyer 2020). Plastics can enter the environment either as large items, called macroplastics, or as small particles, named microplastics (MP). These small particles are called primary MP when are intentionally produced as such (e.g. for cosmetics). MP found in the environment can also be the result of physical and chemical fragmentation of macroplastics, in this case they are classified as secondary MP.

1.2.1 Bioplastics

As the accumulation of MP in virtually all the environmental compartments keeps raising awareness within our society, the demand of a more sustainable alternative rises. To date most of the employed plastics are petroleum-based and non-degradable, so bioplastics should represent a more environmentally friendly substitute. A plastic is defined as bioplastic when it is bio-based, biodegradable or both. When a material is produced, at least partially, from biomass it can be considered bio-based. A plastic can be considered biodegradable when it undergoes biological degradation under appropriate environmental conditions (Goel et al. 2021). Biodegradable plastics can be, but are not necessarily, also bio-based (Figure 1).

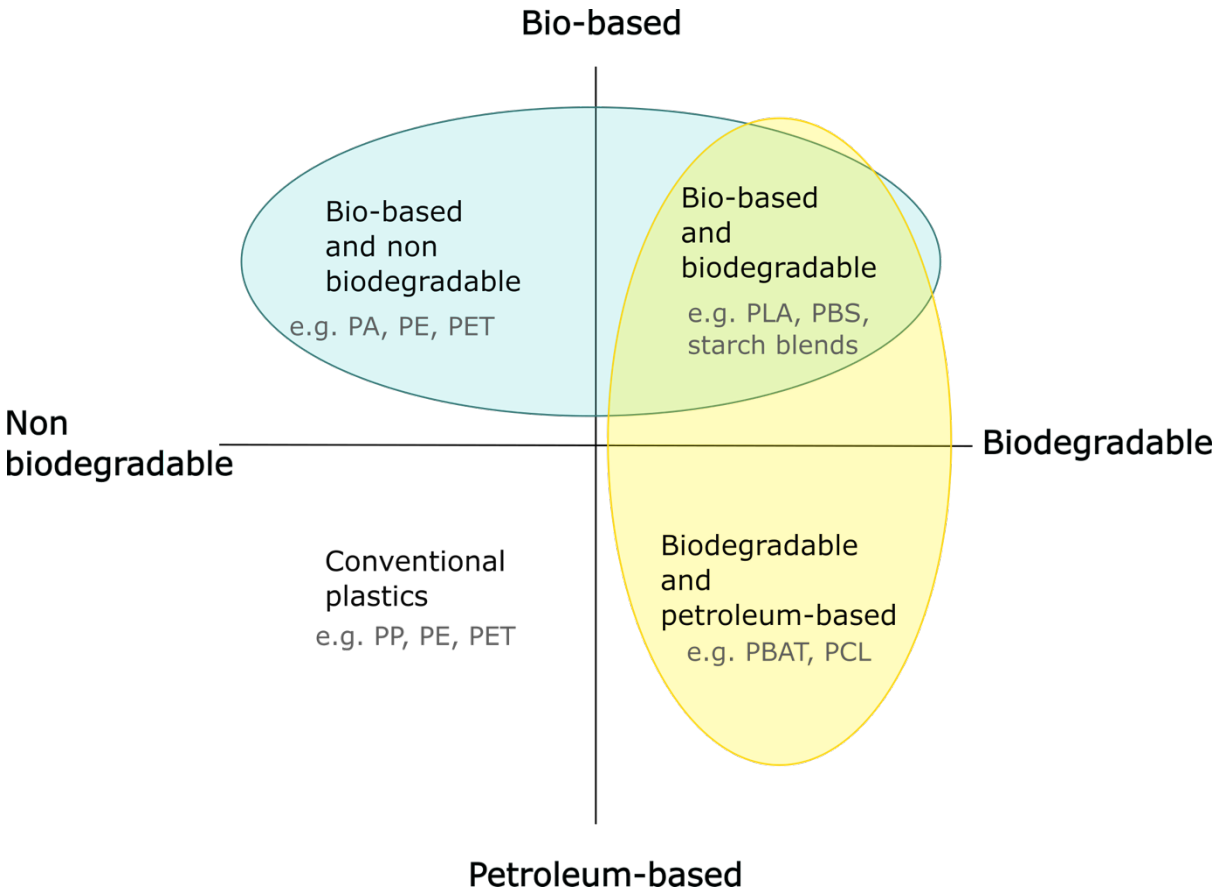


Figure 1: plastic classification based on their composition and degradability. The ellipses surround the bioplastics, in yellow are marked the biodegradable ones and in blue those that are bio-based. (Adapted from Plastics Europe 2022).

Of the 460 Mt of plastic globally produced in 2019 (OECD 2022) 2.3 Mt are labelled as bioplastics. According to a report redacted by Plastics Europe (Plastics Europe 2022) 2.3% of the 57 Mt of plastics produced in Europe in 2021 are bio-based, marking an increase of 0.4 Mt compared to the previous year.

Biodegradable polymers have recently gained a lot of interest, as they were promised as one of the solutions to reduce plastic environmental contamination. Nevertheless, these polymers can only degrade under specific conditions, that are often not met in the environment. Rather, they have been found to fragment in a faster rate compared to conventional plastics (Steiner et

al. 2022), hence contributing to MP accumulation. Effects on the biota of MP from bioplastics to date remains understudied.

1.2.2 Defining microplastics

The term MP was first introduced in 2004 (Thompson et al. 2004), however this paper does not provide a proper definition for these particles. To date, MP are mostly categorized by size. The most widely accepted definition is the one proposed by NOAA (2008) (Arthur et al. 2009) that defines as MP any solid particle with the larger dimension below 5 mm. The lower size limit is often determined by the detection limits of the instrument employed by different research groups, so there is less consensus, even though the upper limit for nanomaterials, 100 nm, can be considered the lower limit for a particle to be considered a MP (Hartmann et al. 2019). In an attempt to establish a common nomenclature for plastic particles Hartmann and coauthors (2019) suggested defining MP as insoluble particles of size 1-1000 μm. This last definition has then been adopted by ISO (ISO 24187:2023). Therefore, for this Thesis' work I will refer to this size classification of MP.

1.2.3 Environmental implications

Nowadays MP have been detected ubiquitously in all environmental compartments, from the Artic (Morgana et al. 2018) to the Antarctic (Rota et al. 2022). It is found in the atmosphere (Gasperi et al. 2018), in the oceans, in ponds, rivers, lakes, glaciers, sediments and soil (Akdogan and Guven 2019). To date there are several methods available for MP detection and quantification. The most employed detection techniques include microscopy, Fourier Transformed Infrared Spectroscopy (FT-IR), Raman Spectroscopy and thermal analyses (Baruah et al. 2022). The detection limit varies greatly among these techniques. Briefly, microscopic and spectroscopic methods can give information on the particle's size and

polymeric composition, for the first one only considering analyses carried out with scanning electron microscope (SEM) coupled with energy dispersive X-ray spectroscopy (EDS). μFT-IR and Raman analyses provide similar information on the chemical identification of the polymers but have different detection limits (ca. 10 μm for μFT-IR and below 1 μm for Raman). Lastly thermal analyses can provide simultaneous information on the polymer and additives, however this is a destructive method that ends up with the complete degradation of the considered sample and provides no information on the particle size (Shim et al. 2017). Often multiple methods are used in parallel to estimate MP abundance and composition in complex matrixes (i.e., environmental samples like water, soil or sediments).

MP environmental concentrations vary greatly according to the chosen method for detection and quantification, the considered compartment, geographical location, land use, development level and economic structure, the weather and even time of the year, spanning from few to thousands of particles (Rios Mendoza and Balcer 2019; Li et al. 2020; Stanton et al. 2020; Talbot and Chang 2022; Li et al. 2023). For example, 3.5×10^{3} particles L^{-1} were detected in the sediments of Lake Huron (US), while only 1.2×10^{-4} particles L^{-1} in the water of Lake Hovsgol from a scarcely populated region in Mongolia (Li et al. 2020). However, quality assurance and quality control (QA/QC) protocols, if enforced, are often not disclosed, hindering the reliability of the reported data.

At the early age of MP research, reflecting the societal concerns, investigations of plastic and MP prevalence and associated effects focused on the marine environment and biota. Quickly the number of investigations showing the negative effects of MP began to increase. While initial studies focused on the marine ecosystems, it is nowadays not the case any longer. Freshwater systems have been recognized not only as a path for plastic contamination to the ocean, but as important sinks themselves (Imhof et al. 2012; Global Plastic Outlook, OECD 2022), so the number of studies concerning these ecosystems increased. Once in the

environment MP can interact with and alter different environmental matrixes; in a water body MP can be detected on the surface, throughout the water column and in the sediment (Bhardwaj et al. 2024). Given MP prevalence, animals living in contaminated systems interact with these particles throughout their lives.

Reports of entanglements and deaths by starvation of charismatic megafauna (Figure 2) due to digestive tracts filled with plastic items or to suffocation helped raising awareness on the issue (e.g., Stelfox et al. 2016). When interacting with the biota, shape, size, polymer type (Schwarzer et al. 2022), plastic associated chemicals (Zimmermann et al. 2019; Zimmermann et al. 2020b) and surface characteristics (Ramsperger et al. 2020; Wieland et al. 2024) have been found to play an essential role in determining the possible effects.



Figure 2: individual of *Aldabrachelys gigantea* ingesting a red string of plastic (Prison Island, Zanzibar; © Simona Mondellini).

MP have been found to be taken up by several organisms across trophic levels, e.g., marine mammals (Nabi et al. 2022), birds (Masiá et al. 2019; Carlin et al. 2020), fishes (Thiele et al.

2021), mussels, planktonic and benthonic species (Setälä et al. 2014), unicellular organisms (Bulannga and Schmidt 2022), etc. Once ingested, MP have been proved to affect survival (Bosker et al. 2019), life history (Messinetti et al. 2018; Schwarzer et al. 2022), reproduction (Sussarellu et al. 2016), behavior (De Felice et al. 2019), metabolic pathways (De Felice et al. 2024), stress and immune responses (Imhof et al. 2017; Limonta et al. 2019) and oxidative status (Xia et al. 2020) of several organisms. Nevertheless, despite some studies on biodegradable and/or bio-based materials have recently been published (Lambert and Wagner 2017; Zimmermann et al. 2020a; De Felice et al. 2024), these investigations mainly focus on non-degradable, petroleum-based MP.

1.3 Water soluble polymers

While plastics are recognized as a global threat to human health and the environment, the vast majority of these investigations suggests that only insoluble polymers are a cause of concern. This, together with a terminological ambiguity (Hartmann et al. 2019), has led to the exclusion of WSP from recent regulation designs addressing the issue of MP pollution (Tarring et al. 2024a). Given that these compounds are not REACH registered (Regulation EC No 1907/2006), scarce information are available on their production volumes. Nevertheless, indirect estimations can be made basing on the educts of polymer synthesis that are REACH registered (Huppertsberg et al., 2020). This method permitted to estimate production rates of millions of tons per year (e.g. PEG ~ 10^6 t/a; PAA ~ 10^5-10^6 t/a).

Despite the massive production rates, WSP prevalence in the environment has not been investigated until recently. WSP are employed in a variety of different fields that facilitate their direct and indirect immissions into the environment. WSP are in fact employed as e.g. flocculants in water treatment plants, for oil recovery, in pharmaceutical and personal care products (PPCP) as thickeners and densifiers, in paint, coatings, fertilizers, in the textile

industry (Huppertsberg et al. 2020; Tarring et al. 2024a), just to mention few of their applications. Their presence in the environment is, therefore, certain.

Given their properties WSP can completely dissolve in water, hence, they do not wash up on shores as MP do (Huppertsberg et al. 2020), so their contamination is not as apparent.

A limit in the investigation of WSP effects and prevalence is the lack of suitable analytical techniques for their detection. So far the methodologies that can be employed are gel permeation chromatography (GPC), matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) (Tarring et al. 2024a), nanostructured LDI-TOF MS (NALDI-TOF MS) (Wang et al. 2021), and continuous-flow off-line pyrolysis gas-chromatography/mass-spectrometry (Py-GC/MS) (Antić et al. 2011; Vidovic et al. 2022). Nevertheless, the organic matter in real environmental samples still poses a limit for these methodologies. Given the detection limit, information regarding WSP environmental prevalence are scarce, nevertheless, some studies already highlight their presence in freshwaters. For instance, PVP, widely used in biomedical and food industry (Julinová et al. 2018), has been detected in a concentration range that spans from 0.9 to 7.1 mg L^{-1} in an effluent of a waste water treatment plant (WWTP) in Germany (Antić et al. 2011); PEG, also employed for biomedical purposes, in PPCP and in the automotive industry, was detected in fresh falling snow with a concentration of 2.19 μg L^{-1} (Wang et al. 2021). Lastly, more recently Pauelsen and co-authors (2023) developed a sensitive methodology (size exclusion chromatography – electrospray ionization – high resolution mass spectrometry; SEC-ESI-HRMS) for the detection of low MW compounds that allowed them to detect PEO in the effluent of WWTP and surface waters in 18 different locations in Germany with concentrations respectively of 20 and below 11 μg L^{-1}.

Once in the water compartment degradation might take place, both in WWTP and in the environment, but it is a slow process that requires a very specific range of conditions. WSP

can undergo mechanical (via turbulent flow), chemical (hydrolysis), UV, thermal- and bio-degradation. Biodegradation is strictly linked to the molecular weight (MW), with higher MW being harder to degrade; it can be carried out by e.g., bacteria, fungi and algae, often it is performed by specific strains that have been selectively domesticated (Wang et al. 2023). Abiotic degradation also requires specific condition of turbulence, salinity, pH, temperature and illumination. Degradation time can vary greatly in the environment, for example PEG can be completely degraded in freshwater, but the time spans from 14 to 65 days according to its MW with lower degradation rates in seawater in aerobic conditions (Bernhard et al. 2008; Wang et al. 2023). A similar trend was observed for PVA that was found to reach 21% degradation in 13 days in freshwater inoculated with WWTP's bacteria (Alonso-López et al. 2021). PEG can also be degraded in anaerobic condition, but the entire process can take up to 165 days (Traverso-Soto et al. 2016). Other polymers, like PVP, are not degradable under environmental conditions (Julinová et al. 2018).

1.3.1 Environmental implications

Given the wide employment and often poor degradation rates of WSP, their interaction with the biota is certain. The investigation of their ecotoxicological implications are only at their infancy, few studies are in fact now available researching their potential effects towards the biota. For example, Nigro and co-authors (2022) evaluated the toxicity of PVOH (0.001, 0.5 and 1.0 mg L^{-1}) for 14 days on *D. magna* and for 5 days on *Danio rerio* and identified no significant negative effects in terms of life history and neurotoxicity. Nascimento and co-authors (2021) investigated the potential toxicity of PEG (72 hours, 5 and 10 mg L^{-1}) on *Physalaemus cuvieri* tadpoles and observed an alteration of the oxidative status of the exposed organisms, together with an increased activity of acetylcholinesterase and butyrylcholinesterase. In another recent work Nigro et al. (2024) exposed *D. magna* to

PVOH, PAA, PVP and PEG (0.001, 0.5 and 1.0 mg L^{-1}) and evaluated their potential effects on life history, heartrate, swimming activity, neurotoxicity and energetic biomarkers. Their investigation highlighted an increase in mortality and neurotoxicity when the daphnids were exposed to PEG and that all the WSP they investigated altered the heartrate, the swimming behavior, the metabolic pathways and energy allocation. The same research group exposed *D. rerio* to the same concentrations of PVP, PEG and PAA (Binelli et al. 2024) and recorded a general protein upregulation for all the WSP and an altered oxidative status for the zebrafish exposed to PAA and PVP. Lastly Tarring et al. (2024b) were able to identify PVP within the tissues of *D. magna* using gel permeation chromatography (GPC) after a 48 hours exposure to 50 mg mL^{-1} of the selected compound. They were however not able to identify a specific amount within the organism, because the concentration was comprised between the limit of detection (LOD) and the limit of quantification (LOQ) of the instrument (0.02 – 0.5 mg mL^{-1}), confirming the interaction with the WSP.

2. Knowledge gaps and objectives

1) Assessment of the potential effects of petroleum-based and biodegradable bio-based MP on freshwater organisms

The so-called bioplastics have been promised as a potential solution to the environmental problem caused by plastic pollution. Nevertheless, these polymers, do not biodegrade in environmental conditions and they have been found to produce an even higher number of MP compared to petroleum-based plastics. Like traditional plastics, bioplastics are manufactured with a range of plastic associated chemical, many of which can leach into the environment and contribute to their overall toxicity. Despite these concerns, current and emerging regulations fail to include bioplastics in risk assessment frameworks, largely due to a supposed lack of relevant ecotoxicological data.

1.1) Effects of MP originated from petroleum based and biodegradable-biobased plastics in unicellular eukaryotic organisms.

Knowledge gaps:

Unicellular eukaryotic organisms are essential for the ecosystem dynamics. In fact, they connect primary production, detritus, other microorganisms and higher trophic levels. Despite their fundamental biological role, studies on MP uptake and possible effects on their population dynamics are currently lacking (both regarding petroleum based and bioplastics). The few studies already published on the topic do not include environmentally relevant concentrations and neglect the potential transfer of MP via predation from different organisms on the lower levels of the food web.

Objectives:

- Prove the uptake and trophic transfer of fluorescent PS-MP in different size ranges among different unicellular eukaryotic organisms (from *P. caudatum* and *T pyriformis* that served as preys, to the selected predator *A. Proteus*);
- Expose *P. caudatum* to different MP, both from biodegradable bio-based and petroleum-based plastics, in an array of concentrations;
- Confirm MP uptake via fluorescence microscopy and Raman Spectroscopy;
- Determine whether the formation of an eco-corona/biofilm might influence the outcome of MP exposure on *P. caudatum.*

1.2) Effects of MP originated from petroleum based and biodegradable-biobased plastics in *Daphnia magna.*

Knowledge gaps:

Numerous studies focused on MP ecotoxicological assessment are carried out using *D. magna* as model organism. Nevertheless, different clonal sensitivities of the employed *Daphnia* are hardly ever taken into account when it comes to MP investigations, hampering studies comparability. Furthermore plastics have a complex chemical make up: different plastic associated chemicals could in fact be responsible for MP's ecotoxicological effects. Hence it is fundamental to investigate the drivers of MP toxicity, distingushing between the effects of the MP as they are, their extracts and the exctacted particles containing less chemicals.

Objectives:

- Expose *D. magna* to MP from biodegradable bio-based and petroleum-based plastics to compare their potential effects;
- Show that the employed *D. magna* clone can influence the outcome of MP exposure studies considering sublethal parameters;
- Investigate the effects of extracted plastic associated chemicals obtained from both bioplastics and petroleum-based polymers on *D. magna.*

2) Water soluble polymers

Knowledge gaps:
WSP are a highly employed class of polymers whose demand accounts for approximately 6% of the global polymer market. Despite being used in a variety of consumer products and biomedical and industrial sectors, investigations concerning their potential effects on aquatic biota are lacking.

Objectives:

- Provide a first assessment of the ecotoxicological effect of five commonly used WSP on *D. magna*'s life history.

3. Synopsis

3.1 Summary of the articles

3.1.1 Effects of microplastic from petroleum-based and bioplastic on freshwater invertebrates

3.1.1.1 Effects on unicellular freshwater organisms

Article I - ***Effects of petroleum-based and biodegradable bio-based microplastics versus natural control particles on Paramecium caudatum, combined with in situ Raman spectroscopic detection***

Article I had multiple objectives. One major aim I wanted to address in this Article was to investigate the potential effects of several MP, including both petroleum-based and biodegradable bio-based plastics, on the unicellular freshwater organism *Paramecium caudatum*. The second aim of this article was to elucidate whether pristine and environmentally conditioned MP could elicit different responses on *P. caudatum.* Finally, as third aim of this Article, I aspired to verify the internalization of the selected particles within the ciliates' cytoplasm, which led to the development of a novel μ-Raman spectroscopic approach.

Unicellular eukaryotic organisms are fundamental components of the ecosystem, constituting the base of aquatic the food web. Although understanding the effects of MP contamination on these organisms is crucial for predicting ecosystem-level impacts, this group remains significantly understudied. Moreover, the exclusive use of pristine particles in experiments limits the ecological relevance and applicability of the findings. To better simulate the

plastisphere typical of freshwater environments, my collegues and I preconditioned all the particles used in this study by incubating them for two weeks in water collected from an outdoor pond and in MilliQ.

To address the first two aims I conducted a 72-hour individual exposure to three concentrations of PLLA, PDLLA, PBS, P3HB, PA, PET, PS, PVC, LDPE and PP MP, along with cellulose, kaolin, grinded mussel shells and Tween20 as control groups. Each exposure was performed using pristine and environmentally conditioned particles. The results highlighted no difference between the effects induced by freshwater- and MilliQ- incubated particles and no concentration-dependent effect, reflecting the non-selective feeding behaviour of *P. caudatum.* Surprisingly, no particle exerted a significantly negative effect. On the contrary, the exposure to biodegradable bio-based MP, PET, cellulose and kaolin was associated with an increase in cell proliferation, possibly suggesting that *P. caudatum* may utilize these materials as a carbon source.

Verification of particle uptake via μ-Raman spectroscopy was performed following an overnight incubation. All particles, apart from cellulose and kaolin, were identified within the exposed individuals. While not every particle type was detected in every cell, the successful identification of all MP types within the ciliates marks an important step toward the development of dose–response models. The spectroscopic method established in this study paves the way for future quantitative analyses of MP uptake in *P. caudatum* and other ciliates.

Overall, the findings that my colleagues and I recorded in this Article were unexpectantly positive. Additional investigations would be necessary to elucidate the mechanisms of action underlying this seemingly positive effect and to explore their broader ecological significance.

Article II - *Size dependent uptake and trophic transfer of polystyrene microplastics in unicellular freshwater eukaryotes*

In Article II, I employed *Tetrahymena pyriformis, P. caudatum* and *Amoeba proteus* as model organisms to investigate the uptake and potential trophic transfer of differently sized fluorescently labelled PS-MP (specifically 0.5 µm and 6 µm beads and fragments < 30 µm) in the lower trophic levels of freshwater ecosystems.

Given the high environmental abundance of MP in smaller size ranges, these particles are available to a broad spectrum of suspension feeders, including unicellular eukaryotic organisms, which are often non-selective in their feeding behaviour. As a result, these organisms tend to ingest MP, and, since they represent the base of the food web, can serve as vectors for MP transfer to higher trophic levels.

In this Article, my colleagues and I selected *T. pyriformis* and *P. caudatum* (both suspension feeders), to investigate size dependent MP uptake and to act as prey in a simplified food web. *A. proteus* was selected as the predator in this system to investigate whether it independently internalize MP, which particle size it could ingest, and if MP uptake increases through predation on the two ciliates.

Size dependent uptake was proved for all three model organisms. *P. caudatum* individuals internalized particles up to 27.7 µm, while *T. pyriformis* ingested MP up to 10 µm. Although both ciliates exhibited high initial uptake rates, the number of individuals containing MP declined markedly after 24 hours. *A. proteus* was only able to internalize MP on its own through pinocytosis, which limits uptake to smaller particles (0.5 and 6 µm) that correspond to the diameter of its pinocytic channel (approximately 6 µm). Interestingly, these smaller MP remained detectable within the cytoplasm up to 14 days post-exposure. Larger MP fragments were detected inside *Amoeba* only following predation on the two ciliates. MP

bioaccumulation in the lower trophic levels could be of particular concern, as not only it could alter their population dynamics, but also cascade over higher trophic levels.

3.1.1.2 Effects on *Daphnia magna*

Article III - *Same but different: The response to petroleum and biodegradable bio-based microplastics deviates between Daphnia magna clones*

In Article III, I investigated the effects of one petroleum-based (PET) and two biodegradable bio-based (PLA and PBS) MP on two different *D. magna* clones. Biodegradable and bio-based plastics are often presented as "environmentally friendly" alternatives to conventional petroleum-based plastics. However, when released in the environment, they can generate equal or even greater quantities of MP, which might negatively affect the organisms. Despite increasing recognition of this issue, investigations on their potential effects are still scarce.

For this study I chose *D. magna* as model organism, as its use is widely accepted in ecological and ecotoxicological investigations. Its amictic parthenogenetic reproduction should ensure the production of genetically identical individuals, which should enable to obtain a homogeneous response to contaminants and ensure comparability among studies. Despite this desirable characteristic, studies on *Daphnia* often report diverse results. Such discrepancies could arise from differences in the physicochemical properties of the tested MP or from varying clonal sensitivities among *Daphnia* strains.

To address this, my colleagues and I conducted a 21-day chronic toxicity test using two *D. magna* clones (BL2.2 and Aig). Individuals of both clones were exposed to two concentrations of each polymer and of microcrystalline cellulose, used here as particle control. The exposure was conducted under limited food availability, to resemble a more

environmentally relevant scenario. As endpoints, we selected to record survival, reproductive output and neonates' sex, juvenile body length growth rate and body length at the end of the exposure.

The results showed that, while mortality was similar between the two clones, there were significant differences in sublethal endpoints. Furthermore, concentration-dependent adverse effects from both the petroleum-based and biodegradable bio-based MP were oberved, regardless of the *Daphnia* clone. These findings underline the urgent need for further research into the ecotoxicological effects of biodegradable bio-based plastics, which so far appear to be at least as harmful as traditional petroleum-based materials.

Article IV - *Extracted chemicals from microplastics exert increased adverse effects on Daphnia magna compared to the pristine particles*

In Article IV I explored the potential drivers of MP toxicity, by testing one petroleum-based (PET) and two biodegradable-biobased (PLA and PBS) MP, alongside microcrystalline cellulose as a particle control. To investigate the role of plastic-associated chemicals, the selected MP underwent a solvent-based extraction protocol in methanol. The resulting extracts, containing leached compounds, were then transferred into DMSO for use in *D. magna* exposures.

Ten *D. magna* individuals were exposed for 10-day, under food limiting conditions, to the following treatments: unextracted MP, extracted MP (eMP), MP extracts, DMSO (solvent control), a blank control and a food level control provided with food *ad libitum*. Every treatment was replicated five times. As endpoints my colleagues and I considered any alteration in body length at day six, the number and sex ratio of the offspring, the level of reacted oxygen species (ROS) and of lipid peroxidation (LPO).

The results obtained highlight that body length was primarily affected by the plastic-associated chemicals, with the extracts of PLA and PET exerting the worse effects. Reproductive outcomes were more complex, with all three treatments (MP, eMP, and extracts) exerting adverse effects on offspring number. Regarding biochemical endpoints, no significant changes were observed in ROS or LPO levels across treatments, except in the *ad libitum* fed group, which showed lower ROS and significantly higher LPO, likely a consequence of increased lipid reserves.

Overall, drawing definitive conclusions about the mechanisms driving MP toxicity remains challenging. However, the data suggest that plastic associated chemicals contribute substantially to observed effects, while the presence of particles themselves also appears to have an impact over the reproductive output.

3.1.2 Effects of water soluble polymers on *Daphnia magna*

Article V - ***Beyond microplastics: Water soluble synthetic polymers exert sublethal adverse effects in the freshwater cladoceran Daphnia magna***

In Article V, I explored the potential toxicity of five commonly used WSP (i.e., PVOH, PVP, PAA, PEO and PEG) using *D. magna* as a model organism. The study included an acute heart rate assay, a 48-hour immobilization test, and a 21-day chronic exposure. While no acute effects were detected, the chronic test revealed significant sublethal impacts. During the 21-day chronic toxicity test I monitored any alteration in survival, in body size (both width and length) when the organisms reached sexual maturity and at the end of the exposure, any change in terms of fitness (number of produced offspring and number of reproductive cycles), and ROS levels at day 21. While no increased mortality was recorded, PVOH, PEO and PEG reduced the average number of produced offspring, whereas all WSP except PEG caused a decrease in the number of reproductive cycles. Morphological parameters recorded at the end of the exposure were also affected, with PVOH causing a decrease both in length and in width, and PVP determining an increase in body length. Analyses of the ROS levels indicated an altered oxidative status for all considered polymers. So, while these WSP did not induce any acute alteration, they did cause changes in life-history and morphological parameters. These findings suggest that WSP, despite being considered safe, can affect key life-history traits and physiological parameters in *D. magna*. This is concerning, as changes in body size may increase vulnerability to predation, while impaired fitness could disrupt population dynamics and propagate effects through the food web. Overall, in this Article I highlight the need to investigate the potential effects of WSP, which should warrant greater attention in regulatory framework alongside conventional MP.

3.2 Discussion

Plastic pollution is now a recognised global environmental and societal problem. Despite polymer contamination and the consequent effects of exposure on the biota have been object of extensive research, numerous knowledge gaps are still to be addressed. Moreover, some of the efforts introduced to mitigate the environmental impact connected to plastic contamination, like the introduction in the market of environmentally friendly alternatives to conventional petroleum-based polymers, have added to the complexity of the MP-related issue.

For instance, little is known about the effects of MP uptake at the lower levels of the food web, the potential similarities in the ecotoxicological effects of petroleum-based and bioplastics, or the environmental risk posed by water soluble polymers, which, though invisible to the naked eye, represent an emerging concern. Even when employing one of the most used model organisms for ecological and ecotoxicological investigation, i.e., *Daphnia magna,* research often overlooks a critical aspect: its clonal reproduction. While this trait makes *D. magna* highly suitable for controlled experiments, differences in clonal sensitivity may influence results, adding an additional layer of complexity to MP-related investigations.

Therefore, the aim of my dissertation was to address two main knowledge gaps: 1) to investigate the effect of petroleum-based and biodegradable bio-based plastics on freshwater unicellular organisms and invertebrates, and 2) to evaluate the potential threat represented by WSP on the freshwater cladoceran *D. magna.*

To address the first identified knowledge gap, I initially focused on the lower levels of the food web, starting with unicellular eukaryotic organisms. These organisms play a pivotal role within freshwater communities by linking primary production, detritus and other microorganisms with higher trophic levels (Mansano et al. 2016).

Despite their ecological importance in freshwater ecosystems, few studies so far dealt with the possible implications of MP uptake at this level of the food web, and with their potential trophic transfer (Gupta et al. 2017; Bulannga and Schmidt 2022). MP-driven effects on their population dynamics could, in turn, influence the bacterial communities they prey upon, as well as the primary consumers that feed on them, with the potential to disrupt the structure and functioning of the entire freshwater food web.

To establish a foundation for investigating MP transfer in a simplified artificial food web, I first examined the uptake of MP fragments by *P. caudatum.* This ciliate is well suited to be used in laboratory conditions, and its fast reproduction rates make it ideal for standardized toxicity testing. The tested MP fragments included both biodegradable bio-based (PLLA, PDLLA, PBS, P3HB) and petroleum-based MP (PA, PET, PS, PVC, LDPE, PP), together with three natural control particles (cellulose, kaolin and mussel shells), in both pristine form and environmentally conditioned (incubated in freshwater from an outdoor pond). All employed particles were selected in a similar size range (< 20 µm).

This study enabled the assessment of the effects of various polymers on cell proliferation, expanding beyond PS, which is the most studied MP in similar investigations (e.g., Scherer et al. 2017; Liang et al. 2021; Zhang et al. 2021). Interestingly, biodegradable bio-based MP determined an unexpected significant increase in cell proliferation, whereas paramecia exposed to petroleum-based MP had proliferation rates comparable to that of the control treatment. Additionally, by incubating the used particles both in ultra-pure and in freshwater, I evaluated potential differences in toxicity of pristine and environmentally conditioned particles (Ramsperger et al. 2020). Particle incubation was carried out for two weeks prior to exposure, and the formation of an eco-corona/biofilm was confirmed via SEM imaging. This step was included as previous studies reported that environmentally conditioned particles exhibited higher cellular internalization rates (e.g., Ramsperger et al. 2020), which could

translate into more severe effects for the exposed organisms. Despite no significant differences were highlighted in this study concerning the particle incubation, reflecting the non-selective feeding behaviour of *P. caudatum*, this approach could represent an essential step in formulating a more realistic risk assessment. Lastly, particle uptake was confirmed using a novel µ-Raman method, which did not require the use of any chemical fixation or of fluorescent dyes, establishing a valuable tool for future studies aiming to create a real dose-response curve.

To further investigate trophic interactions, I recreated a simplified food web, involving two prey species (*P. caudatum* and *T. pyriformis*) and one predator (*A. proteus*). To facilitate visualization, I employed fluorescently labelled PS beads and fragments in different sizes. Initially, PS-MP were first administered separately to each of the selected organisms, so to confirm size-dependent uptake. Subsequently, *A. proteus* was exposed to MP-fed preys.

Size-dependent particle uptake was confirmed for all three eukaryotes: *P. caudatum* was able to ingest the largest fragments (up to ca. 27 µm), *T. pyriformis* took up particles up to 10 µm, while *A. proteus*, despite its greater size, took up only the beads in the smaller size fraction (0.5 and 6µm) when exposed directly to MP. This reflects the feeding behaviour of the chosen model organisms, and it is connected to their average size. The two ciliates, in fact, are unselective suspension feeders, thus the size of the particles that they are able to take up is strictly related to their own body size (in our cultures *T. pyriformis* has an average size of 40 µm, while *P. caudatum* of approximately 200 µm). The number of observed internalised MP within the ciliates was highest 2 hours post exposure but declined rapidly thereafter; no MP-carrying individuals were detected 24 hours after exposure. This indicates that MP release takes place and that the egested MP are not re-ingested a second time.

A. proteus (which in our cultures has an average size of ca. 500 µm), on the other hand, employs two distinct feeding mechanisms: phagocytosis and pinocytosis. Phagocytosis

enables the amoeba to feed selectively, often avoiding non-motile particles, such as MP, by relying on the chemical and mechanical stimuli that are characteristic of a living prey. In contrast, pinocytosis is a non-selective uptake mechanism that allows the internalization of particles smaller than the diameter of the pinocytic channels, estimated to be around 6 µm (Klein et al. 1989). This unselective process also leads to the uptake of small MP, as confirmed by the obtained results. Furthermore, the observed increase in pinocytic uptake following the experimenrtal alteration of the exposure medium to increase pynocitosis rates provides additional support for this interpretation. Therefore, we can conclude that the MP detected inside *A. proteus* were taken up passively via this last mechanism. MP taken up via this route could be detected within the amoebae cytoplasm up to 14 days post exposure. This outcome may be explained by the fact that the selected PS-MP did not alter the cytoplasmic osmotic balance, and therefore did not trigger particle egestion. Larger particles (fragments < 30 µm) were not phagocitated by *A. proteus*, despite attempts to induce uptake via protein coating. Nevertheless, when fed MP-laden preys, larger MP were also detected within the cytoplasm of *A. proteus.* However, in this case, retention time was shorter than for MP taken up via pinocytosis, as most individuals expelled the particles within 72 hours. While active excretion explains this observation, it can also be hypothesized that this outcome resulted from an asymmetrical cell division of *A. proteus* (Prescott, 1955), which could have diluted the proportion of MP-laden individuals.

Despite in this study ecotoxicological effects were not investigated, the obtained results remain concerning. Ciliophora can take up tens of MP, which, despite the relatively short retention time, has unclear ecological consequences. *A. proteus,* capable of ingesting up to 70 cells per day, could potentially ingest hundreds of MP via predation. Therefore, bioaccumulation of MP in these organisms, has high biomagnification potential, not only

among other unicellular organisms, but also to higher trophic levels (Zhang et al. 2021; Bulannga and Schmidt 2022; Sarker et al. 2022).

Moving up along the freshwater food web, *D. magna* was selected as model organism. This species plays a central role in lentic ecosystems, both by regulating populations of primary producers and by serving as a key food source for a wide range of predators. Therefore, alterations in *D. magna*'s population dynamics could have impacts over entire ecosystems.

D. magna is widely employed in evolutionary, ecological and ecotoxicological research (Brehm et al. 2023), due to the ease at which it can be maintained under laboratory conditions, its sensitivity to toxicants and its ability to reproduce via amictic parthenogenesis under optimal environmental conditions (Sarma and Nandini 2006). This reproductive strategy enables the use of maternal clonal lines, producing offsprings that are genetically identical to the parental organism, which, in turn, should ensure homogeneous responses in exposure studies. However, the clone lines used across laboratories are different, due to different native environments, and are rarely reported in the published works, which could hinder comparability and reproducibility of the studies (Picado et al. 2007). In MP research, the issue is further complicated by the diverse physicochemical properties of the material, which can influence their environmental behaviour and biological interactions (Salehi et al. 2024). Furthermore, plastics are associated with a wide array of intentionally and unintentionally added chemicals, that can contribute to their potential toxicity (Rochman et al. 2019; Da Costa et al. 2023; Wagner et al. 2024; Monclús et al. 2025). As a result, even when comparing the effects of supposedly identical MP in terms of polymeric composition, size and surface characteristics (Ramsperger et al. 2022; Wieland et al. 2024), results could differ also due to the presence of several plastic associated chemicals. Introducing clonal variability into this already complex scenario may render meaningful conclusions difficult to achieve.

To address these challenges, I exposed two *D. magna* clones (Aig, originating from Bavaria, and BL2.2, originating from Belgium) to two biodegradable bio-based MP (PLA and PBS), a petroleum-based MP (PET) and microcrystalline cellulose fragments as particle control. This experimental design was meant to elucidate potential differences in toxicity of different concentrations of petroleum-based and biodegradable bio-based MP, while at the same time evaluating potential differences in the sensitivity of the two selected clones. The study was conducted following the OECD guidelines for chronic exposures (OECD No. 211), with one modification: the organisms were not fed *ad libitum* (2 mg C L^{-1}), but instead they were given 0.5 mg C L^{-1} of the green algae *Acutodesmus obliquus.* This change was introduced to recreate a more environmentally realistic scenario representative of mesotrophic lakes during summer months. The results obtained in this study highlight that, even if mortality is comparable between the two clones, these are significantly different in all considered sublethal responses. In particular, the Aig clone appears to be significantly more sensitive to the experimental conditions than BL2.2. Despite the clones having different sensitivities it was still possible to assess MP induced effects. Results highlighted a concentration dependent negative effect of all three polymers compared to cellulose and that biodegradable bio-based MP, despite being marketed as an environmentally friendly solution to the issue represented by plastic pollution, can be equally toxic to conventional materials. These results contrast with what was observed for *P. caudatum*, in which case biodegradable bio-based MP induced a seemingly positive effect on asexual division. Differences between these two model organisms are however to be expected, due to their distinct physiology and levels of biological complexity. Furthermore, an increase in ciliates cellular proliferation, despite being seemingly positive, may lead to unforeseen consequences at population and community levels. These observations provide a valuable

foundation for future investigations aimed at better understanding broader ecological implications connected to MP contamination.

To disentangle the drivers of MP toxicity I designed a follow up experiment, employing the same MP (PBS, PLA and PET) and control particle (microcrystalline cellulose fragments), maintaining an equal nutrition level described above (0.5 mg C L^{-1}). In this work I compared the effects of one concentration of MP, eMP and extracts. The employed eMP and extracts were produced via solvent-based extraction from the selected MP.

To date, over 16,000 chemicals have been identified as being intentionally or unintentionally added to plastics, of these 4,219 are recognized as chemicals of concern (Wagner et al. 2024; Monclús et al. 2025). Nevertheless, only 6% of these compounds are currently regulated by Multilateral Environmental Agreements (e.g. Basel, Stockholm and Minamata Conventions; Montreal Protocol) (Monclús et al. 2025). These compounds leach out during the entire life cycle of plastics and interact with organisms, exerting largely unknown effects. Thus, the exposure experiments performed to test the effects of MP, eMP and extracts were conducted on *D. magna*, specifically on the clone BL2.2, previously identified as the most suitable due to its lower mortality rates. By using this approach, it was possible to assess both particle- and chemical-mediated effects under controlled laboratory conditions, offering valuable insights into the potential impacts of MP on *D. magna*.

Unlike the previously described experiment conducted on single individuals, this study involved five replicates per treatment consisting of ten organisms each. Next to the above-mentioned treatments, I included a blank, a DMSO, and a food level control fed *ad libitum*, to account for the solvent and nutrition related effects.

The results of this work further highlighted the complexity of MP mode of action. GC/MS analyses revealed the presence of various plastic associated chemicals, even though only four could be unequivocally identified based on their Kovats indices (Kováts 1958). While

none of the detected chemicals was previously reported as toxic alone, the exposure to their mixture could have unpredicted negative effects (Silva et al. 2022). Morphological parameters were primarily affected by the extract treatment, emphasizing the contribution of plastic associated chemicals in MP toxicity. However, all particle components from the selected polymers (MP, eMP and extracts) negatively affected the number of produced offspring, indicating that both particle- and chemical-mediated mechanisms can influence reproductive outcomes.

Next to life history parameters, the levels of ROS and LPO were also investigated. MP exposure can cause an imbalance in the daphnids' oxidative status, which, in turn, could determine the disruption of macromolecules like lipids (De Felice et al. 2022). In this experiment, however, the biochemical analyses of ROS and LPO levels did not reveal any significant alteration. The organisms' defences against oxidative damage are physiologically costly. While changes in ROS and LPO levels have previously been reported following MP exposure (e.g., Roy et al 2023; De Felice et al. 2024; Oduro et al. 2024), the absence of measurable oxidative damage might reflect a reallocation of energy in favour of antioxidant and detoxifying machinery. This energetic trade-off may have reduced the resources available for growth and reproduction (Barata et al. 2005), consistent with the observed results.

The control treatments, consisting of a blank, cellulose control (as MP, eMP and extracts), DMSO and food level control, did not negatively affect the exposed organisms. The only consistently different treatment was the food level control, in which, in line with our expectation, the animals grew larger and produced higher number of offspring. This control group also exhibited lower ROS levels but significantly higher LPO levels, potentially linked to an higher lipid content in these animals compared to those exposed to a restricted feeding regimen, which therefore might have had a lower pool of peroxidable substrate

(Yang et al. 2021). These findings highlight that MP exposure under environmentally relevant conditions may lead to stronger effects than those typically reported in current literature. While classical experimental designs have been pivotal in advancing our understanding of MP effects, these results underscore the need for further studies incorporating realistic exposure scenarios.

Lastly, the second aim of this dissertation was addressed by investigating the effects induced by five commonly used WSP. This class of synthetic polymers has, to date, received relatively limited attention compared to conventional plastics. One reason may be the terminological ambiguity surrounding the term plastic (Hartmann et al. 2019), which refers exclusively to solid items, thereby excluding WSP from both scientific discourse and current regulatory frameworks (Tarring et al. 2024a, 2024b). Furthermore, WSP contamination is inherently less visible than MP pollution, making it less likely to draw attention despite its potential environmental relevance (Huppertsberg et al. 2020). To this must be added the current limitations in detecting WSP within environmental matrices, which hinder the determination of their environmental concentrations and, consequently, impair accurate hazard assessment. In this study *D. magna* individuals were acutelly and chronically exposed to PVOH, PVP, PAA, PEO and PEG. These WSP are widely used for a variety of everyday applications, which facilitates their entry into WWTP and ultimately ensures their presence in the environment, making the interaction with aquatic biota certain.

Despite the results of my work did not highlight any acute alteration following the exposure to the selected WSP, such as increased mortality or effects on the heartrate, sublethal effects were recorded following a chronic exposure. These included significant changes in morphological parameters, reproductive output and levels of ROS.

D. magna covers an essential role in lentic communities, being prymary consumers of phytoplankton and serving as s key food source for secondary consumers (Diel et al. 2020). As such, it is essential for the transfer of carbon and energy within freshwater food webs. Alterations in daphnids population may disrupt both the top-down control of algal proliferation, and the bottom-up regulation of higher trophic levels. Consequently, the recorded negative effects of both MP and WSP could not only impair *D. magna*'s population dynamics, but also cascade over higher trophic levels, ultimately affecting the structure and function of freshwater ecosystems (Bosker et al 2019).

To conclude, while the field of MP research has expanded significantly over the past two decades, several critical knowledge gaps remain unaddressed. With this Dissertation I aimed at adding new insights to the current body of knowledge concerning polymers ecotoxicological effects by:

1) providing new evidence on the impacts of MP uptake at the basal level of freshwater food webs, showing that not only unicellular eukaryotes are able to internalize MP of different sizes, but that they may also represent an important pathway for biomagnification to higher trophic levels;
2) showing that petroleum-based and biodegradable bio-based plastics and their extracts exert comparable effects on *D. magna*, while highlighting the importance of environmentally relevant experimental designs. These findings indicate that biodegradable bio-based plastics should not be regarded as a straightforward solution to plastic contamination and highlight the need for them be included by policy makers in new regulatory designs. Moreover, results obtained with different *D. magna* clones reveal variability in their response to MP exposure, emphasising the necessity to report lineage information to ensure study comparability;

3) reporting, for the first time, the negative effects of WSP on *D. magna*, highlighting the urgent need to extend the current research efforts also on the investigation of these so far neglected class of polymers.

It is noteworthy that persistent inconsistencies in definitions and terminology continue to hinder not only the comparability of studies but also the effectiveness of regulatory efforts, e.g., by deliberately excluding bioplastics and WSP from the latest regulatory designs. So, while these are intended to mitigate the effects of MP pollution, they may result in the development and consequent introduction in the environment of newer, but equally dangerous, materials. Moreover, the current directives indirectly encourage the use of biodegradable and WSP given the supposed lack of relevant ecotoxicity data, thereby possibly increasing the issue.

In the present thesis, I aim to address part of this issue by conducting a more comprehensive investigation into the effects of MP on unicellular eukaryotic organisms, as well as by demonstrating the environmental threats posed by both biodegradable bio-based polymers and WSP. This work, therefore, contributes to the growing body of knowledge on polymer pollution and may serve as valuable resource for future research, as well as for policymakers who may one day consider the inclusion of these compounds in regulatory frameworks addressing polymer contamination.

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4. Authors' contribution

Article I- Effects of petroleum-based and biodegradable bio-based microplastics versus natural control particles on *Paramecium caudatum*, combined with *in situ* Raman spectroscopic detection

Authors: Michael Schwarzer*, **Simona Mondellini***, Vinay Kumar Bangalore-Narayana, Simon Wieland, Sven Ritschar, Marvin Kiene, Julian Brehm, Holger Kress, Martin G.J. Löder, Magdalena M. Mair, Christian Laforsch

(*contributed equally to this work)

Contributions:

MS and **SM**: Conceptualization, Methodology, Investigation, Data curation, Writing - Original Draft, Writing - Review & Editing, Formal analysis, Visualization, Project administration, Validation. VKBN: Investigation, Methodology, Validation, Formal Analysis, Visualisation, Writing - Review & Editing, Data Curation. SW: Investigation, Methodology, Visualization, Writing - Review & Editing. SR: Formal analysis, Writing - Review & Editing. MK and JB: Writing - Review & Editing, Formal analysis. HK: Project administration, Funding acquisition. MGJL: Writing - Review & Editing, Validation, Project administration, Supervision. MMM: Data Curation, Formal analysis, Validation, Writing - Review & Editing. CL: Conceptualization, Resources, Writing - Review & Editing, Validation, Project administration, Supervision, Funding acquisition.

Status: Published (DOI: 10.1016/j.jhazmat.2026.141575)

Individual contributions of authors with shared first authorship: Michael Schwarzer and Simona Mondellini contributed equally to this article and therefore share first authorship. Both authors were involved throughout the research process, including conceptualization, investigation, data analysis, validation, and all writing phases of this manuscript (creation of the original draft, review, and editing).

Simona Mondellini took the lead in developing sample-processing methodologies and in data curation, including the creation and organization of the raw dataset. Michael Schwarzer led

the microscopic investigations, including those of the organisms, and the creation and editing of schematic visualizations.

Validation of the results and interpretation of the findings were carried out collaboratively. Michael Schwarzer additionally took primary responsibility for coordinating research activities and managing task distribution.

Article II - Size dependent uptake and trophic transfer of polystyrene microplastics in unicellular freshwater eukaryotes

Authors: Simona Mondellini*, Michael Schwarzer*, Matthias Völkl*, Julia Jasinski, Valérie Jérôme, Thomas Scheibel, Christian Laforsch, Ruth Freitag

(*contributed equally to this work)

Contributions:

SM, MS and MV: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. JJ: Investigation. VJ: Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. TS: Writing – review & editing, Resources, Project administration, Funding acquisition. CL: Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. RF: Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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Individual contributions of authors with shared first authorship: Simona Mondellini, Michael Schwarzer, and Matthias Völkl contributed equally to this article and therefore share first authorship. All three authors were involved across the entire research process, including

conceptualization, methodology development, investigation, formal analysis, validation, and all the phases of manuscript preparation (creation of the original draft and review and editing). Simona Mondellini was primarily responsible for conducting uptake experiments on organisms and quantifying particle uptake. Michael Schwarzer led the acquisition and processing of microscopic images, including organism imaging, particle uptake, and visualization of trophic transfer, as well as SEM micrographs. He also took primary responsibility for project administration, including coordinating research activities and managing individual responsibilities.

Matthias Völkl was primarily responsible for managing and organizing particle-related datasets.

Overall, the study was developed through close collaboration among all authors, with shared responsibility for data interpretation and result validation.

Article III - Same but different: The response to petroleum and biodegradable bio-based microplastics deviates between *Daphnia magna* clones

Authors: Simona Mondellini*, Michael Schwarzer*, Marvin Kiene, Gabriël Olthof, Dipannita Ghosh, Seema Agarwal, Martin G.J. Löder, Matthias Schott, Martin Wagner, Christian Laforsch

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SM and MS: Conceptualization, Methodology, Investigation, Data curation, Writing - Original Draft, Writing - Review & Editing, Formal analysis, Visualization, Project administration, Validation. MK: Writing - Review & Editing, Formal analysis, Visualization. GO: Conceptualization, Investigation, Writing - Review & Editing. DG: Investigation,

Writing - Review & Editing. SA: Project administration, Funding acquisition, Resources, Writing - Review & Editing. MGJL and MS: Investigation, Writing - Review & Editing, Validation, Project administration, Supervision. MW and CL: Conceptualization, Resources, Writing - Review & Editing, Validation, Project administration, Supervision, Funding acquisition.

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Individual contributions of authors with shared first authorship: Simona Mondellini and Michael Schwarzer contributed equally to this article and therefore share first authorship. Both authors were involved throughout the research process, including conceptualization, investigation, data analysis, validation, and all writing phases of this manuscript (creation of the original draft, review, and editing).

Michael Schwarzer was responsible for the acquisition and processing of microscopic images, including organism imaging and SEM micrographs, as well as the creation and editing of schematic visualizations. He also led the collection of morphological data.

Simona Mondellini was responsible for statistical analysis and interpretation of the data, as well as data curation, including the creation and organization of raw datasets. She also took primary responsibility for project administration, including coordinating research activities and managing individual responsibilities.

Overall, the study was conducted through close collaboration, with both authors contributing to data interpretation and validation of results.

Article IV - Extracted chemicals from microplastics exert increased adverse effects on *Daphnia magna* compared to the pristine particles

Authors: Simona Mondellini*, Michael Schwarzer*, Matthias Schott, Marvin Kiene, Bettie Cormier, Dipannita Ghosh, Martin G.J. Löder, Seema Agarwaal, Martin Wagner, Christian Laforsch

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SM and MS: Conceptualization, Methodology, Investigation, Data curation, Writing - Original Draft, Writing - Review & Editing, Formal analysis, Visualization, Project administration, Validation. MS: methodology, Investigation, Data curation, Formal analysis, Writing – Review & Editing. MK: Writing - Review & Editing, Formal analysis, Visualization. BC: Methodology. DG: Investigation, Writing - Review & Editing. SA: Project administration, Funding acquisition, Resources, Writing - Review & Editing. MGJL: Investigation, Writing - Review & Editing, Validation, Project administration, Supervision. MW and CL: Conceptualization, Resources, Writing - Review & Editing, Validation, Project administration, Supervision, Funding acquisition.

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Michael Schwarzer was primarily responsible for acquiring and processing microscopic images, including organism imaging and SEM micrographs, as well as creating and editing schematic visualizations. He also contributed to data curation by creating raw datasets.

Simona Mondellini led the statistical analysis and interpretation of the data and was primarily responsible for project administration, including coordinating research activities and managing individual responsibilities.

Methodological development was carried out collaboratively, with Simona Mondellini leading the chemical extraction of microplastic particles in DMSO for exposure experiments and the biochemical analyses of the exposed organisms, and Michael Schwarzer leading the chemical extraction in methanol for chemical characterization and organism measurements.

Overall, the study was conducted through close collaboration, with both authors contributing to data interpretation and validation of results.

Article V - Beyond microplastics: Water soluble synthetic polymers exert sublethal adverse effects in the freshwater cladoceran *Daphnia magna*

Authors: Simona Mondellini, Matthias Schott, Martin G.J. Löder, Seema Agarwaal, Andreas Greiner, Christian Laforsch

Contributions:

SM: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Formal analysis, Visualization, Project administration, Validation. MS: Conceptualization, Methodology, Investigation, Writing - Review & Editing, Formal analysis, Validation. MGJL: Conceptualization, Resources, Writing - Review & Editing, Validation, Project administration, Supervision, Funding acquisition. SA: Project administration, Funding acquisition, Resources, Development of Reference Material. AG: Project administration,

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Article I

Effects of petroleum-based and biodegradable bio-based microplastics versus natural control particles on *Paramecium caudatum*, combined with *in situ* Raman spectroscopic detection

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Effects of petroleum-based and biodegradable bio-based microplastics versus natural control particles on *Paramecium caudatum*, combined with *in situ* Raman spectroscopic detection

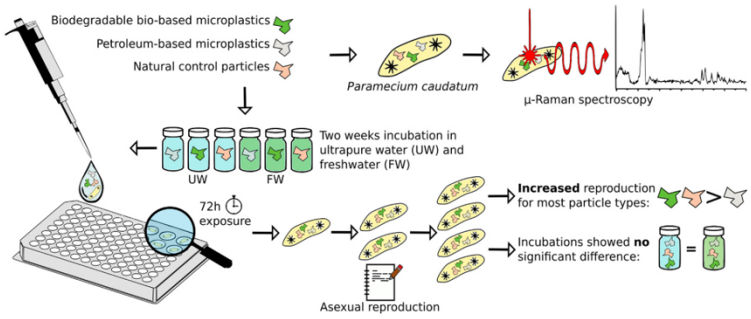
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HIGHLIGHTS

- Particle identification within paramecia can be achieved using μ-Raman spectroscopy.
- Reproduction in *P. caudatum* increases with the presence of specific particles.
- Paramecia may try to digest biodegradable bio-based microplastics, as well as PET.
- Freshwater incubated particles have effects similar to pristine particles.

GRAPHICAL ABSTRACT



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ABSTRACT

The impacts of microplastics (MPs) are becoming increasingly concerning. Although many ecotoxicological studies have examined potential effects of MPs on organisms, most have tested only a limited range of pristine plastic types, which do not reflect the properties of environmentally conditioned plastics. This limits the extent to which the results can be applied to real-world situations. Additionally, understanding the ecological impact of MPs requires studies that begin at the lower levels of the food web. In freshwater ecosystems, unicellular ciliophora are a key part of these trophic levels. Studying the effects of MPs on this group is essential for understanding their overall impact on the ecosystem. This study aimed to address both issues by examining MP uptake and the impact of environmentally conditioned MPs on the ciliophora *Paramecium caudatum*. A 72-hour exposure was conducted using six petroleum-based (PB-) and four biodegradable bio-based (BB-) MP types at three concentrations, albeit higher than those found in the environment, along with three types of particle controls. All particles were incubated in ultrapure and freshwater to compare the effects of pristine versus environmentally conditioned MPs. Verification of particle uptake was performed with μ-Raman spectroscopy, confirming particle uptake without the need for fluorescent dyes, except for two control particles. The exposure experiments showed increased reproduction in all treatments with BB-MPs and control particles, except for one, whereas results for PB-MPs were inconsistent. No significant differences were observed between different particle incubation conditions. Our findings indicate that MP effects depend on plastic type, regardless of environmental conditioning, and that uptake by *P. caudatum* alters the Raman spectra of BB-MPs and PET particles.

1. Introduction

Over the past twenty years, it has become evident that environmental pollution caused by microplastics (MPs, 1–1000 μm, ISO 24187:2023, [1]) affects every part of the environment and the living organisms within it. As a result, the potential ecotoxicological effects of MP pollution have gained more attention, leading to increased research on how MPs impact different species. Originally focusing on marine species [2,3], studies now also include organisms from other environments [4,5], such as freshwater and soil ecosystems [6–10].

So far, most published studies have focused on pristine petroleum-based MPs (PB-MPs), such as polystyrene (PS), polyethylene (PE), polypropylene (PP), and polyvinylchloride (PVC) [11–14]. As a result, pollution caused by PB-MPs continues to raise public awareness. Consequently, the demand for seemingly safer alternatives, like bio-based and/or biodegradable (BB-) plastics such as polylactic acid (PLA), polyhydroxyalkanoates (PHAs), polybutylene succinate (PBS), polybutylene adipate terephthalate (PBAT), polycaprolactone (PCL), and starch-based plastics; see also Goel et al. [15], steadily increases in the market [16]. With the rising production of BB-plastics, these polymers are likely to significantly contribute to plastic waste and MP pollution in the future [17]. However, research on the effects of BB-MPs has received limited attention so far (e.g., [18–20]), and only a few studies report concerning results (e.g., [21-23]). Especially with the increase in BB-plastic production, more comparative research on the biological effects of PB- and BB-MPs is urgently needed.

To date, most studies on the effects of MPs have tested only a limited variety of PB-plastic types, mainly as pristine particles (e.g., [19, 24–26]). However, once MPs enter the environment, their surface quickly interacts with their surroundings. They are rapidly coated by a complex matrix of biomolecules from dissolved organic matter, humic and fulvic acids, extracellular polymeric substances (EPS), and inorganic materials such as minerals and ions, which together can form an eco-corona [27,28]. When particles are large enough, they can also be colonized by microbial communities, forming a biofilm known as the "plastisphere" [29]. All these factors can alter the behavior and toxicity of environmentally conditioned MPs and increase the likelihood of uptake by organisms and cells [30–32]. Therefore, exposing organisms in the laboratory to pristine MP particles that do not resemble the properties of environmentally conditioned MPs is unlikely to accurately reflect what occurs in the environment [33]. This limits the ability to extrapolate experimental results to real-world situations.

Regarding the studied biota, current laboratory investigations of MP effects on freshwater organisms mainly rely on various model species, such as the green algae *Acutodesmus obliquus* [34], *Chlorella vulgaris* [35], or animals like *Daphnia magna* [19,36,37], *Dreissena polymorpha* [38], *Lumbriculus variegatus* [39], *Gammarus fossarum* [40], *Cyprinus carpio* [11], or *Danio rerio* [41]. However, understanding the ecological impact of MPs requires more studies focusing on the lower levels of the food web. One group often overlooked are unicellular freshwater ciliophora [26,42–44]. Ciliophora are vital to aquatic ecosystems because they serve as a key link between primary producers and higher trophic levels [45,46]. While Ciliophora have been used as model organisms to study the effects of substances such as antibiotics [47], pesticides [45], and metals [48,49], comprehensive research on the effects of MP exposure on these organisms is still lacking. The few studies available on MP interactions with freshwater Ciliophora have used pristine PB-MP particles [26,42–44,50,51–58]. However, a detailed analysis of how different types of MPs, including both PB- and BB-MPs, as well as a comparison between pristine particles and environmentally conditioned MPs, is necessary for realistic risk assessments and for understanding potential ecosystem-level effects.

This study aimed to examine the potential effects of MPs on the freshwater ciliophora *Paramecium caudatum*. The organisms were exposed to various PB- and BB-MP fragments (all d_{50} ~20 μm). To assess environmentally relevant properties of MPs, we compared the effects of pristine MPs with those of environmentally conditioned MPs. To isolate the effects of particle presence alone, we included natural (non-plastic) control particles ([7,59], [60]) in additional treatments with cellulose fragments, kaolin (i.e., mineral particles), and mussel shell fragments (MS). Furthermore, our study aimed to confirm the uptake of all PB-, BB-MPs, and control particles within a single experimental setup without the need for fluorescence marking or fixation reagents, by developing an advanced μ-Raman spectroscopy method.

This method was developed based on previous studies that detected MPs in tissues and cells [61–64]. This approach enabled us to identify each type of plastic particle within the cells, thereby establishing an initial tool capable of creating a true dose-response curve for future research.

2. Materials and methods

2.1. Paramecium caudatum culture

The freshwater Ciliophora *P. caudatum* originated from a well-established in-house cultivation, which was originally purchased from Carolina Science GmbH (Berlin, Germany). The Ciliophora were kept in 500 mL Erlenmeyer Duran flasks (DURAN group, Wertheim, Germany) filled with Z medium [26,65] diluted 1:4 in ultrapure water (PURELAB Flex, ELGA LabWater, Veolia Water Technologies, Celle, Germany). The

culture was maintained in static conditions in dark cabinets and fed *ad libitum* with an infusion of dried celeriac root (*Apium graveolens*). Individuals for the experiments were randomly selected from cultivations at the logarithmic growth phase.

2.2. Pristine microplastics and control particles preparation

Fragments from ten different polymers and three natural control particles were selected for the experiments, all in sizes ~ 20 µm (d_{50}) (Figure SI 1–11). Six out of the ten MP types were chosen from petroleum-based polymers (PB-MP; Figures SI 1–6), while the remaining four were biodegradable-biobased (BB-MP; Figures SI 7–10). The PB-MPs selected for this experiment were Low-density Polyethylene (LDPE, Lupolen 1800 P, LyondellBasell Industries, Basell Polyolefine GmbH, Wesseling, Germany), Polystyrene (PS, PS 158 N, INEOS Styrolution Group, Frankfurt am Main, Germany), Polyamide (PA, PA66, Ultramid A27 E, BASF SE, Ludwigshafen, Germany), Polypropylene (PP, Moplen HP526J, LyondellBasell Industries, Basell Polyolefine GmbH, Wesseling, Germany), Poly(vinyl chloride) (PVC, S 3268, Vinnolit GmbH & Co. KG, Ismaning, Germany), and Poly(ethylene terephthalate) (PET, NEOGROUP, Klaipėda FEZ, Lithuania).

The BB-MPs selected for this experiment were Poly-3 (hydroxybutyrate) (P3HB, Biomer, Schwalbach, Germany), Poly (butylene succinate) (PBS, BioPBSFZ91PM, Mitsubishi Chemicals, Tokyo, Japan), and two variants of Poly(lactic acid) (PLA): Poly(D/L lactide acid) (PDLLA, NatureWorks, Nebraska, USA) and Poly(L lactide acid) (PLLA, TotalEnergies Corbion, Netherlands). These two variants differ in the stereospecificity of the lactide monomer and in their crystalline structures. PDLLA is amorphous, while PLLA has a crystalline structure. The MP fragments, bot BB- and PB-, were produced in-house by milling and sieving (by the Macromolecular Chemistry II, University of Bayreuth; CRC 1357 Microplastics, https://www.sfb-mikroplastik.uni-bayreuth.de/en/index.html and https://www.limnoplast-itn.eu/). A detailed procedure is reported in the SI.

As natural control particles, kaolin, cellulose, and ground mussel shells (MS), obtained from the quagga mussel (*Dreissena bugensis*), were included. The MS were prepared as previously described by Schwarzer et al. [66]. Kaolin fragments were purchased from Gebrüder Dorfner GmbH & Co. (Hirschau, Germany). Cellulose fragments were produced in-house from cellulose fibers for column chromatography (length 0.02–0.1 mm; Carl Roth GmbH + Co. KG, Karlsruhe, Germany, CAS No. 9004–34–6), using a Retsch CryoMill (Retsch GmbH, Haan, Germany) to obtain the desired size range (see Figure SI 11).

Size distributions of plastic particles and cellulose were determined using a combination of laser diffraction (LD) and dynamic image analysis (DIA; FLOWSYNC, Microtrac MRB, Montgomeryville and York, USA). The size distributions of the used MS and Kaolin particles are reported in Schwarzer et al. [66], and measurements were not repeated. All size distributions measured for this study are reported in SI Figures 1–11. For size distributions of MS and kaolin, see the supplementary information from Schwarzer et al. [66].

2.3. Microplastic and control particles incubation

All particles were incubated for two weeks in either freshwater (FW) coming from an artificial outdoor pond or ultrapure water (UW). The incubation was carried out as previously described by Ramsperger et al. [67] with a few adaptations. For each particle type, 20 mg of particle powder were incubated in 20 mL glass vials (autosampler vials; neoLab, Heidelberg, Germany). To maintain the natural composition of the incubation medium, the vials were centrifuged (2000 g, 20 min., room temperature) every three days, and the supernatant was replaced with the same volume of either FW or UW (MilliQ water). Such a procedure may affect biofilm formation but appears necessary to prevent particle aggregation and to keep biofilm formation under control [32]. Where necessary, Tween20 (0.001 %) was added to prevent particle aggregation (applied to PP and LDPE). The same procedure was applied to control medium vials without adding particles (one vial containing only UW, one containing only FW, both with and without Tween20).

2.4. Scanning electron microscopy analyses

The morphology and surface characteristics of the MP particles were analyzed using a scanning electron microscope (SEM, FEI Apreo Volumescope, Thermo Fisher Scientific, 5 kV, working distance 2 mm, T1 in-lens detector). For sample preparation, 100 µL of each particle suspension was pipetted onto a small piece of silicon wafer (approx. 1 cm) and left to sediment for 1 h. To preserve the biofilm, the particles were fixed using Karnovsky's fixative; 2 % PFA and 2.5 % glutaraldehyde in 1 × PBS), dehydrated in an increasing ethanol series (30, 50, 70, 80, and 90 % for 30 min each, 95 % and absolute ethanol for 1 h each), and dried in hexamethyldisilazane (HMDS) overnight. Then, the silicon wafers were placed on carbon conductive tabs (Ø 12 mm Plano GmbH, Wetzlar, Germany) and fixed to aluminum stubs (Ø 12 mm, Plano GmbH, Wetzlar, Germany). This procedure has been shown to preserve the biofilm structure on particle surfaces in previous studies [67], but alterations in surface structure cannot be ruled out. Samples were subsequently coated with a 4 nm-thick platinum layer (208HR sputter coater, Cressington, Watford, UK). It should be noted that a 4 nm platinum layer could alter nano-surface structures while it substantially improves sample conductivity [68]. The stubs were finally transferred into a desiccator and stored under vacuum until the micrographs were acquired.

2.5. Raman spectroscopic analyses

2.5.1. Instrument specifications

All Raman spectroscopic measurements were performed using a micro-Raman spectrometer (WITec Alpha 300 RA+, Ulm, Germany) equipped with a UHTS 300 spectrometer and a back-illuminated Andor Newton 970 EMCCD camera. A frequency-doubled Nd-YAG laser with a wavelength of 532 nm was used as the excitation source. The exciting laser radiation was coupled to a Zeiss microscope through a wavelength-specific single-mode optical fiber. The laser beam was focused onto the sample by means of a 50 × long working distance [numerical aperture (NA) = 0.7, lateral resolution ca. 500 nm] and 100 × (NA = 0.9, lateral resolution ca. 300 nm) Zeiss objective. The focal length of the spectrometer is 300 mm, and it is equipped with a diffraction grating with a groove density of 600 lines per millimeter, providing a spectral resolution of ~3–4 cm^{-1}. The laser power used was approximately 5–15 mW at the fiber for all measurements. Raman scattered light was detected by a Peltier-cooled complementary metal oxide semiconductor–based CCD with a sensor size of 1600 × 200 px. The instrument was operated by the integrated Witec Control Five software (version 5). All spectra were acquired in the 3600–500 cm^{-1} spectral range. The acquisition was mainly carried out using the Raman imaging mode, in which each cell was imaged entirely at a specific lateral resolution. The acquired data were preprocessed using instrument-integrated software that allowed compensating for cosmic radiation and other background signals. Each Raman Image required an acquisition time of ~ 20 min under the 50x magnification and comprised a data set constituting a total of approximately 12,500 individual spectra. The Raman images were processed using the Witec integrated Project software (version 5.3) and were subjected to k-means cluster analysis coupled with spectral demixing to visualize the spatial distribution of different components within the Raman images (MP signature and *Paramecium* cell component signature).

Each cell component spectrum represents a combination of different elements, such as proteins, lipids, carbohydrates, and secondary metabolites (e.g., carotenoids). The used algorithm identified a spectrum as "Component 1" based on how different it was from the spatially averaged spectrum obtained from the image. This means that each cell component had distinct combinations of biomolecules, along with

consistent variation in the intensity of a specific peak. Finally, a component with a signature resembling a polymer spectrum, along with residual biochemical signatures, was detected as another individual component.

This process enabled the separation of spectral data by grouping them according to similarities within the dataset and minimizing spectral variance within each cluster [69]. The application of k-means has been previously shown for hyperspectral images of MPs analysis [70] and even nanoparticles in cells [71]. The advantage of this method for data analysis is that no prior knowledge of the data set analyzed is necessary, whereas other applied algorithms have to be trained for the specific detection [62] of, e.g., polymers like in random forests [72,73]. This implies that k-means may also help to identify patterns in a sample that trained algorithms may not recognize [74]. This may become relevant when analyzing particle uptake in environmental samples.

2.5.2. Sample preparation

For Raman analysis of MP uptake by paramecia, cells were exposed overnight to 100,000 particles mL^{-1} of the pristine MPs of each treatment, respectively. After the exposure, the cells were transferred onto a glass slide in 10 µL of the exposure medium. Glass fragments from crushed cover slides were placed on the microscopy slide as spacers to prevent the cells from being pressed, and a cover slide (18 ×18 mm) was placed on top. The so-prepared microscopic slides were sealed with transparent nail polish (trend it up nail polish, dm-drogerie markt GmbH + Co. KG, Germany) and, once the cells stopped moving, were analyzed using a Raman microscope.

2.6. 72-hour exposure

Individuals of *P. caudatum* were exposed in static conditions to three different concentrations of the selected particles (500, 5000, and 50,000 particles mL^{-1}). These concentrations were selected to cover a wide range, which could potentially reveal any concentration-dependent effects. However, it should be noted that all concentrations are higher than those reported in freshwater environments [75]. Each treatment was replicated fifteen times and randomly distributed in 96-well plates (Eppendorf Group, Hamburg, Germany). The well plates were maintained in dark boxes and placed on an orbital shaker (KS125 basic IKA LABORTECHNIK, IKA Werke GmbH & Co. KG, Staufen, Germany) at 1.5 mot min^{-1} to reduce particle settling and promote particle uptake. Each well initially contained one individual *P. caudatum* cell in 250 µL of the treatment suspension in Z medium, with 2 $mgCL^{-1}$ of the algae *Acutodesmus obliquus*. A detergent-free incubation control (same as particle treatment but without added particles) and a Tween20 control (same as particle treatment without particles but with added Tween20 0.001 %) were also included in the experiments. The chosen concentration of Tween20 was kept low enough to prevent detachment of any biofilm, which could occur at higher concentrations (>0.1–1 %) of the surfactant due to disruption of interfacial and extracellular matrix interactions [76]. Concentrations below 0.01 % were not reported to inhibit biofilm formation [76]. To minimize dilution effects from the incubation medium, all stock suspensions were previously diluted in Z medium before being added to the exposure wells. The organisms were then checked at three time points every 24 h (24, 48, and 72 h) using a stereo microscope (Leica MS5, Leica Microsystems, Wetzlar, Germany), and the number of living/free-moving paramecia was recorded.

2.7. Statistical analyses

Asexual reproduction of paramecia exposed to MPs and natural control particles was analyzed by counting the number of paramecia in the wells after 24, 48, and 72 h of exposure. In a first analysis, we wanted to know whether the different particle treatments changed the rate of binary fission compared to the controls. To this end, we fit a generalized linear model with Conway-Maxwell-Poisson distributed error term and log link to the number of paramecia, including particle type (control, Tween control, cellulose, kaolin, MS, PLLA, PDLLA, PBS, P3HB, PA, PET, PS, PVC, LDPE and PP), incubation media (FW and UW), and interaction of particle type and incubation media and time nested in particle type as fixed factors (glmmTMB package version 1.1.12; [77]). Residuals were checked with the package DHARMa version 0.4.7 [78] with simulation-based diagnostics to assess model fit and dispersion. The overall effects of the predictors and interaction terms were evaluated via a type II ANOVA calculated by the *Anova()* function from the package *car* version 3.1–3 [79]. Estimated marginal means for the different particle types and custom pairwise comparisons between particle types and controls were conducted using the package *emmeans* version 1.11.2–8 [80]. For the comparison of particle treatments with controls, emmeans calculated marginal means for mean exposure times (i.e., 48 h) and mean particle concentrations. LDPE and PP responses were compared to the particle-free Tween control, because Tween was used in these two MP treatments as a detergent to get the particles into suspension. All other polymer particles and the natural control particles were compared to the particle and detergent-free control. P-values for pairwise comparisons were corrected for multiple comparisons based on Benjamini and Hochberg [81]. To test for particle type differences in concentration dependency and reproduction over time, we fit a second model to the data, excluding all control data. In this second model, particle type, concentration, time, incubation media, and the interaction of the latter three with particle type were included as fixed effects. Again, a Conway-Maxwell-Poisson error distribution with logit link was used, adding an additional dispersion parameter (quasi-poisson). Estimated marginal means were again derived from the model using *emmeans*. All analyses were done in R version 4.5.1 (R Core Team 2022). Data preparation was done with the help of the *dplyr* package version 1.1.4 [82].

3. Results

3.1. MP characterization and biofilm formation

After exposure to FW for 2 weeks, we observed structures typical of biofilm formation on the surface of the MP particles (e.g., PDLLA and PET) and natural control particles (e.g., MS). These structures were not visible on particles incubated in UW (Fig. 1). Structures included amorphous shapes, which likely consisted of biomolecules and remnants of organisms present in the freshwater. Additionally, cells of various shapes and sizes were present on the particles' surface, likely representing both prokaryotic and eukaryotic organisms. In samples prepared from freshwater without particles, we observed various prokaryotic and eukaryotic organisms, such as *Vorticella spp.* (Figure SI 13). Overall, the SEM images show that upon environmental exposure, the MP particles developed a biofilm, consistent with earlier results [67,83,84]. For SEM images of all particles and overview images, see Figures SI 12 & 13.

3.2. Particle uptake

Particle uptake was confirmed using Raman spectroscopy. All particle types were detected within the cells, except for cellulose and kaolin. Raman images acquired from individual cells were reconstructed from the k-means clustering analysis to visualize particle uptake. Fig. 2 shows the uptake of LDPE MP particles. The clustering analysis reveals the presence of an average of 5–6 clusters (color-coded spectra) corresponding to the LDPE particles, different cell components, and the medium in which the cells were suspended. The same approach was applied to visualize the uptake of the other particles (Figures SI 14–16).

The uptake of fluorescent PS MPs was also confirmed via Raman spectroscopy (Figure SI 17) and fluorescence microscopy (Figure SI 18; for a detailed method description, see SI and [26]). Raman Imaging data also revealed a change in the spectral profiles of the BB-MP particles within *P. caudatum* cells when compared to the spectra obtained from

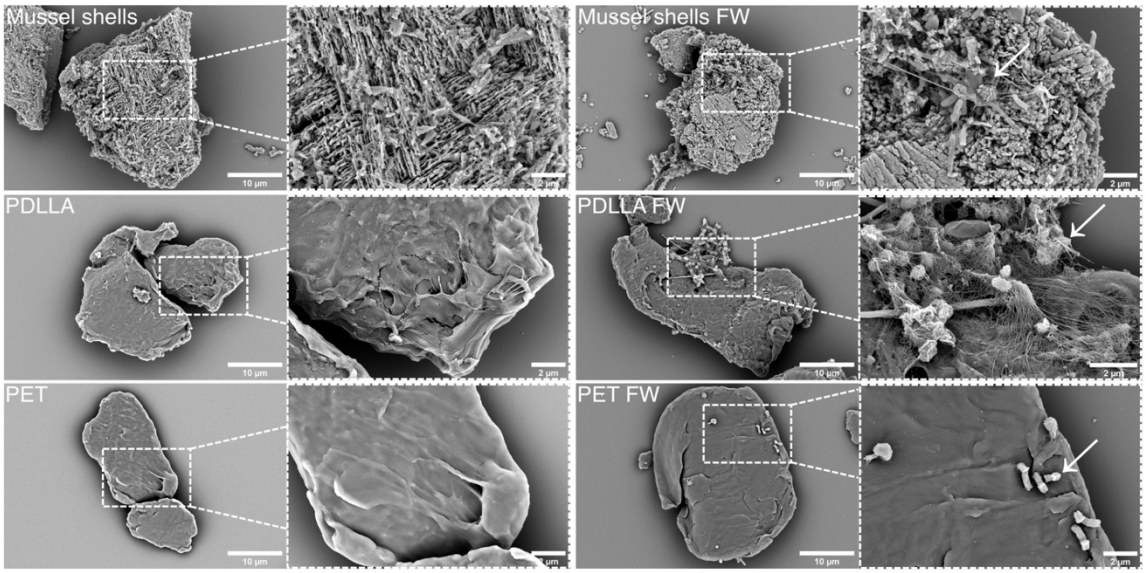


Fig. 1. SEM images of selected incubated particles (Mussel shells – MS, PDLLA – BB-MP, and PET – PB-MP). The two left columns (left: particle overview; right: close-up of the same particle) show the UW-incubated particles, while the two right columns (left: particle overview; right: close-up of the same particle) show the freshwater (FW) ones. After incubation in FW for 2 weeks, all MP particles and natural control particles developed a biofilm. The biofilms included organisms of various sizes, likely comprising both prokaryotic and eukaryotic species (indicated by white arrows in the 4th column). Furthermore, the particles were covered with amorphous structures, which likely consisted of biomolecules and remnants of freshwater organisms. For SEM images of all particles, see Figures SI 12 & 13.

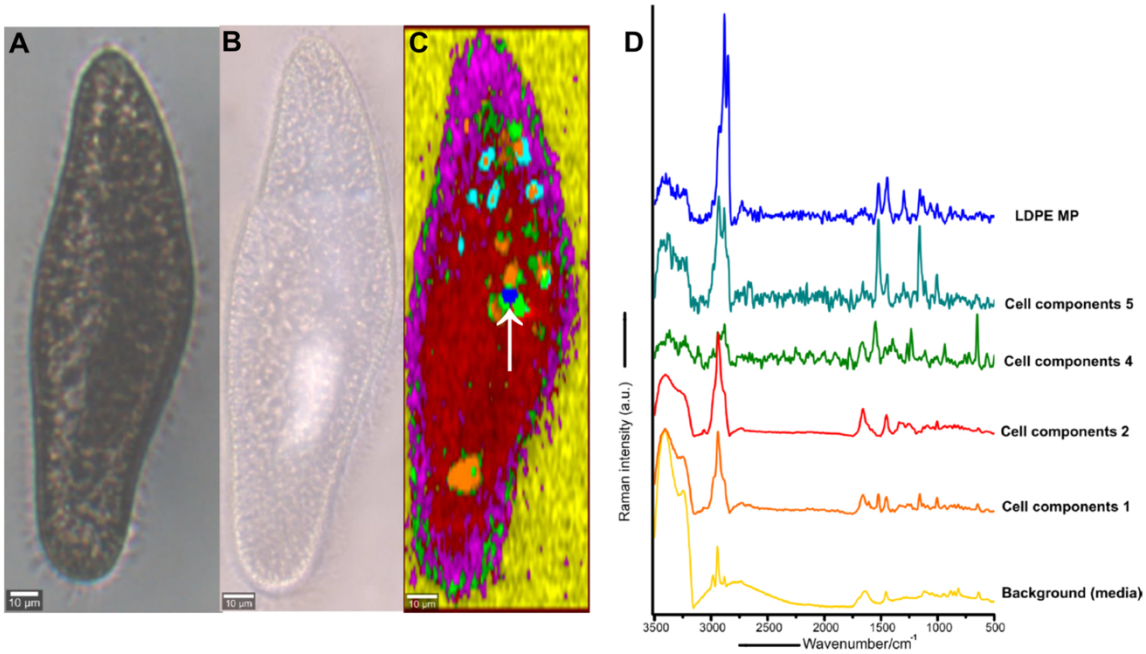


Fig. 2. Analysis of particle uptake by Raman Imaging; A) Bright-field microscopic image of the paramecium, taken at 10x magnification; B) Bright-field microscopic stitched image of the paramecium, single tiles were taken at 50x magnification, using the objective of the Raman microscope; C) Raman image reconstructed from the k-means cluster analysis and spectral demixing depicting the different components (LDPE MP, background and cell components 1–5) color coded according to the spectra shown in (D) respectively. White arrow = LDPE MP; D) Mean Raman spectra of different cellular components separated by the data analysis, along with the spectrum for LDPE MP used for particle depiction, color-coded in blue, and the mean spectra of the background representing the media in which the paramecia were reared.

the same pristine BB-MP particles (Fig. 3; for reference spectra of every particle type, see Figures SI 19–22).

Specifically, the changes in PBS MP compared to its reference show a baseline increase and a general broadening from 3500 to 500 cm^{-1}, indicating oxidation and fluorescence from organic residue attachment. The reduced sharpness and intensity in the C═O stretching

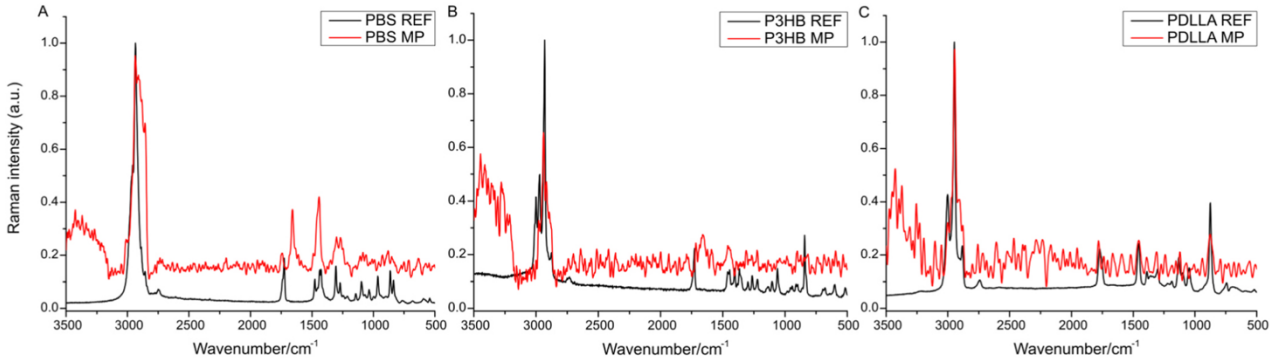


Fig. 3. Changes in the μ-Raman spectra of BB-MP particles after uptake by *P. caudatum*, compared to their references. Reference spectra are shown in black, and the altered spectra of the MP particle inside *P. caudatum* are shown in red. (A) PBS; (B) P3HB; (C) PDLLA.

(~1735 cm^{-1}) ester bond region suggest hydrolysis of ester linkages and partial depolymerization. A decrease in intensity of the backbone region (~1100–1200 cm^{-1}) involving C–O–C and C–C vibrational modes provides evidence of chain scission and backbone degradation. The changes in PDLLA MP in comparison to its reference show changes in the regions ~2950–3000 cm^{-1} C–H stretching region (CH_3, CH, CH_2), with notably higher intensity and slight broadening, indicating chain scission leading to more terminal groups or partial oxidation. Additionally, the decrease or shift in intensity of the ~1760–1750 cm^{-1} C═O stretching (ester group) in PDLLA MP suggests ester bond cleavage or hydrolysis of the PDLLA backbone. Changes in P3HB MP, compared to its reference, include increased baseline and noise. A broad elevation across the spectrum suggests possible fluorescence from organic residues. The reduced sharpness of the C═O peak (~1725 cm^{-1}) and slight broadening imply ester bond hydrolysis or cleavage of carbonyl groups. The decreased intensity in the C–O–C region (1050–1150 cm^{-1}) indicates chain scission and reduced polymer integrity. The loss of a well-defined peak at ~850 cm^{-1} points to material fragmentation. Finally, the broadened CH stretching (~2950 cm^{-1}) region, which is less defined, reflects local chain disorder and altered hydrogen environments resulting from degradation.

3.3. Effects of MPs on asexual reproduction of Paramecium caudatum

Overall, asexual reproduction (binary fission) in particle treatments was higher than in the corresponding particle-free controls, but the strength of these effects differed among different particle types (Fig. 4; Conway-Maxwell Poisson regression, Wald chi-square test: χ^2_{14} = 791.23, $p < 2.2e\text{-}16$). All BB-MPs and three out of six PB-MPs (PET, LDPE, and PP) increased binary fission compared to the corresponding particle-free control ($p < 0.05$ for all comparisons; for full results and values for marginal mean estimates see supplementary tables S1 and S2). However, LDPE and PP increased binary fission only when incubated in FW ($z_{ratio_LDPE} = 3.43$, $p_{LDPE} = 0.001$, $z_{ratio_PP} = 2.51$, $p_{PP} = 0.02$), but not when incubated in UW ($z_{ratio_LDPE} = 1.72$, $p_{LDPE} = 0.13$, $z_{ratio_PP} = 0.29$, $p_{PP} = 0.77$). Among the natural control particles, binary fission was significantly higher in treatments with cellulose (FW: $z_{ratio} = 6.18$, $p < 0.0001$, UW: $z_{ratio} = 5.26$, $p < 0.0001$) and kaolin (FW: $z_{ratio} = 6.05$, $p < 0.0001$, UW: $z_{ratio} = 5.91$, $p < 0.0001$), while the number of cells in treatments with MS fragments did not differ significantly from the control (FW: $z_{ratio} = 1.35$, $p = 0.25$, UW: $z_{ratio} = 0.52$, $p = 0.71$). Tween20-exposed individuals had reproduction rates similar to those of individuals exposed to the Tween-free control, revealing no adverse effects of this surfactant (see SI for discussion on Tween20).

In our detailed comparison of effects on reproduction in response to different particle types (excluding controls), we found that the number of paramecia increased over time (Conway-Maxwell Poisson regression, Wald chi-square test: $\chi^2_2 = 785.20$, $p < 2.2e\text{-}16$) and this increase

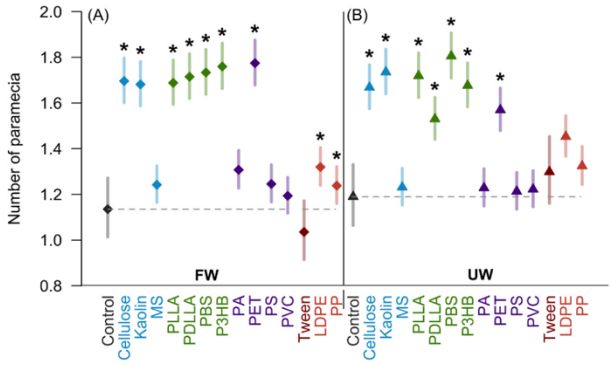


Fig. 4. Estimated marginal means (and 95 % confidence intervals) of numbers of *P. caudatum* in an experiment comparing the effects of different MP particles with natural control particles and blank controls. (A) Particles incubated for two weeks in natural freshwater (FW). (B) Particles incubated for two weeks in ultrapure water (UW). LDPE (low-density polyethylene) and PP (polypropylene) treatments contained Tween20 and are compared to the Tween control (Tween). All other particles are compared to the detergent-free control (Control). Significant differences are indicated by asterisks ($p < 0.05$). Blue: natural control particles, green: BB-MPs, purple: PB-MPs, red: PB-MPs with Tween20.

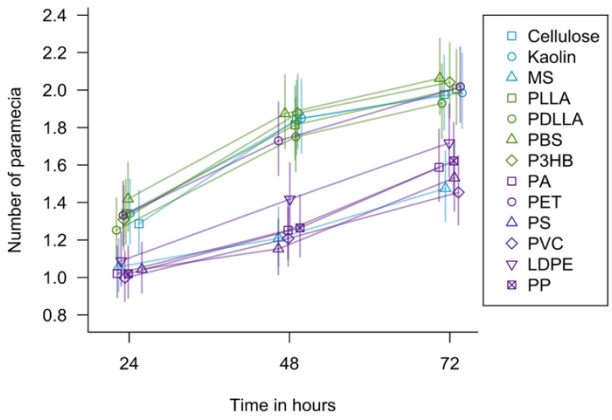


Fig. 5. Asexual reproduction of individuals of *P. caudatum* over time in response to different MPs (green: BB-MPs, purple: PB-MPs) and natural control particles (blue). Estimated marginal means are shown with 95 % confidence intervals.

differed among different particle types (interaction time x particle type: $\chi^2_{24} = 39.58$, $p < 0.02$; Fig. 5).

Asexual reproduction also differed across particle concentrations, but no clear dose-dependent pattern (i.e., no consistent increase in effect size with increasing MP concentration) was observed ($\chi^2_2 = 7.44$, $p = 0.024$; Fig. 6). In addition, the response across the three particle concentrations differed among different particle types (interaction concentration x particle type: $\chi^2_{24} = 89.33$, $p < 1.9e\text{-}9$).

Overall, asexual reproduction did not differ significantly between particles incubated in FW and particles incubated in UW ($\chi^2_1 = 1.03$, $p = 0.31$; Fig. 7), but the direction of effect differed among different particle types (interaction incubation x particle type: $\chi^2_{12} = 28.70$, $p = 0.004$).

4. Discussion

This study aimed to assess the potential effects of pristine MPs (PB- and BB-MPs) and natural control particles on the reproduction of *P. caudatum*, compared to the same particles after environmental conditioning. To determine particle uptake and potential alterations of MPs after ingestion, MPs were identified inside the organisms using Raman spectroscopy.

4.1. Particle identification using μ-Raman imaging

Using μ-Raman imaging, most particles were identified within *P. caudatum*, and their signals could be distinguished from those of the surrounding biological material. Our analysis showed the uptake of all MP types to which the paramecia were exposed, as well as that of the natural control particle MS. The lack of identification of kaolin and cellulose could have had several reasons. First, cellulose may not have been detected during Raman analysis because the spectra were too similar to those of the cellular components, preventing a clear distinction of the cellulose signal from the surrounding cytoplasm. Thus, the Raman spectra of *Paramecium* cells and cellulose show notable similarities, particularly in peaks related to carbohydrate structures, as both contain significant amounts of polysaccharides [85,86]. Furthermore, Raman scattering intensity can vary significantly with polarization and orientation, especially in anisotropic polymers and crystalline materials such as the cellulose particles used in this study. As a result, randomly oriented particles may produce weak or inconsistent spectral signatures, making identification difficult [87]. Additionally, weak signals may be obscured by fluorescence from surrounding biological material excited by the laser, resulting in a signal several orders of magnitude stronger

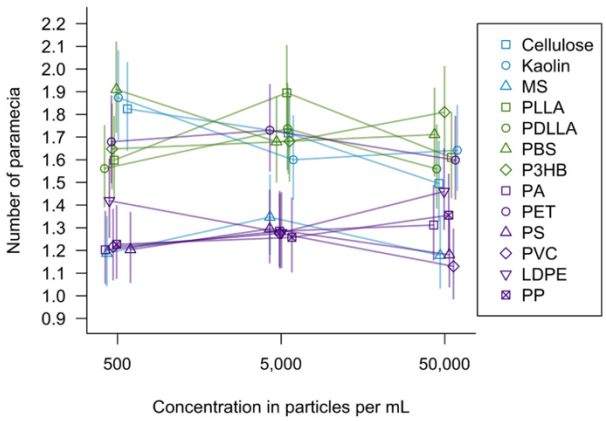


Fig. 6. Asexual reproduction of individuals of *P. caudatum* in response to different concentrations of MPs (green: BB-MPs, purple: PB-MPs) and natural control particles (blue). Estimated marginal means are shown with 95 % confidence intervals.

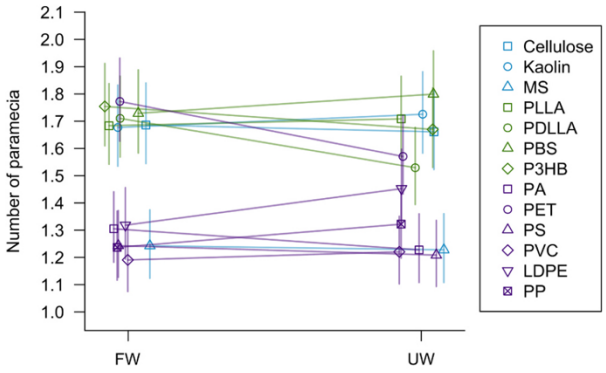


Fig. 7. Asexual reproduction of individuals of *P. caudatum* in response to MP particles (green: BB-MPs, purple: PB-MPs) and natural control particles (blue) incubated in either natural FW or UW prior to their use in exposure bioassays. Estimated marginal means are shown with 95 % confidence intervals.

than that of the target particles, which makes their identification nearly impossible [88]. To complicate matters further, cellulose particles may have been digested during the cyclosis process.

Some protists, such as those found in the gastrointestinal tract of ruminants [89] or termites [90], are known to be capable of digesting cellulose. Although this has not yet been demonstrated experimentally, cellulose digestion is also likely to occur in *P. caudatum*, as it has already been shown for other Ciliophora, such as *Diploplastron affine* [91]. Also, Ciliophora feed on various algae and phytoplankton that are small enough to be ingested and whose cell walls are partly composed of cellulose [52,92]. Given that paramecia lack any hardened structures to crush these algae, as is the case in other phytoplankton-feeding organisms [93], it can be inferred that they can break down the algal cell walls chemically [94]. However, due to the limited literature on this topic, further research is necessary to clarify this aspect.

Other factors that may have contributed to the lack of particle detection for kaolin and cellulose include their different material densities and agglomeration tendencies [60]. For instance, kaolin has a higher density (~2.6 g cm^{-3}; [66]) than the MP particles used in this study (lowest density: LDPE ~0.9 g cm^{-3}; highest density: PET ~1.4 g cm^{-3}; [95]), potentially leading to increased settling of particles at the bottom of the wells despite the gentle shaking throughout the exposure. This could have reduced the availability of kaolin particles to the paramecia, as the experiment was conducted under static conditions, meaning the exposure medium was not exchanged during the exposure period. However, MS, whose uptake was confirmed by the Raman analyses (Fig. SI 14), has a similarly high density (~2.9 g cm^{-3}; [66]) as kaolin. Additionally, cellulose has a density (~1.3 g cm^{-3}; [66]) comparable to that of MPs, and cellulose particles were also not identified in our Raman analyses. Thus, density alone cannot explain why we have not detected kaolin and cellulose particles inside the cells.

Another factor to consider when detecting kaolin is its smaller size compared to other particles. Kaolin had a d_{50} of less than 2 μm [66], which may have made it more difficult to identify within the cells. Similar challenges in detecting small particles have been reported for the identification of small PS MPs in *Eisenia fetida* by Kniese et al. [62]. Since 50 % of all kaolin particles were close to the detection limit of the Raman instrument used (~1–2 μm), and considering the surrounding cellular components, particle detection and identification may have been compromised in the exposed individuals. However, this does not apply to cellulose, which had larger particles ($d_{50} = 26.07$ μm; Figure SI 11) and should not have limited Raman identification. Additionally, other particles used in this study had a size distribution similar to that of cellulose (Figures SI 1–10) and were identified within the exposed organisms (Figures SI 14–17). Therefore, it remains unclear why kaolin and cellulose uptake and/or identification were absent.

Besides kaolin and cellulose, all other particles were identified in *P. caudatum*. Interestingly, our analyses revealed that the Raman spectral profiles of the BB-MP fragments inside the cells differed from those of the raw material. This occurs because the chemistry of BB-MPs tends to change due to factors such as hydrolysis and chain scission resulting from processes during cyclosis within the organisms (e.g., oxidative stress and enzymatic digestion), which can break chemical bonds. Hence, in such cases, new Raman peaks may appear, and existing peaks may exhibit reduced intensity and a shift in the Raman spectra. During cyclosis, vacuoles are acidified to support the digestion of food particles [96], thereby providing conditions for, e.g., acid phosphatase activity [97–99]. Therefore, the onset of an acidic environment within the vacuole after particle uptake might have altered the Raman spectra of the BB-MPs in our measurements, while the spectra of the PB-MP particles remained stable. This is supported by studies reporting that both weathering and acidic environments can break chemical bonds and lead to the adsorption of biomolecules from the vacuole onto the particle. If this occurs within the vacuole due to acidity, it can alter the Raman spectral profiles of the BB-MPs [100–103]. This may explain the differences observed in the spectral profiles of the ingested BB-MPs compared to their references.

Although not all particle types were identified within the Ciliophora in the present study, detecting all MP particles inside the cells using μ-Raman spectroscopy represents a first step toward developing dose-response models. This means that with the method established here, quantifying MP uptake could be feasible in future experiments with *P. caudatum* or other unicellular eukaryotes. Such data could then help determine realistic effect concentrations and establish a threshold dose-response relationship, providing clear implications for MP risk and hazard assessment [104].

However, it should be noted that applying μ-Raman spectroscopy in this way presents certain challenges for researchers. Some of these will be discussed below. (1) Depth-dependent signal attenuation: Raman signals weaken as they penetrate deeper into the cell due to scattering and absorption, which may cause an underestimation of particles located at greater depths of the sample. (2) High-resolution mapping is time-consuming: Raman imaging requires scanning point by point, making it slow for large areas or many cells. (3) Lack of linear correlation: Raman signal intensity does not always have a direct linear relationship with MP mass or volume because of differences in particle size, shape, and orientation. (4) Analyzing statistically meaningful numbers of paramecia can be tedious due to slow imaging speeds, complicating population-level quantification, which is essential in dose-response studies. (5) Smaller particles (such as nanoplastics) may generate weaker signals, and irregular shapes can scatter light unpredictably, impacting detection efficiency. (6) Surface modifications or coatings: Environmental weathering or biofouling of MPs can change Raman signatures, making identification more difficult. (7) Particle agglomeration inside cells can also pose a major obstacle to quantification [105–108].

4.2. Particle effects on asexual reproduction

We observed significant effects of the different particles tested, related to particle type and concentration, but independent of biofilm presence. The only treatment differing from this pattern was UW-incubated PET. The overall pattern was an increase in reproduction among paramecia exposed to particles. Specifically, both BB-MPs and PET-MPs had a significant positive impact on reproduction, regardless of environmental conditioning. PB-MPs, however, showed binary fission rates similar to those of the respective controls, except for PET.

An increase in reproduction, particularly upon exposure to BB-MPs, may suggest that paramecia try to digest these particles to use them as a carbon source. This was also hypothesized for, e.g., terrestrial earthworms, where similar positive effects of BB-MPs (PLLA and PCL) on the reproduction of *Eisenia fetida* were observed [109]. Additionally, degradation of biodegradable PLGA by marine microbial plankton (including Ciliophoran organisms) was also implied in a recent study [110]. Similarly, it may be possible that *P. caudatum* can digest these plastics, as suggested by alterations in their Raman spectral profiles. Increased reproduction was also noted in individuals exposed to PET. Despite PET being a non-biodegradable polymer, several microorganisms and enzymes capable of degrading it have been previously isolated [111]. Hence, as with the BB-MPs, albeit speculative, our results could suggest that *P. caudatum* may also try to utilize PET as a carbon source. This aspect, however, would require further investigation in future studies, using, for example, ^{13}C-labelled polymers [109] or amino acid stable isotope fingerprinting [112] to track the carbon's fate. Therefore, because there is no experimental evidence proving that these organisms can digest these plastics, the idea of using these particles, especially non-degradable PET, as a carbon source remains speculative.

Another important factor that may explain the increased reproduction in the particle treatments in our experiments is that some unicellular eukaryotes, such as *Tetrahymena*, multiply much faster when particulates are present in the medium [113]. Thus, higher particle concentrations (including non-nutritional PS particles; Rasmussen and Modeweg 1973) increase filtration rates [114] and, consequently, stimulate the formation of food vacuoles. In this context, an increased number of food vacuoles was observed to lead to increased growth rates [115]. Generally, Ciliophorans are adapted to particle-rich environments, which supports sustained population performance in such settings [114,116]. This, along with possible digestive processes, could explain our findings of increased reproductive rates in most particle treatments, even though no studies specifically report this phenomenon in *Paramecium*. However, if particle-stimulated growth were the only reason for the positive outcomes in this study, it should have been observed across all experimental treatments, but this was not the case. In fact, the test organisms were exposed to similar concentrations of the other particle types (i.e., PVC, PS, MS, and LDPE and PP in the UW treatments), and none of these showed such an increase in reproduction. Thus, the positive effects of reproductive stimulation might have been suppressed by other factors that could not be disentangled in the present study (e.g., leaching chemicals from the plastic particles), suggesting a negative effect of these MP types on asexual reproduction.

As no significant difference was observed between most FW- and UW-incubated particles, we could assume that the present biofilm does not contribute significantly to the food demand of the paramecia, hence, the positive effect observed in terms of reproduction probably comes from the particles themselves. Nevertheless, as previously stated, not all MPs induced this positive effect. Therefore, either some MPs, but not all, could serve as a carbon source for the exposed paramecia, or some MPs might induce negative effects, negating the positive effect observed with particle presence alone.

The lack of significant differences in incubation type on the reproduction rates of *P. caudatum* likely also reflects the non-selective feeding behavior of these organisms [51,117]. The feeding strategy of *Paramecium* sp. involves indiscriminate suspension feeding, which might explain why particle surface characteristics, including the presence of a biofilm, do not influence uptake. However, more detailed research is necessary to examine how particle surface characteristics affect uptake by *Paramecium* sp. and their potential impacts. This is because, even if uptake remains unaffected, particles coated with a biofilm may exhibit enhanced sorption capabilities for pollutants, increasing their hazard potential [33,118]. Therefore, although our study did not observe differences in reproduction with the presence of a biofilm, pollutants adsorbed by the biofilm on particles in the environment could still cause significant adverse effects on the biota.

Similar to our study, positive effects of MP exposure on the population growth of the Ciliophora *Blepharisma japonicum* were also observed for polystyrene (PS) microbeads [50]. In their study, the authors hypothesized that hormesis might explain this observation. This phenomenon describes a positive response to low concentrations of a

contaminant or a naturally occurring element (e.g., suspended minerals), while higher doses may produce the opposite effect [119,120]. In MP ecotoxicology research, hormesis has been suggested for various aquatic organisms, including *Ceriodaphnia dubia*, *Eriocheir sinensis*, and *Amphibalanus amphitrite* [121]. Therefore, we cannot rule out the possibility that this could also apply to *P. caudatum*, which might explain the positive effects observed in our study. However, the wide range of concentrations used in our study (500–50,000 particles mL^{-1}) showed no dose-response pattern, which would be expected if hormesis were the cause.

To rule out that the observed effects in this study were solely due to general particle exposure, three natural control particles were included in the experimental design. Considering these particles, independently of the environmental conditioning, a significant increase in reproduction was observed when *P. caudatum* was exposed to cellulose and kaolin. In contrast, those exposed to MS had a reproduction rate similar to that of the particle-free control. This finding indicates that MS was the most suitable particle control, as it was intended to be on the same level as the particle-free control, and supports the hypothesis that the positive effect on the reproductive outcome is particle type dependent. Additionally, MS was the only particle control for which uptake was proven (see paragraph 4.1). While an increase in reproduction by cellulose particles may be explained by their digestion (see paragraph 4.1), the increase in reproduction by kaolin particles was unexpected. As discussed above, the presence of particles alone could have already been advantageous enough to produce this result, although it could also be attributed to hormesis. Nonetheless, kaolin is ubiquitous in freshwater ecosystems, so unicellular organisms in the environment are generally exposed to fluctuating concentrations of this material, and positive effects of kaolin in unicellular eukaryotes have already been found in other studies [122].

These previous studies suggest that, in natural environments, the combination of high feeding capacity and omnivorous strategy typical of oligotrich unicellular eukaryotes leads to the uptake of large amounts of non-nutritive particles, such as clays. Therefore, to remain competitive with other bacterivorous organisms, they adapted to high concentrations of mineral particles that might otherwise disrupt their feeding activity. Boenigk et al. (2004) hypothesized that the observed increase in cell proliferation in the presence of mineral particles might be due to organic nutrients bound to the particles' surfaces, thereby providing a higher energy input.

4.3. Ecological relevance

Given that this study was conducted using relatively high concentrations (500–50,000 particles mL^{-1}), which exceed MP concentrations found in the environment (e.g., [75,123,124]), translating the findings to real-world scenarios proves challenging. However, particulates, both natural and anthropogenic, are common in aquatic environments [125] and are likely to exceed the concentrations used in this study. This is important to consider, as one of the main findings of the present study was the reproductive stimulation of *P. caudatum* by the presence of particles. Therefore, this finding is relevant in environments with high suspended particle concentrations, thereby enhancing the study's overall ecological significance. On the other hand, to accurately assess MP effects on *P. caudatum*, additional studies using environmentally relevant concentrations are necessary. The present study represents a first step towards that goal, establishing a promising method for particle quantification using μ-Raman spectroscopy and a convenient approach for testing MP toxicity in *P. caudatum* and other single-celled organisms.

5. Conclusion

This study demonstrated that particle uptake occurs in *P. caudatum* across various types of plastics, including PB- and BB-plastics, as well as natural control particles. Additionally, our results showed that environmental conditioning of particles prior to testing did not affect the asexual reproduction rates of *P. caudatum*. The effects of particle exposure on reproduction were positive, indicating that the presence of most particle types in this study stimulated reproduction and that paramecia may try to digest and potentially use some plastics as a carbon source. However, further research is necessary to understand the mechanisms behind this seemingly beneficial effect. This becomes even more important given the vital role Ciliophora play in ecosystems. For example, longer exposure experiments with individual cells and populations should be conducted to better assess the impacts of PB- and BB-MPs on *P. caudatum*. Moreover, to provide clearer insights for risk and hazard assessments of MP exposure in *P. caudatum*, subsequent experiments involving various plastic types, e.g., to establish a clear dose-response curve, would be helpful. Future studies should include more realistic, environmentally relevant concentrations. The ones selected in this study, in fact, although appropriate for investigating concentration-dependent effects, do not reflect a natural contamination scenario. The Raman spectroscopic method developed in this study qualitatively confirms particle uptake and could also quantify it, reducing the need for potentially toxic fluorescent dyes and fixation chemicals in Ciliophora MP ecotoxicology. However, to quantify particle uptake comprehensively, future studies would need to improve this method by developing workflows that enable higher sample throughput.

Environmental implication

Ciliophora exhibit a wide range of functional diversity and are therefore key components of aquatic food webs. Since they feed on particulates, they may be affected by microplastics. This study compared the effects of bio-based biodegradable microplastics and petroleum-based ones, with and without biofilm, on the reproduction of *Paramecium caudatum*. Results indicated that bio-based biodegradable microplastics, along with PET, promoted reproduction. Furthermore, μ-Raman spectroscopy enables the identification of microplastics within the cytoplasm and the detection of spectral changes of particles after uptake. This could help establish realistic dose-response curves in ecotoxicological studies using unicellular organisms.

CRediT authorship contribution statement

Sven Ritschar: Writing – review & editing, Formal analysis. **Marvin Kiene:** Writing – review & editing, Formal analysis. **Vinay K. Bangalore Narayana:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Visualization. **Simon Wieland:** Writing – review & editing, Visualization, Methodology, Investigation. **Michael Schwarzer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Simona Mondellini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Magdalena M. Mair:** Writing – review & editing, Validation, Formal analysis, Data curation. **Christian Laforsch:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Holger Kress:** Writing – review & editing, Project administration, Funding acquisition. **Martin G.J. Löder:** Writing – review & editing, Validation, Supervision, Project administration. **Julian Brehm:** Writing – review & editing, Formal analysis.

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Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2026.141575.

Data Availability

Data will be made available on request.

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Supplementary material pertaining to Article I

Effects of petroleum-based and biodegradable bio-based microplastics versus natural control particles on *Paramecium caudatum*, combined with *in situ* Raman spectroscopic detection

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(*contributed equally to this work)

Supplementary Information

Effects of petroleum-based and biodegradable bio-based microplastics versus natural control particles on *Paramecium caudatum*, combined with *in situ* Raman spectroscopic detection

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Particle milling and sieving

Pellets of each polymer were mixed with shredded dry ice (1:1 V/V) and then infused in liquid nitrogen for 20 minutes. The obtained mixture was then poured into an ultracentrifugal mill (18000 U/min, 350 µm spacer sieve, 24 tooth rotor, Ultra-Centrifugal Mill ZM 200, Retsch, Haan, Germany). The ground polymer was dried under dry vacuum at 40°C for 24 hours. This process was repeated with increasingly smaller sieves (200-75 µm, <75µm, and finally <20µm, ALPINE Air Jet sieve e200 LS, FRITSCH, Idar-Oberstein, Germany), until the desired size was obtained.

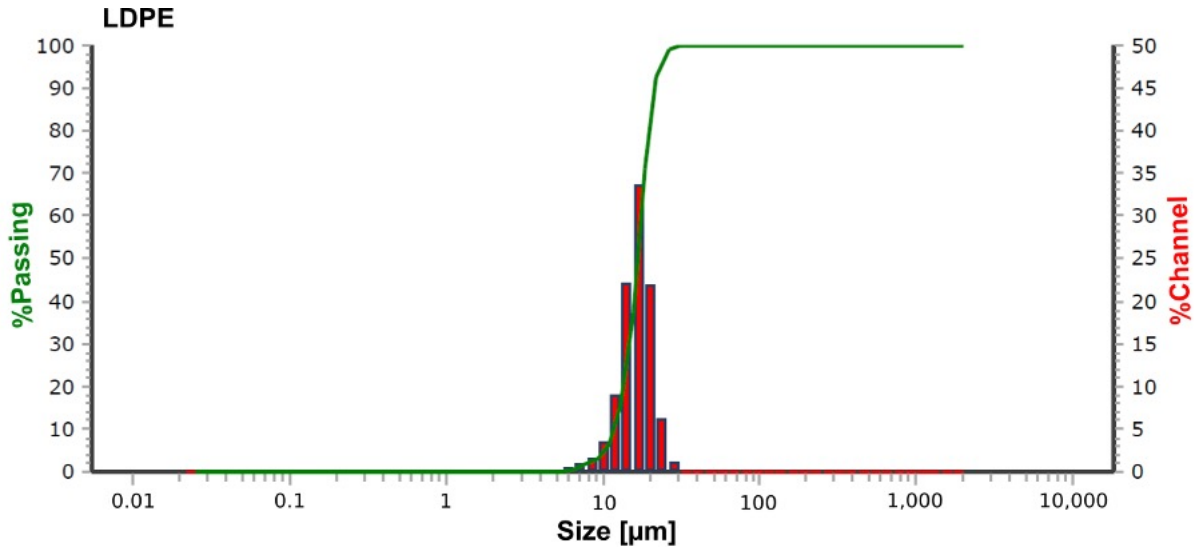


Figure SI 1: Size distribution of LDPE fragments (d_{90}=21.33 µm; d_{50}=16.61 µm). Measurements were conducted using MD and DIA.

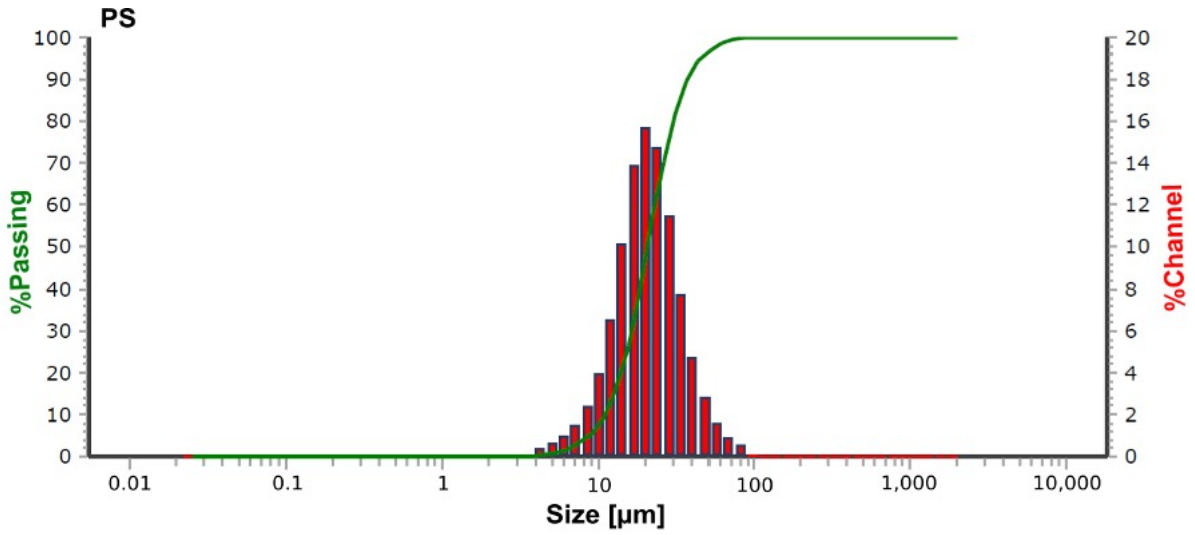


Figure SI 2: Size distribution of PS fragments (d_{90}=37.54 µm; d_{50}=20.67 µm). Measurements were conducted using MD and DIA**.**

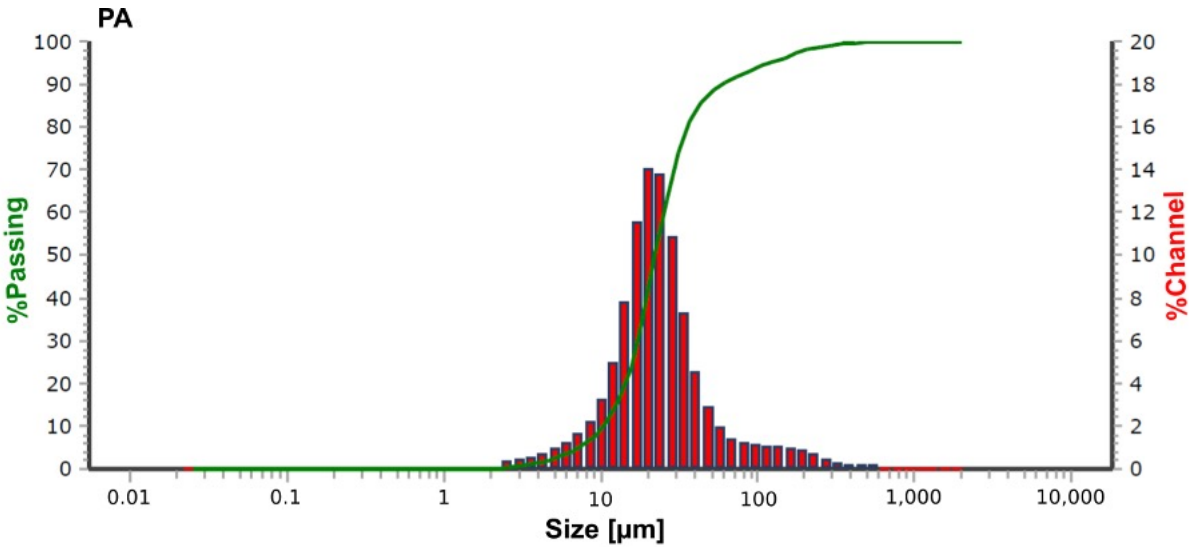


Figure SI 3: Size distribution of PA fragments (d_{90}=59.15 µm; d_{50}=22.17 µm). Measurements were conducted using MD and DIA.

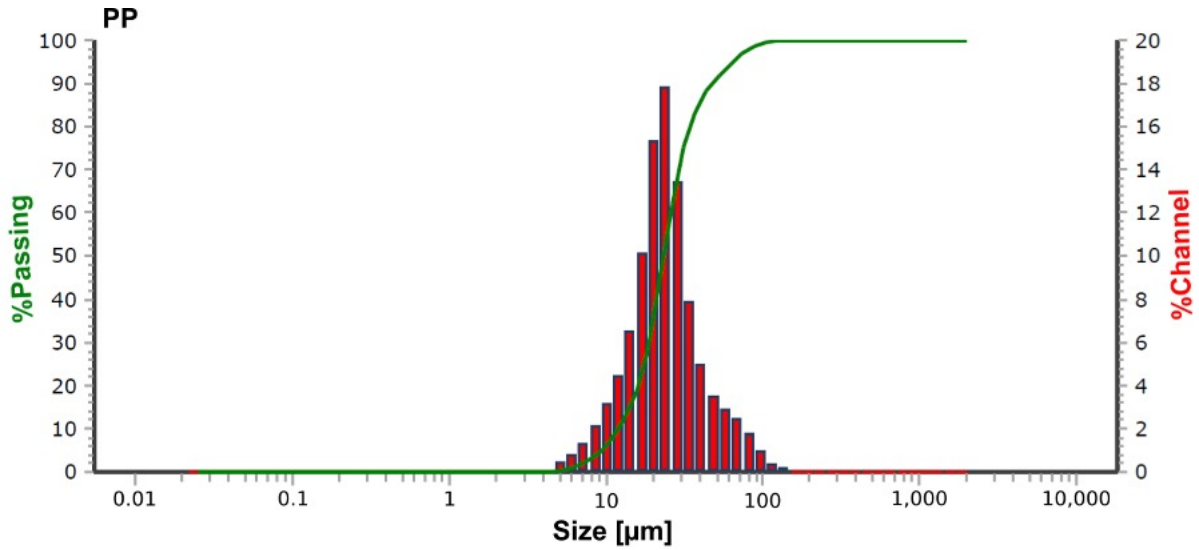


Figure SI 4: Size distribution of PP fragments (d_{90}=47.99 µm; d_{50}=23.28 µm). Measurements were conducted using MD and DIA.

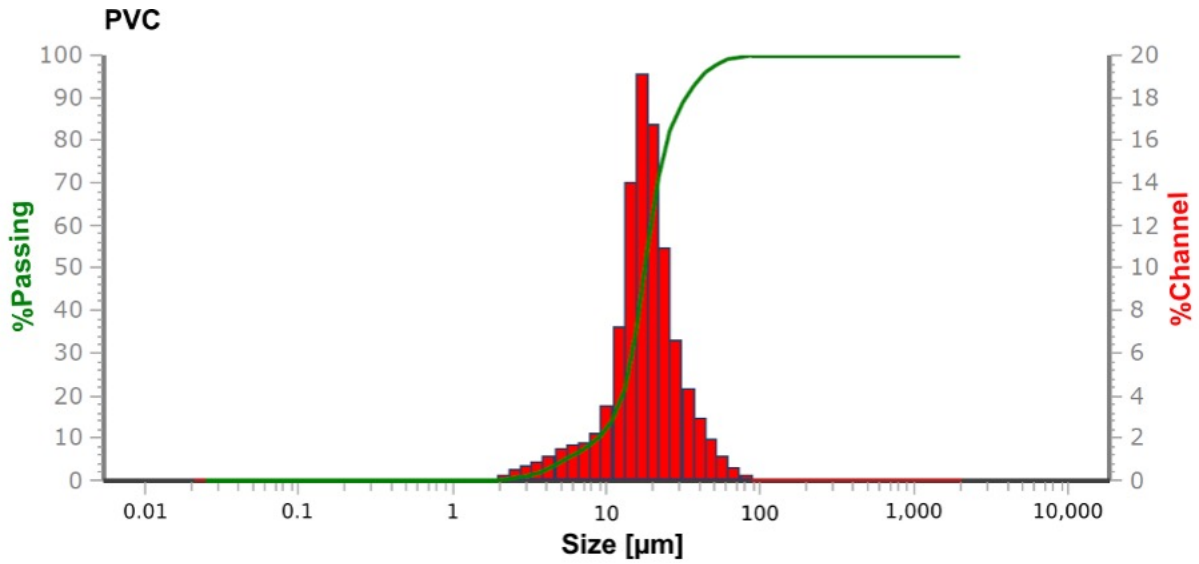


Figure SI 5: Size distribution of PVC fragments (d_{90}=32.38 µm; d_{50}=17.77 µm). Measurements were conducted using MD and DIA.

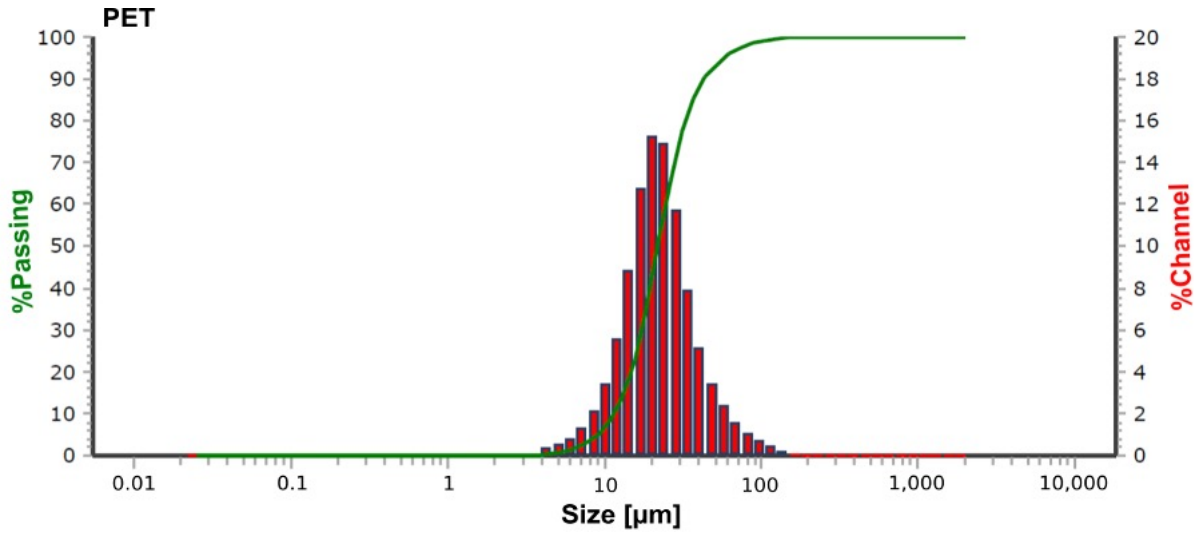


Figure SI 6: Size distribution of PET fragments (d_{90}=43.36 µm; d_{50}=21.82 µm). Measurements were conducted using MD and DIA.

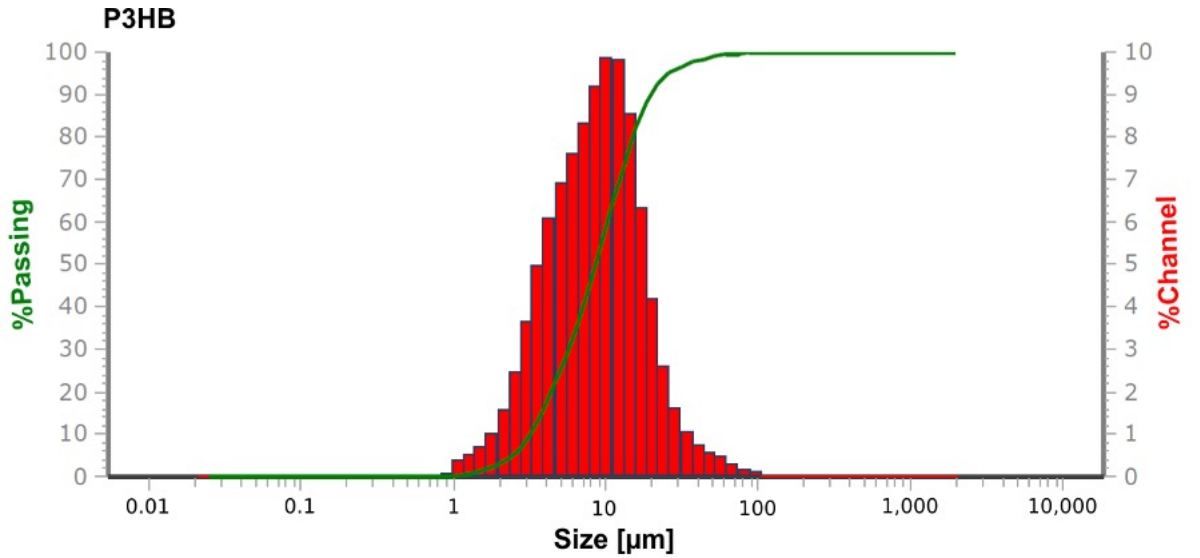


Figure SI 7: Size distribution of P3HB fragments (d_{90}=19.77 µm; d_{50}=8.66 µm). Measurements were conducted using MD and DIA.

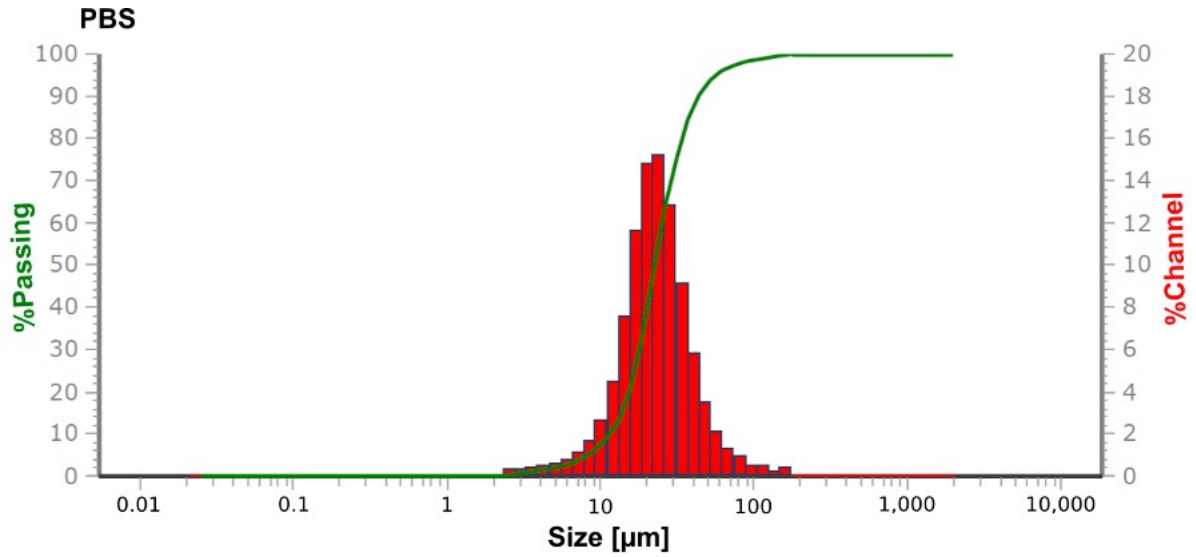


Figure SI 8: Size distribution of PBS fragments (d_{90}=43.6 µm; d_{50}=22.7 µm). Measurements were conducted using MD and DIA.

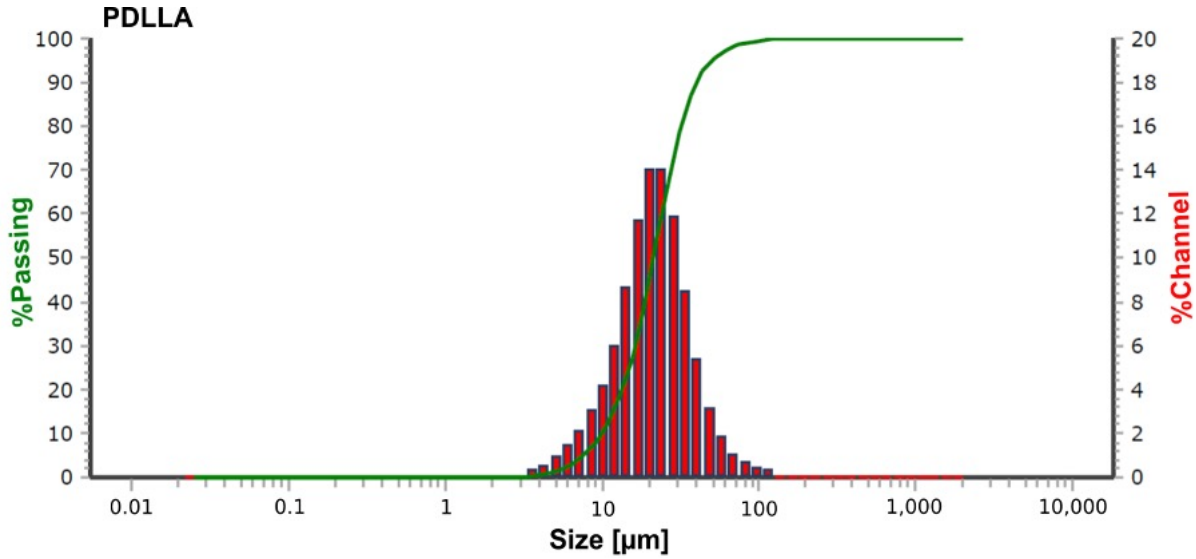


Figure SI 9: Size distribution of PDLLA fragments (d_{90}=40.11 µm; d_{50}=21.25 µm). Measurements were conducted using MD and DIA.

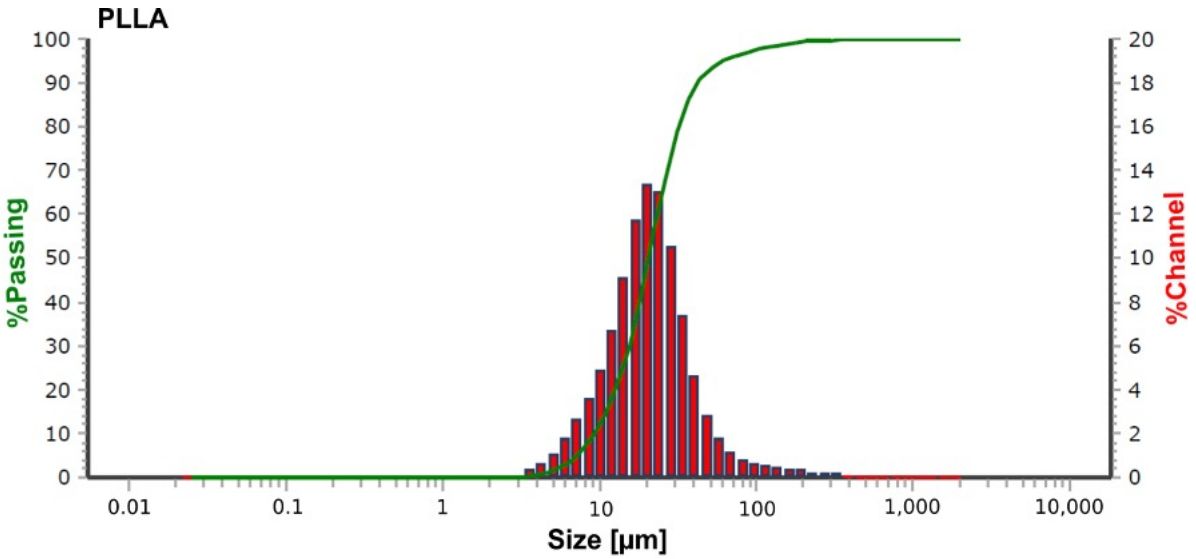


Figure SI 10: Size distribution of PLLA fragments (d_{90}=42.50 µm; d_{50}=20.53 µm). Measurements were conducted using MD and DIA.

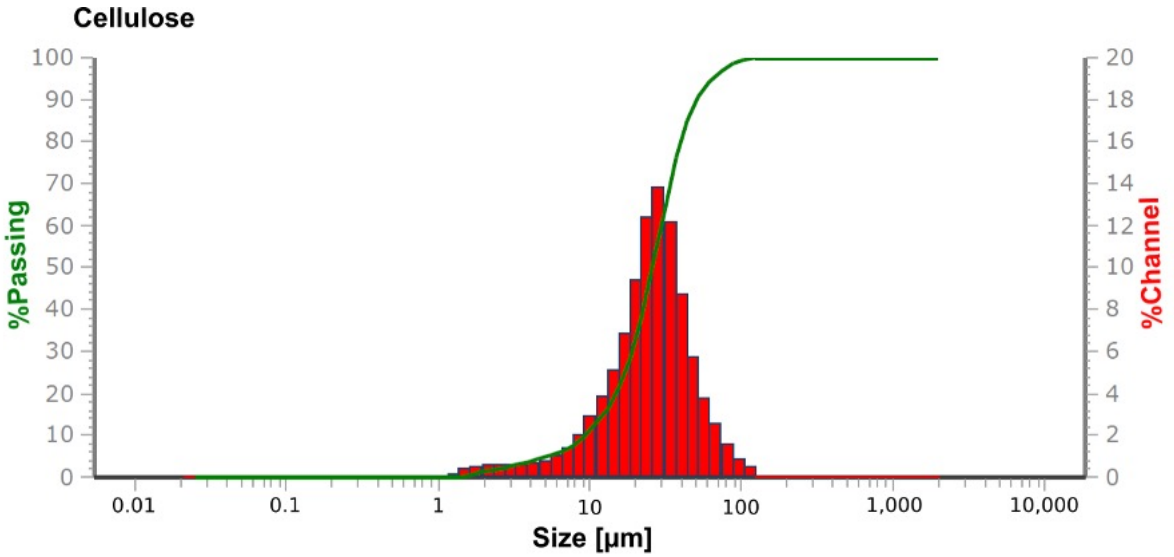
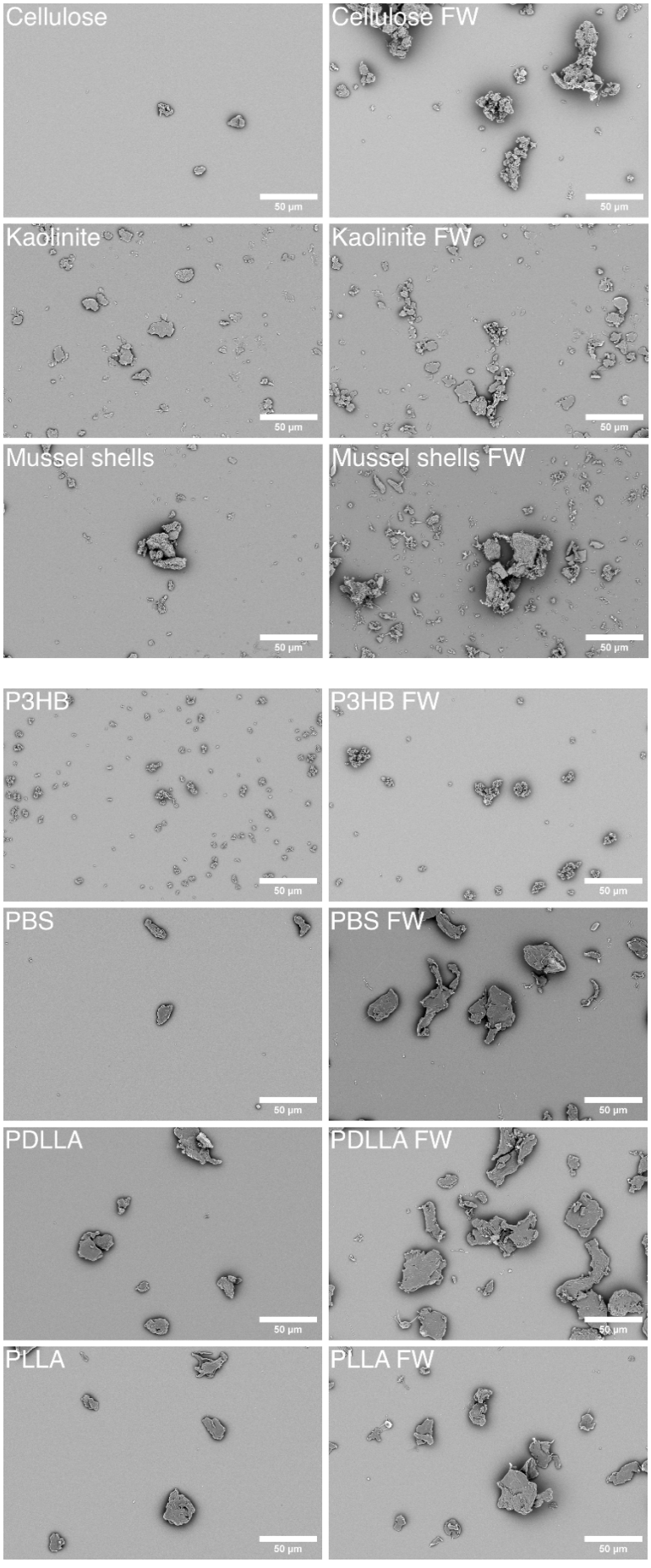


Figure SI 11: Size distribution of cellulose fragments (d_{90}=51.11 µm; d_{50}=26.07 µm). Measurements were conducted using MD and DIA.


Cellulose
Cellulose FW
Kaolinite
Kaolinite FW
Mussel shells
Mussel shells FW
P3HB
P3HB FW
PBS
PBS FW
PDLLA
PDLLA FW
PLLA
PLLA FW
50 µm

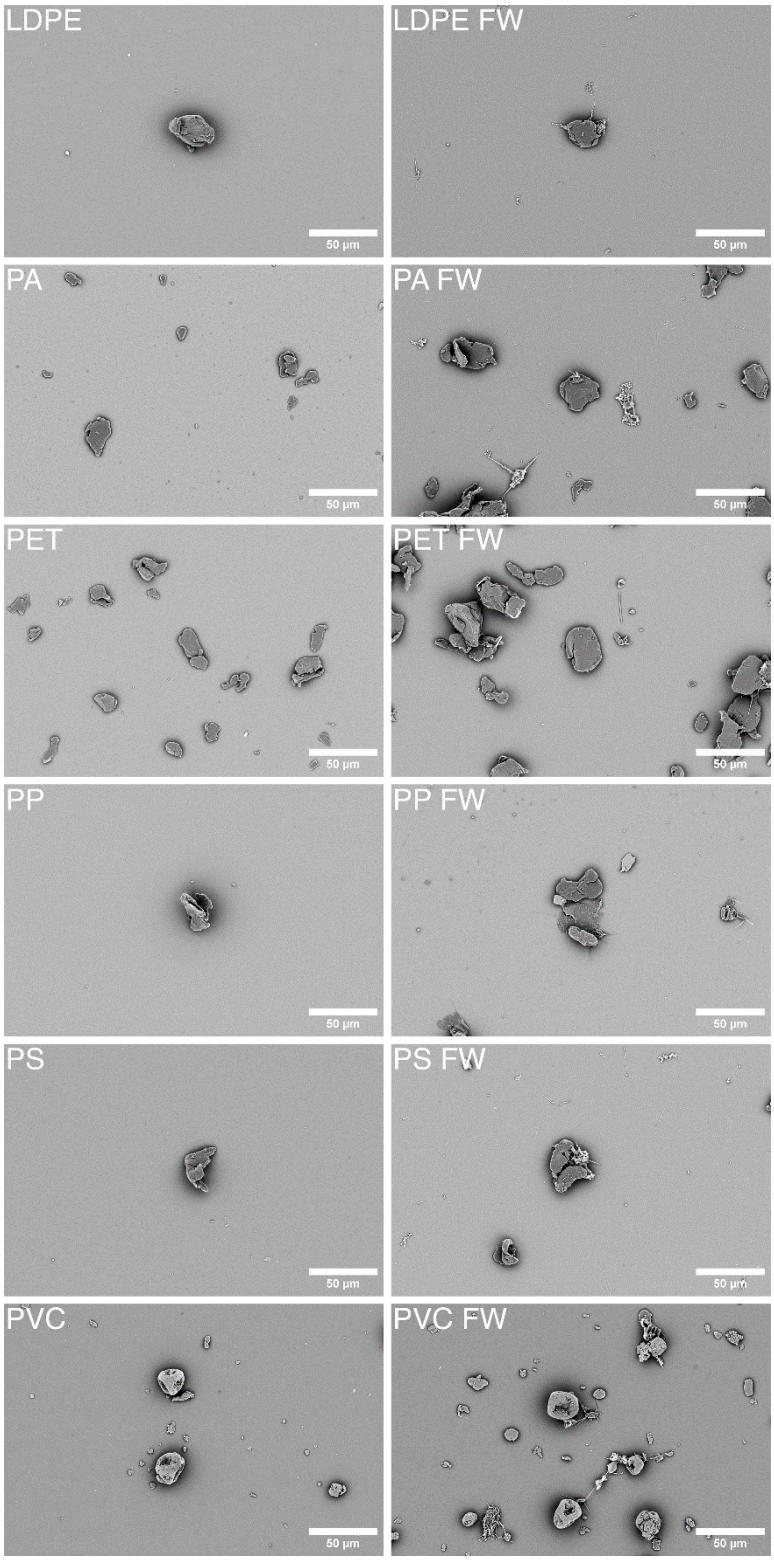
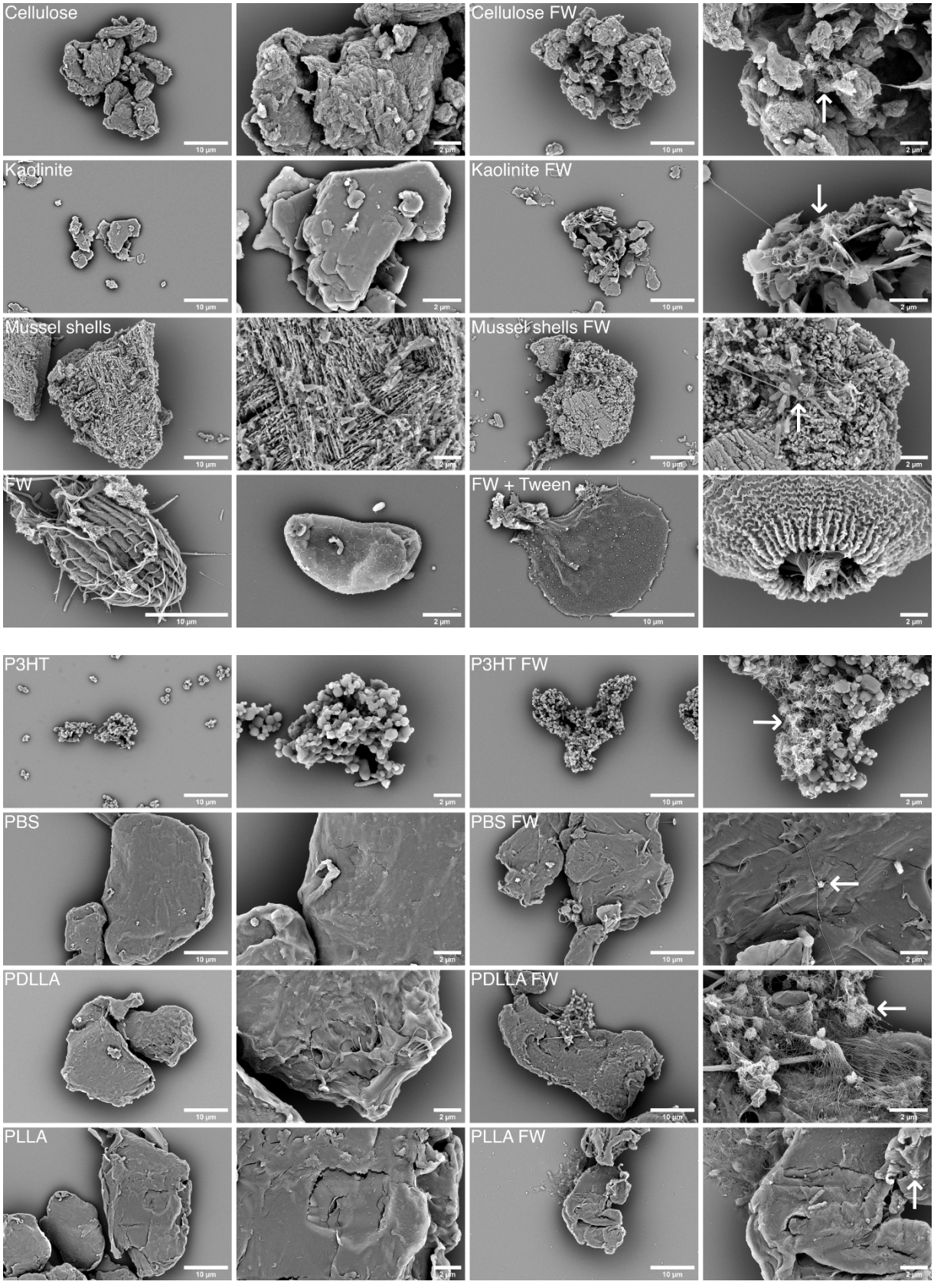


Figure SI 12: Overview SEM images of all incubated MP particles. The left column shows the ultrapure water (UW) incubated particles, and the right column shows the freshwater (FW) incubated particles.


Cellulose
Cellulose FW
Kaolinite
Kaolinite FW
Mussel shells
Mussel shells FW
FW
FW + Tween
P3HT
P3HT FW
PBS
PBS FW
PDLLA
PDLLA FW
PLLA
PLLA FW

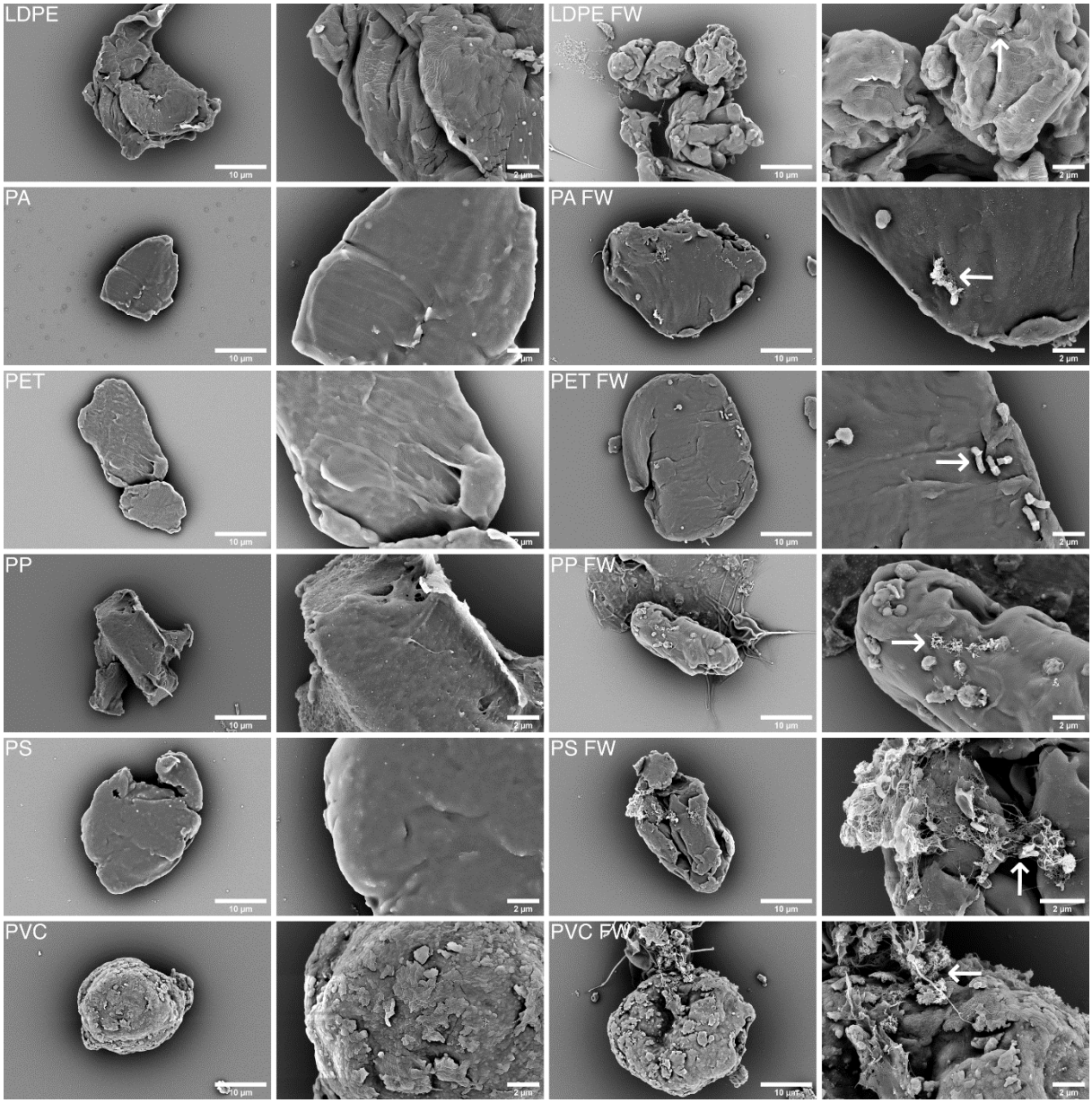


Figure SI 13: SEM images of all incubated MP particles. The two left columns show the ultrapure water (UW) incubated particles, while the two right columns show the freshwater (FW) incubated particles. After incubation in FW for 2 weeks, all MP particles and control particles developed a biofilm (white arrows in the 4th column). The biofilms included organisms of various sizes, likely comprising both prokaryotic and eukaryotic species. Furthermore, the particles were covered with amorphous structures, which likely consisted of biomolecules and remnants of freshwater organisms. In the FW samples, we observed various prokaryotic and eukaryotic organisms, including *Vorticella* spp.

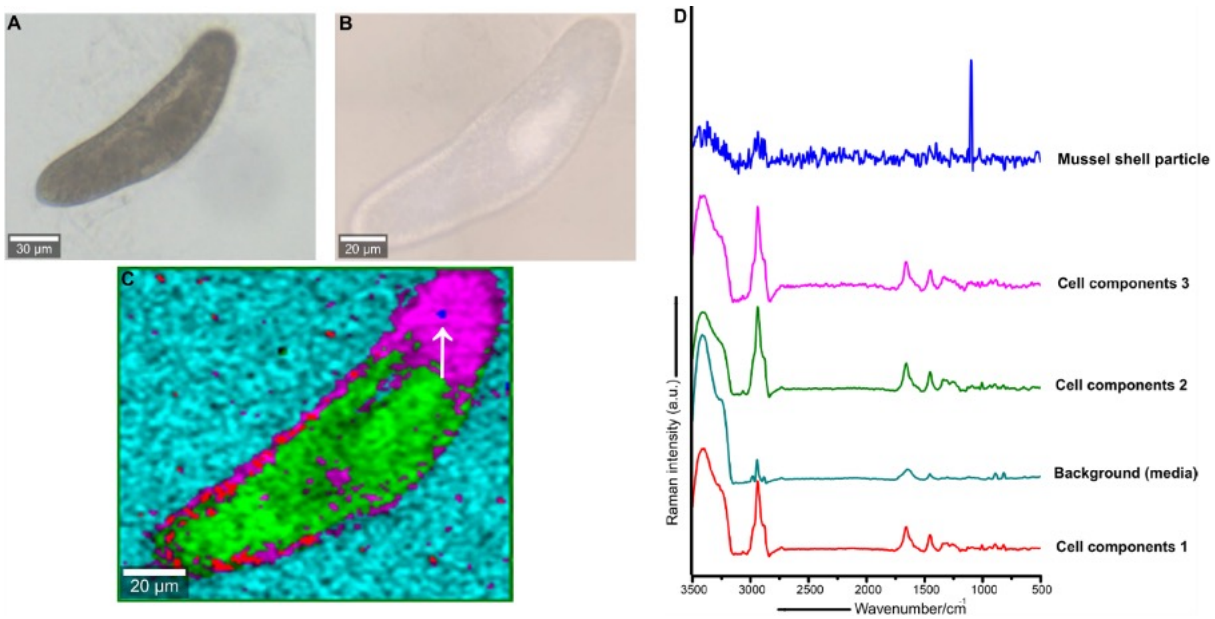
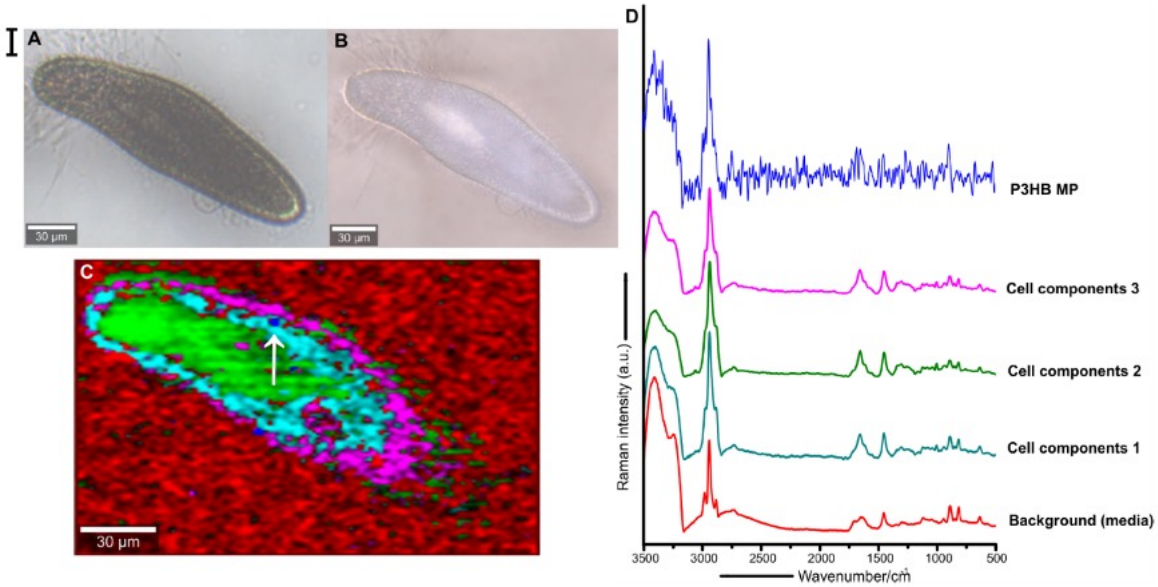
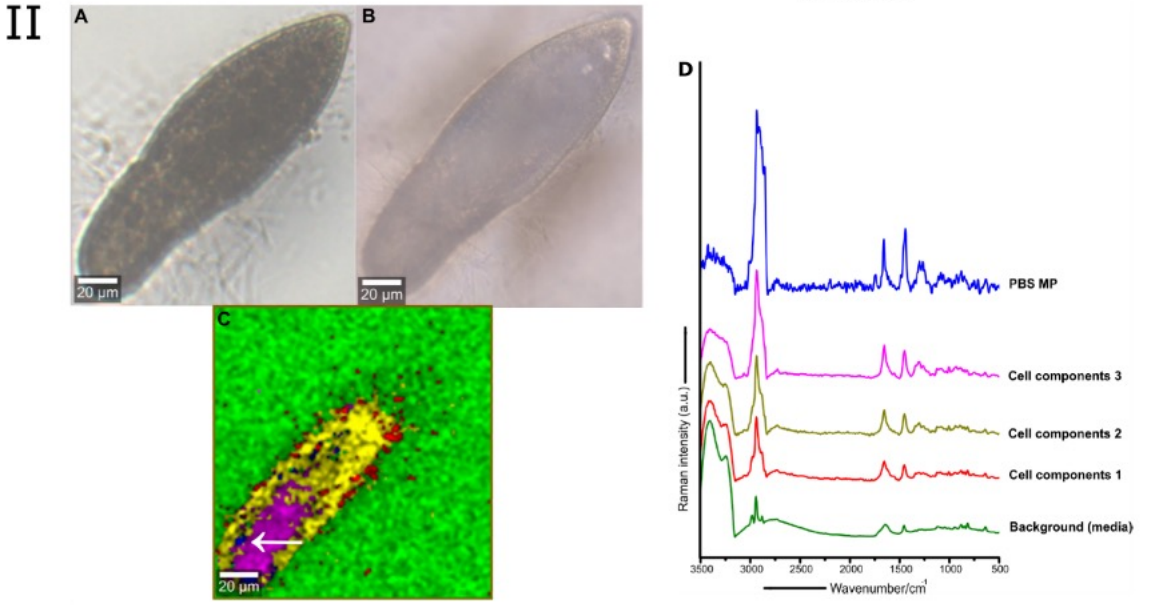


Figure SI 14: Analysis of a mussel shell (MS) particle uptake by Raman Imaging; A) Bright-field microscopic image of the paramecium, taken at 10x magnification; B) Bright-field microscopic stitched image of the paramecium, single tiles were taken at 50x magnification, using the objective of the Raman microscope; C) Raman image reconstructed from the k-means cluster analysis and spectral demixing depicting the different components (MS particle (white arrow), background and cell components 1-5) color coded according to the spectra shown in (D) respectively; D) Mean Raman spectra of different cellular components separated by the data analysis along with the spectrum for MS used for particle depiction color coded in blue and the mean spectra of the background representing the media in which the paramecia were reared.


I
A
B
30 µm
30 µm
C
30 µm
D
P3HB MP
Cell components 3
Cell components 2
Cell components 1
Background (media)
Raman intensity (a.u.)
3500 3000 2500 2000 1500 1000 500
Wavenumber/cm-1


II
A
B
20 µm
20 µm
C
20 µm
D
PBS MP
Cell components 3
Cell components 2
Cell components 1
Background (media)
Raman intensity (a.u.)
3500 3000 2500 2000 1500 1000 500
Wavenumber/cm-1

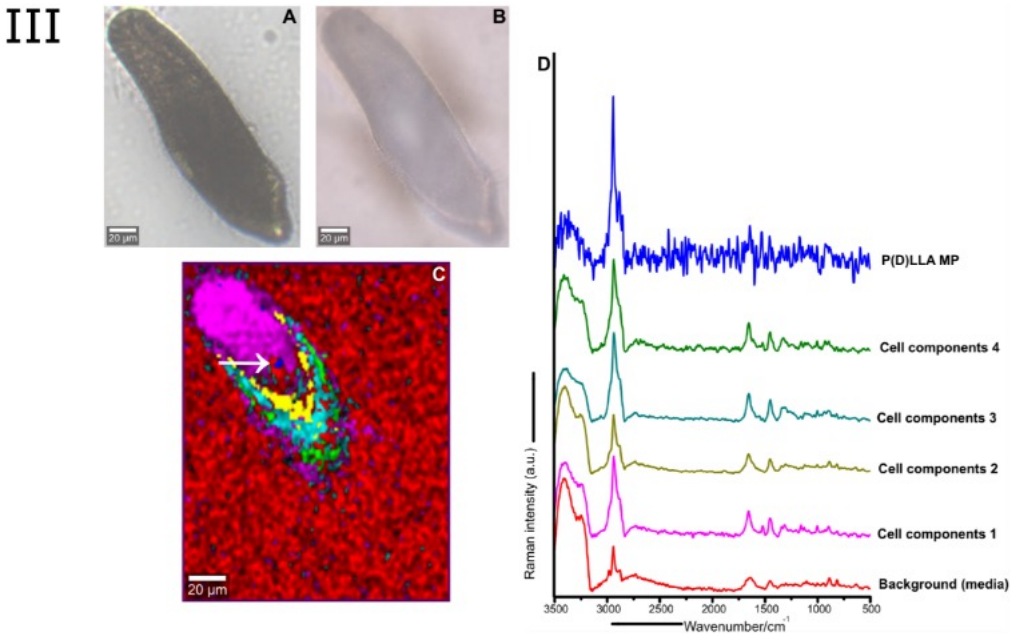
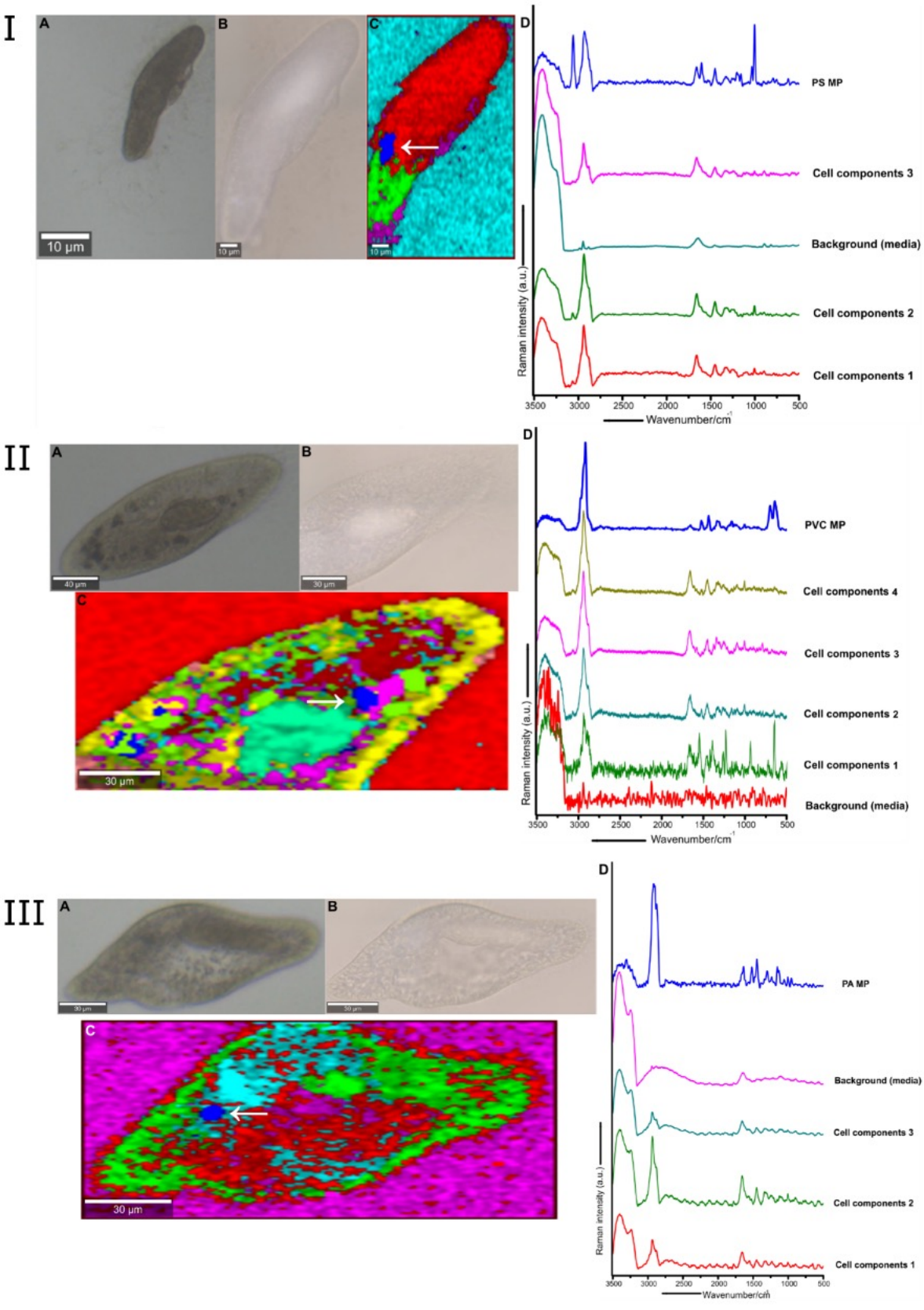

III
A
B
20 µm
20 µm
C
20 µm
D
P(D)LLA MP
Cell components 4
Cell components 3
Cell components 2
Cell components 1
Background (media)
Raman intensity (a.u.)
3500 3000 2500 2000 1500 1000 500
Wavenumber/cm-1

Figure SI 15: Analysis of BB-MP uptake by Raman Imaging; (I) P3HB; (II) PBS; (III) P(D)LLA. (A) Bright-field microscopic image of the paramecium, taken at 10x magnification; B) Bright-field microscopic stitched image of the paramecium, single tiles were taken at 50x magnification, using the objective of the Raman microscope; C) Raman image reconstructed from the k-means cluster analysis and spectral demixing depicting the different components (MP particle (white arrow), background and cell components 1-5) color coded according to the spectra shown in (D) respectively; D) Mean Raman spectra of different cellular components separated by the data analysis along with the spectrum for BB-MP used for particle depiction color coded in blue and the mean spectra of the background representing the media in which the paramecia were reared.


I
A
B
C
10 μm
D
PS MP
Cell components 3
Background (media)
Cell components 2
Cell components 1
Raman intensity (a.u.)
3500 3000 2500 2000 1500 1000 500
Wavenumber/cm-1
II
A
B
C
30 μm
D
PVC MP
Cell components 4
Cell components 3
Cell components 2
Cell components 1
Background (media)
Raman intensity (a.u.)
3500 3000 2500 2000 1500 1000 500
Wavenumber/cm-1
III
A
B
C
30 μm
D
PA MP
Background (media)
Cell components 3
Cell components 2
Cell components 1
Raman intensity (a.u.)
3500 3000 2500 2000 1500 1000 500
Wavenumber/cm-1

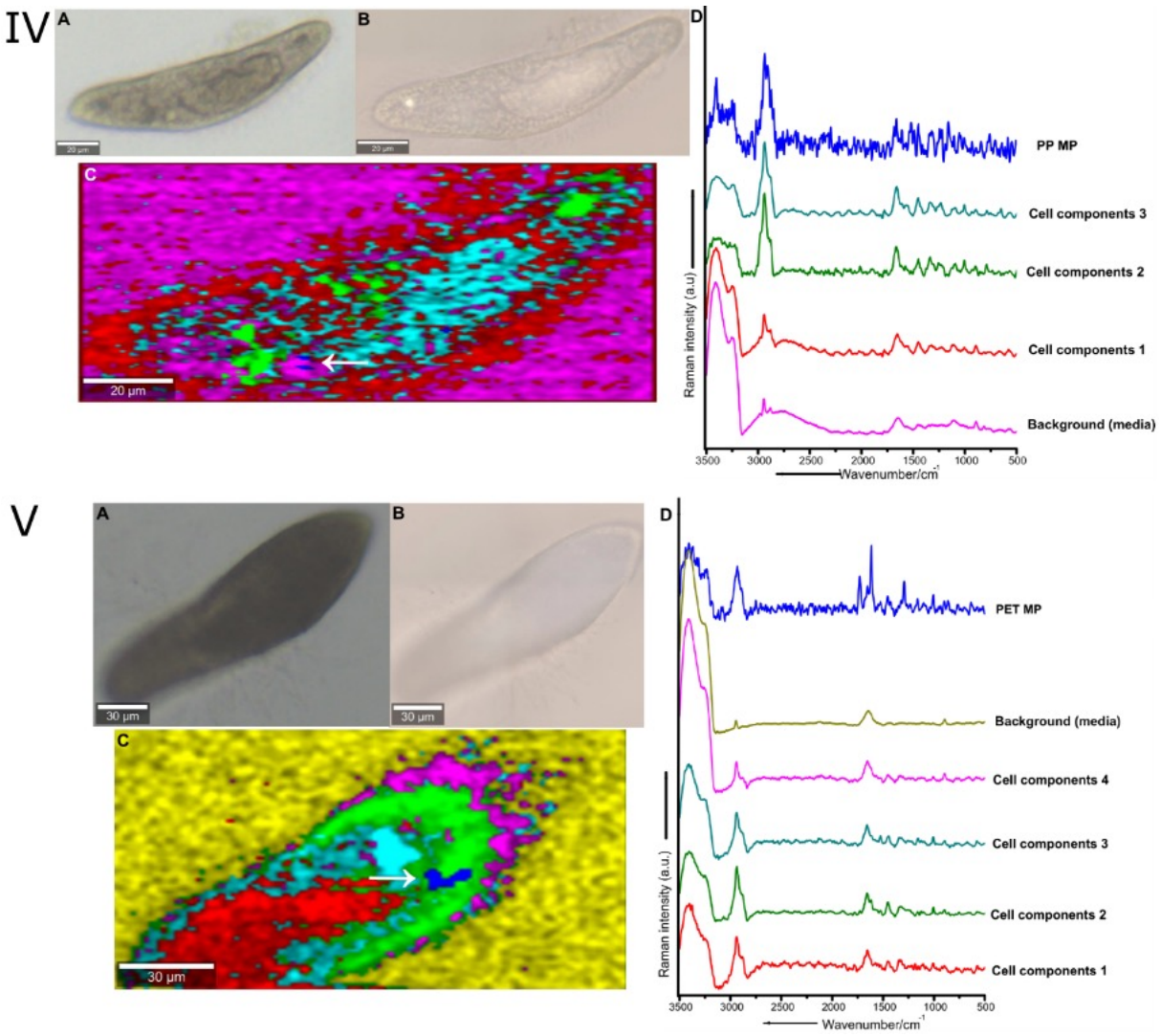


Figure SI 16: Analysis of PB-MP particle uptake by Raman Imaging; (I) PS; (II) PVC; (III) PA; (IV) PP; (V) PET. (A) Bright-field microscopic image of the paramecium, taken at 10x magnification; B) Bright-field microscopic stitched image of the paramecium, single tiles were taken at 50x magnification, using the objective of the Raman microscope; C) Raman image reconstructed from the k-means cluster analysis and spectral demixing depicting the different components (MP particle (white arrow), background and cell components 1-5) color coded according to the spectra shown in (D) respectively; D) Mean Raman spectra of different cellular components separated by the data analysis along with the spectrum for PB-MP used for particle depiction color coded in blue and the mean spectra of the background representing the media in which the paramecia were reared.

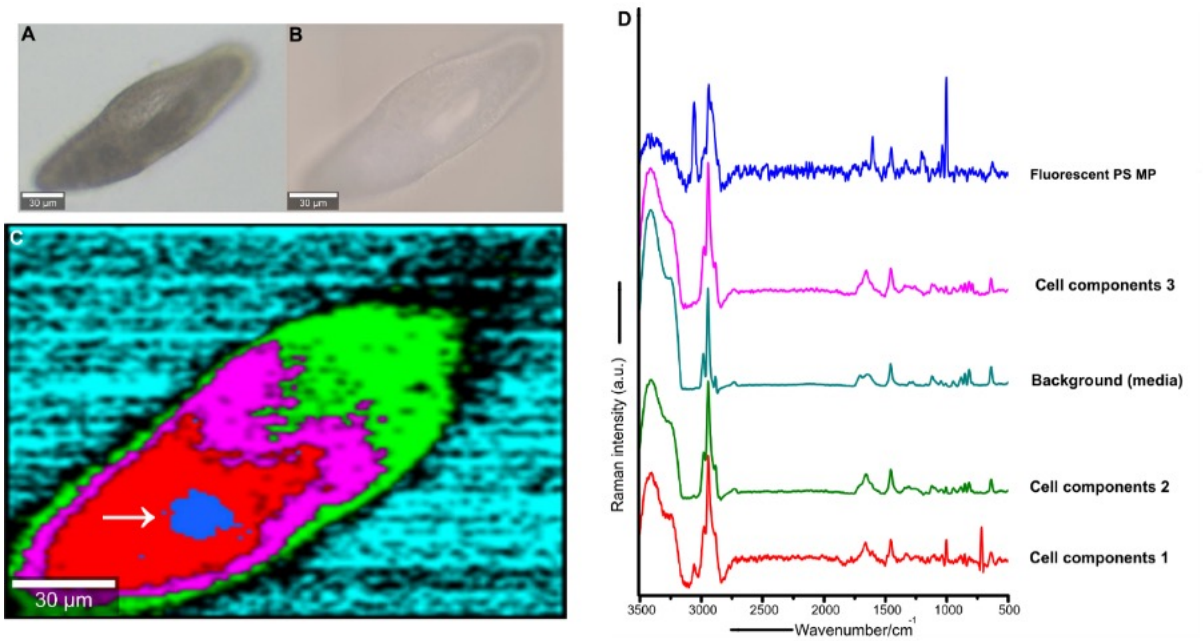


Figure SI 17: Analysis of fluorescent PS MP particle uptake by Raman Imaging; A) Bright-field microscopic image of the paramecium, taken at 10x magnification; B) Bright-field microscopic stitched image of the paramecium, single tiles were taken at 50x magnification, using the objective of the Raman microscope; C) Raman image reconstructed from the k-means cluster analysis and spectral demixing depicting the different components (fluorescent PS MP (white arrow), background and cell components 1-5) color coded according to the spectra shown in (D) respectively; D) Mean Raman spectra of different cellular components separated by the data analysis along with the spectrum for fluorescent PS MP used for particle depiction color coded in blue and the mean spectra of the background representing the media in which the paramecia were reared.

Fluorescence microscopy

Short-term exposures of two hours were performed to demonstrate particle uptake. These exposures involved using fluorescently labeled PS fragments (<20 µm). Individuals of *P. caudatum* were exposed to 10000 particles mL^{-1}. Ten µl of the media containing exposed organisms was then transferred onto a microscopy slide, and the fluorescently labeled PS particles were observed with a fluorescence microscope (Zeiss Imager.Z2m, 1x camera adapter, Carl Zeiss, Jena, Germany). See also Mondellini et al. (2024) for more information.

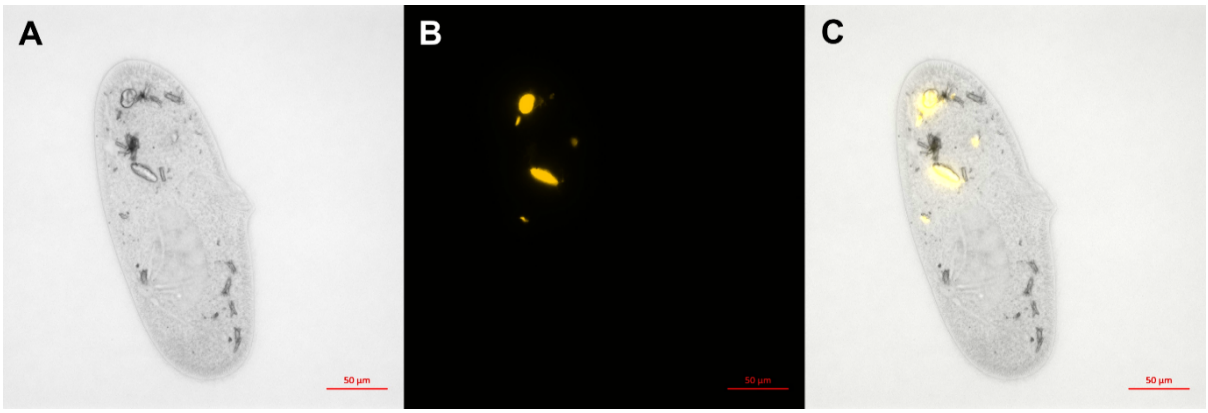


Figure SI 18: Fluorescently labeled PS fragments (<20 µm) ingested by *P. caudatum* observed with fluorescence microscopy. A: bright field, B: epifluorescence, C: merged channels. Scale bars = 50µm.

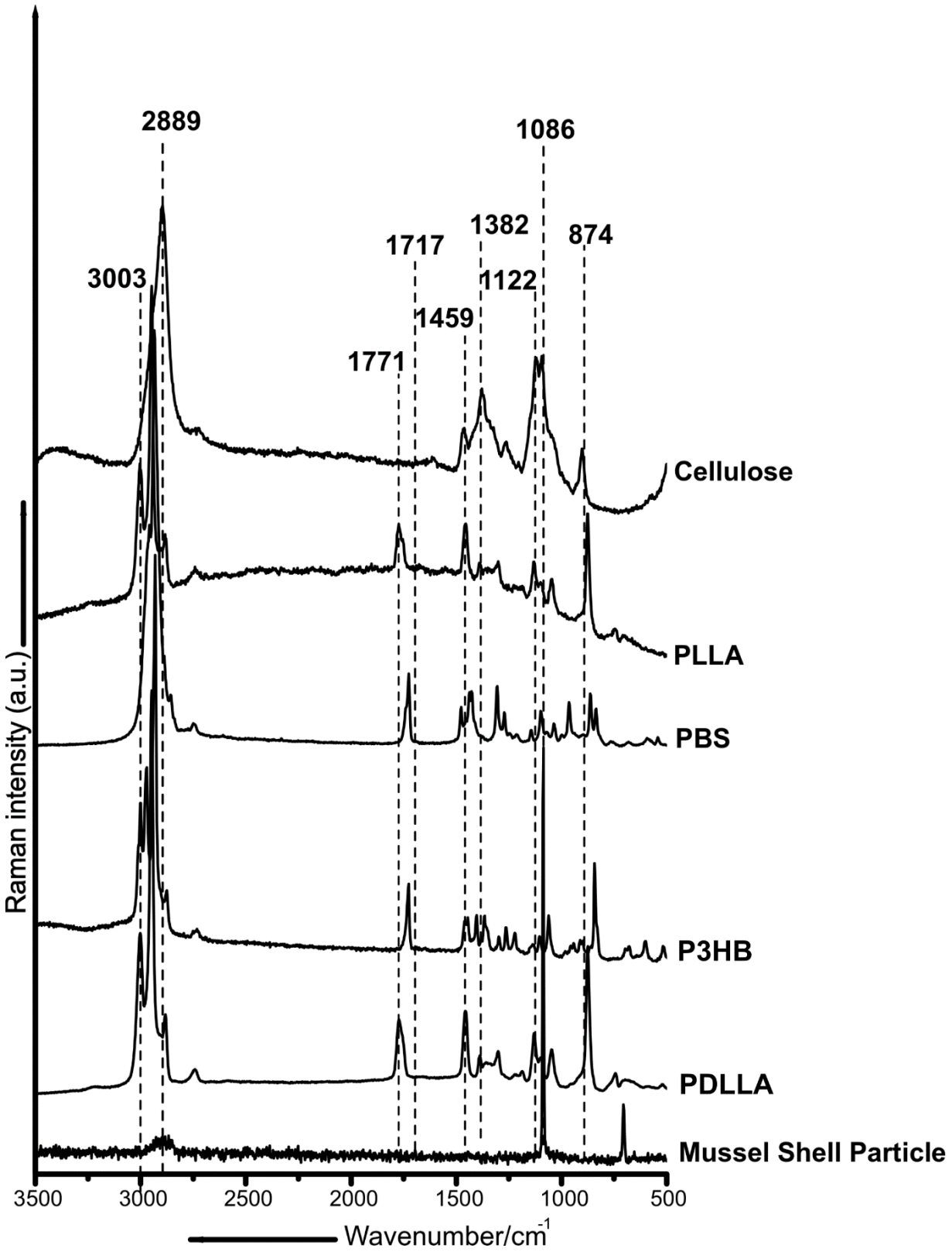


Figure SI 19: Raman reference spectra for BB-MPs and control particles.

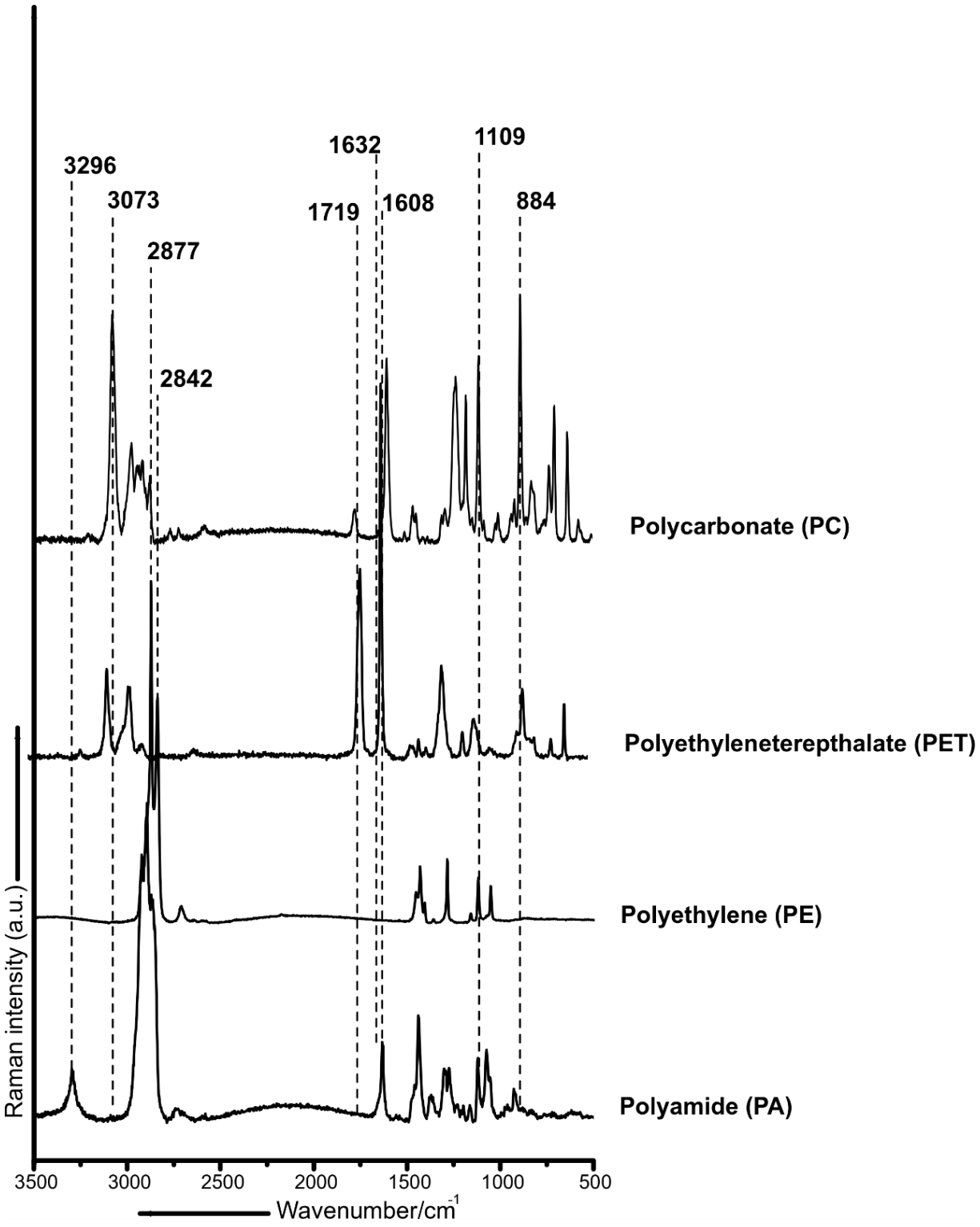


Figure SI 20: Raman reference spectra for PB-MP particles.

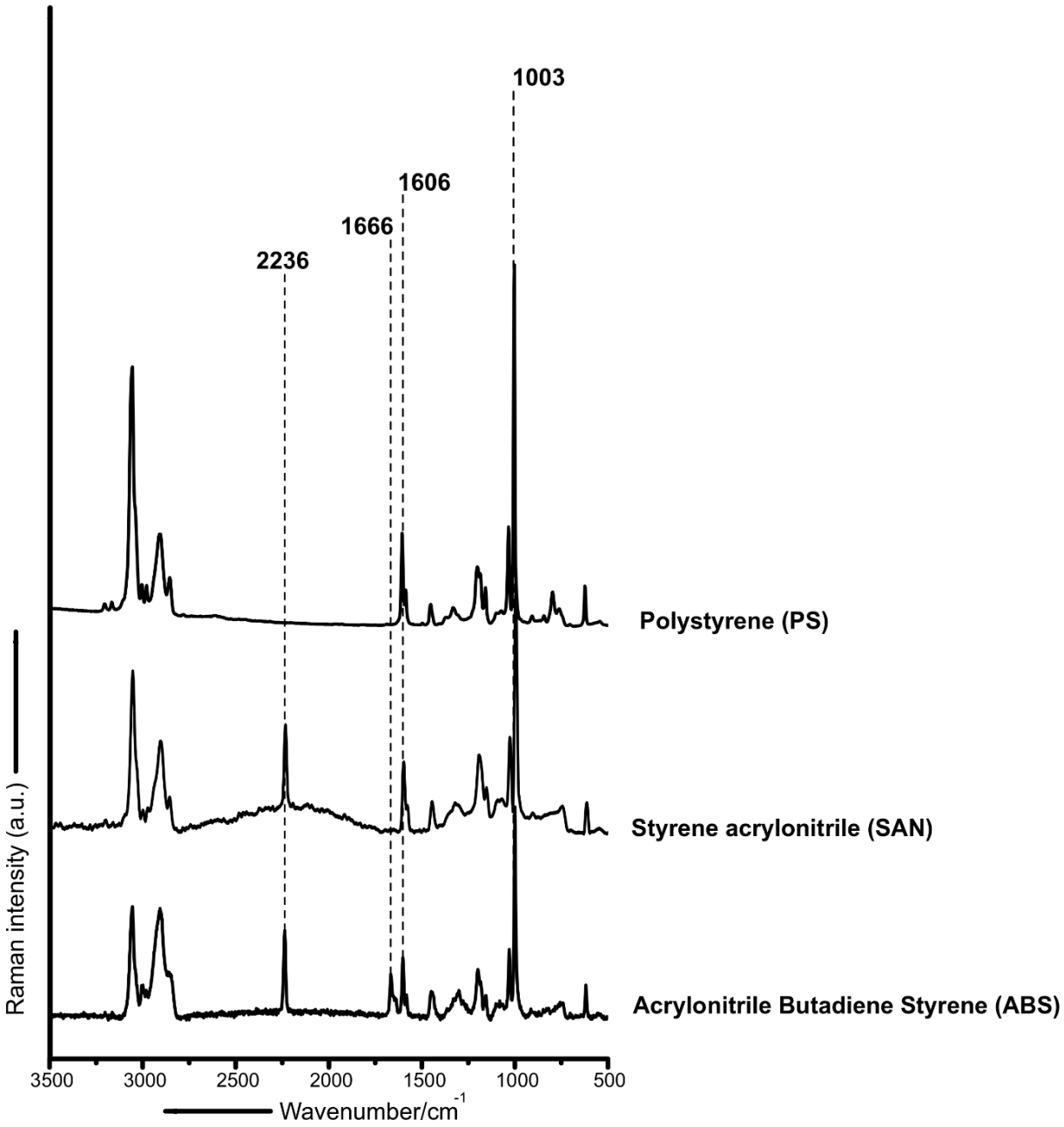


Figure SI 21: Raman reference spectra for PB-MP particles.

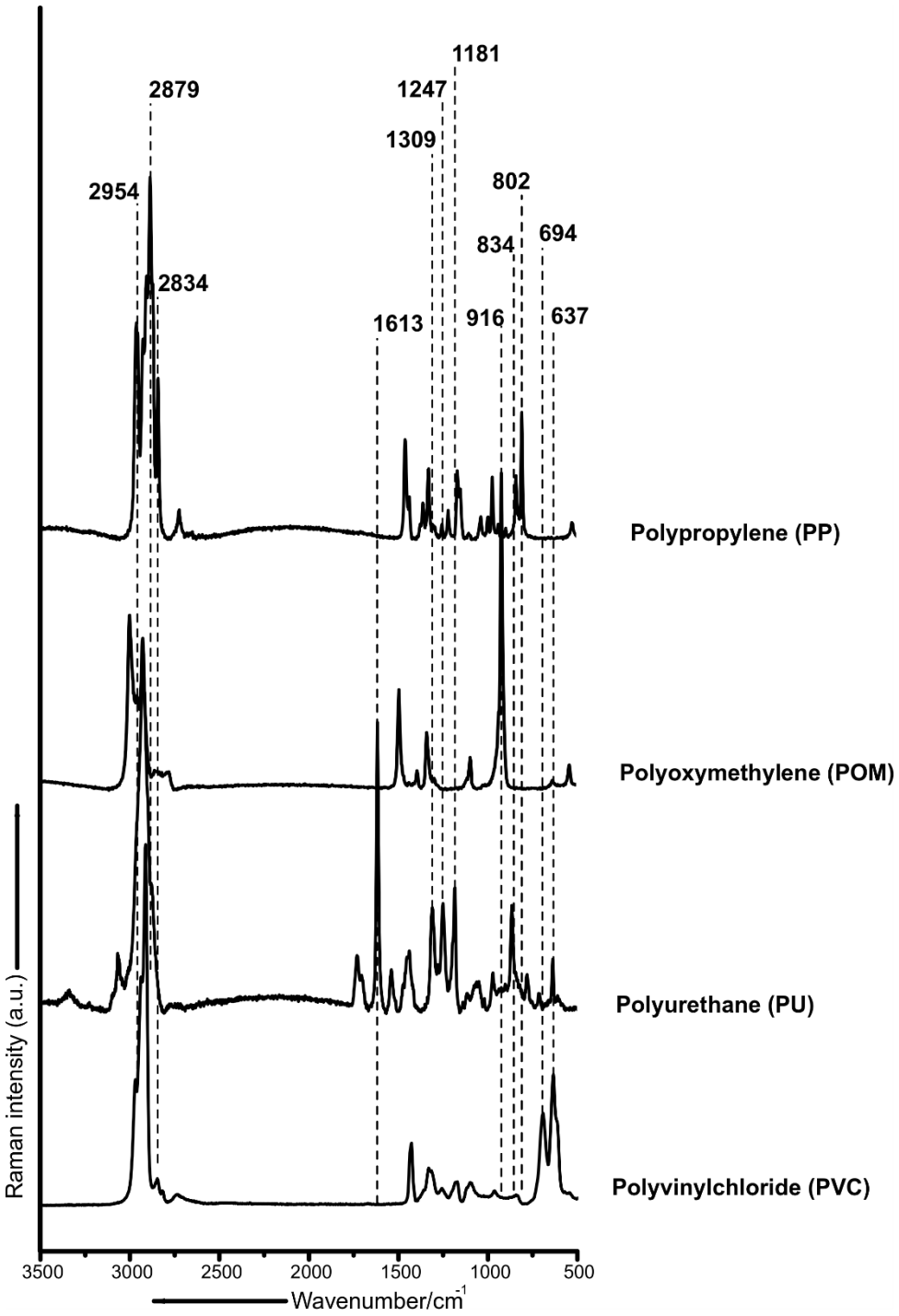


Figure SI 22: Raman reference spectra for PB-MP particles.

Table S1: *Results from contrasts comparing microplastic (MP) treatments with their respective controls. MP treatments where Tween20 was added to prevent particle aggregation are compared to a Tween control, freshwater (FW) MP treatments are compared to the FW control, and ultrapure water (UW) MP treatments are compared to the UW control.*

Contrast	Ratio	SE	df	Null	z ratio	p-value	Significance
Cellulose FW / Control FW	1.49	0.10	Inf	1	6.18	<0.0001	***
Kaolin FW / Control FW	1.48	0.10	Inf	1	6.05	<0.0001	***
MS FW / Control FW	1.09	0.07	Inf	1	1.35	0.2473	
PLLA FW / Control FW	1.49	0.10	Inf	1	6.11	<0.0001	***
PDLLA FW / Control FW	1.51	0.10	Inf	1	6.37	<0.0001	***
PBS FW / Control FW	1.53	0.10	Inf	1	6.54	<0.0001	***
P3HB FW / Control FW	1.55	0.10	Inf	1	6.78	<0.0001	***
PA FW / Control FW	1.15	0.08	Inf	1	2.13	0.0547	
PET FW / Control FW	1.56	0.10	Inf	1	6.93	<0.0001	***
PS FW / Control FW	1.10	0.07	Inf	1	1.39	0.2427	
PVC FW / Control FW	1.05	0.07	Inf	1	0.75	0.5534	
Tween FW / Control FW	0.91	0.08	Inf	1	-1.08	0.3593	
LDPE FW / Tween FW	1.27	0.09	Inf	1	3.43	0.0011	**
PP FW / Tween FW	1.20	0.09	Inf	1	2.50	0.0214	*
Cellulose UW / Control UW	1.40	0.09	Inf	1	5.26	<0.0001	***
Kaolin UW / Control UW	1.46	0.09	Inf	1	5.91	<0.0001	***
MS UW / Control UW	1.03	0.07	Inf	1	0.51	0.7078	
PLLA UW / Control UW	1.44	0.09	Inf	1	5.75	<0.0001	***
PDLLA UW / Control UW	1.29	0.08	Inf	1	3.88	0.0002	***
PBS UW / Control UW	1.52	0.10	Inf	1	6.56	<0.0001	***
P3HB UW / Control UW	1.41	0.09	Inf	1	5.35	<0.0001	***
PA UW / Control UW	1.03	0.07	Inf	1	0.48	0.7109	
PET UW / Control UW	1.32	0.09	Inf	1	4.29	<0.0001	***
PS UW / Control UW	1.02	0.07	Inf	1	0.29	0.7727	
PVC UW / Control UW	1.03	0.07	Inf	1	0.41	0.7362	
Tween UW / Control UW	1.09	0.09	Inf	1	1.08	0.3593	
LDPE UW / Tween	1.12	0.07	Inf	1	1.72	0.1327	
PP UW / Tween	1.02	0.07	Inf	1	0.29	0.7727	

Values derived from a Conway-Maxwell Poisson regression model. Model formula: n.individuals ~ Particle.type*time* + *concentration* + *Particle.type:concentration* + *incubation* + *Particle.type:incubation. SE: standard error, df: degrees of freedom. Significant ratios are marked by asterisks.* *: $p < 0.05$.

***Table S2**: Marginal means for the number of paramecia depending on treatments. Means calculated with R package emmeans for mean exposure time (48 hours) and mean particle concentration based on a Conway-Maxwell Poisson regression.*

Treatment	Incubation type	Time	Response	SE	df	asymp.LCL	asymp.UCL
Control	FW	48	1.14	0.07	Inf	1.014	1.272
Cellulose	FW	48	1.70	0.05	Inf	1.601	1.797
Kaolin	FW	48	1.68	0.05	Inf	1.587	1.781
MS	FW	48	1.24	0.04	Inf	1.165	1.325
PLLA	FW	48	1.69	0.05	Inf	1.594	1.788
PDLLA	FW	48	1.71	0.05	Inf	1.620	1.815
PBS	FW	48	1.73	0.05	Inf	1.638	1.835
P3HB	FW	48	1.76	0.05	Inf	1.663	1.862
PA	FW	48	1.31	0.04	Inf	1.227	1.392
PET	FW	48	1.77	0.05	Inf	1.679	1.876
PS	FW	48	1.25	0.04	Inf	1.167	1.329
PVC	FW	48	1.19	0.04	Inf	1.118	1.275
Tween	FW	48	1.04	0.07	Inf	0.915	1.172
LDPE	FW	48	1.32	0.04	Inf	1.240	1.405
PP	FW	48	1.24	0.04	Inf	1.160	1.321
Control	UW	48	1.19	0.07	Inf	1.064	1.331
Cellulose	UW	48	1.67	0.05	Inf	1.575	1.767
Kaolin	UW	48	1.74	0.05	Inf	1.640	1.836
MS	UW	48	1.23	0.04	Inf	1.154	1.314
PLLA	UW	48	1.72	0.05	Inf	1.624	1.819
PDLLA	UW	48	1.53	0.05	Inf	1.441	1.624
PBS	UW	48	1.81	0.05	Inf	1.709	1.907
P3HB	UW	48	1.68	0.05	Inf	1.583	1.775
PA	UW	48	1.23	0.04	Inf	1.149	1.312
PET	UW	48	1.57	0.05	Inf	1.479	1.665
PS	UW	48	1.21	0.04	Inf	1.135	1.296
PVC	UW	48	1.22	0.04	Inf	1.146	1.304
Tween	UW	48	1.30	0.07	Inf	1.160	1.453
LDPE	UW	48	1.45	0.05	Inf	1.367	1.544
PP	UW	48	1.32	0.04	Inf	1.243	1.410

Model formula: n.individuals ~ Particle.type*time + concentration + Particle.type:concentration + incubation + Particle.type:incubation. SE: standard error, df: degrees of freedom, LCL: Lower confidence limit. UCL: Upper confidence limit.

Discussion of the potential effects of Tween20

As an additional control in the reproduction experiment, a Tween20 exposure group was included. Tween20 is a non-ionic surfactant commonly used in our daily lives and in MP studies as a dispersant for particles with buoyant properties (Khosrovyan and Kahru 2022) to improve their suspensibility. Tween20 modifies liquid properties by reducing surface tension and interfaces (Rebello et al. 2014). In this study, Tween20 was used for the hydrophobic plastics PP and LDPE. It is generally considered a non-toxic substance, especially at the concentrations used in MP studies (0.01 and 0.001 Volume%; e.g., Khosrovyan and Kahru 2022; Esterhuizen et al. 2023), but some studies have reported induced cyto/genotoxicity, apoptosis, and necrosis (e.g., Eskandani et al. 2013; Reitermayer et al. 2018), as well as effects on plankton communities in the case of Tween80 (Yuan et al. 2024). In our study, *P. caudatum* individuals exposed to Tween20 showed no adverse effects from this surfactant.

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Article II

Size dependent uptake and trophic transfer of polystyrene microplastics in unicellular freshwater eukaryotes

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Size dependent uptake and trophic transfer of polystyrene microplastics in unicellular freshwater eukaryotes

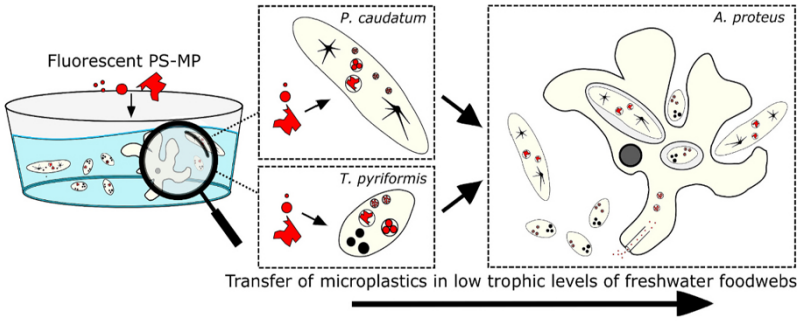
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HIGHLIGHTS

- *P. caudatum* and *T. pyriformis* internalize large MP, relative to their cell size.
- *A. proteus* avoids ingesting MP larger than 6 μm suspended in the water column.
- *A. proteus* ingests larger microplastic particles when encountered during predation.
- Evidence of polystyrene MP transfer in lower trophic levels

GRAPHICAL ABSTRACT



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ABSTRACT

Microplastics (MP) have become a well-known and widely investigated environmental pollutant. Despite the huge amount of new studies investigating the potential threat posed by MP, the possible uptake and trophic transfer in lower trophic levels of freshwater ecosystems remains understudied. This study aims to investigate the internalization and potential trophic transfer of fluorescent polystyrene (PS) beads (0.5 μm, 3.6×10^8 particles/mL; 6 μm, 2.1×10^5 particles/mL) and fragments (<30 μm, 5×10^3 particles/mL) in three unicellular eukaryotes. This study focuses on the size-dependent uptake of MP by two freshwater Ciliophora, *Tetrahymena pyriformis, Paramecium caudatum* and one Amoebozoa, *Amoeba proteus,* serving also as predator for experiments on potential trophic transfer. Size-dependent uptake of MP in all three unicellular eukaryotes was shown. *P. caudatum* is able to take up MP fragments up to 27.7 μm, while *T. pyriformis* ingests particles up to 10 μm. In

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A. proteus, small MP ($PS_{0.5\mu m}$ and $PS_{6\mu m}$) were taken up via pinocytosis and were detected in the cytoplasm for up to 14 days after exposure. Large PS-MP ($PS_{<30\mu m}$) were detected in *A. proteus* only after predation on MP-fed Ciliophora. These results indicate that *A. proteus* ingests larger MP via predation on Ciliophora ($PS_{<30\mu m}$), which would not be taken up otherwise. This study shows trophic transfer of MP at the base of the aquatic food web and serves as basis to study the impact of MP in freshwater ecosystems.

1. Introduction

The extensive usage and careless handling of plastics since the second half of the 20th century led to an increasing accumulation of plastic waste in all environmental compartments (Ilyas et al., 2018; Li et al., 2016). Increasing pollution, not only with macro-, but also microplastics (MP, 100 nm - 5 mm, de Souza Machado et al., 2023), emerges as an ubiquitous hazard for the biota (Bellasi et al., 2020; Imhof et al., 2013; Kernchen et al., 2022). MP can be divided into primary (intentionally manufactured for specific applications) and secondary (fragments from larger plastic objects) plastic particles with variable shapes (spheres, fragments or fibers), sizes, and surface characteristics (Frias and Nash, 2019; Wieland et al., 2024). Due to their small size and ubiquitous presence, MP can be ingested by various organisms (Huang et al., 2021; Imhof et al., 2013; König Kardgar et al., 2023; Liu et al., 2022a; Magni et al., 2020). The impact of MP on aquatic ecosystems has been the subject of many investigations. MP research focused initially on marine compartments, and has nowadays extended to freshwater ecosystems (Li et al., 2023; Range et al., 2023), and the organisms that inhabit them (Triebskorn et al., 2019; Wagner et al., 2014). It has been shown that the ingestion of MP by aquatic organisms has the potential to induce different negative effects, such as impairment of food consumption, growth, and reproduction (Du et al., 2021). Different studies have been carried out on this issue, using both vertebrate and invertebrate organisms (Brehm et al., 2022; Brehm et al., 2023; Eltemsah and Bøhn, 2019; Imhof et al., 2017; Klein et al., 2021; Schwarzer et al., 2022; Xia et al., 2020; Xu et al., 2020). However, information regarding the potential impact of MP on organisms of lower trophic levels in freshwater ecosystems is still scarce. Nevertheless, continuous fragmentation and degradation of MP results in rising numbers of smaller MP particles in the aquatic environment, increasing their bioavailability for smaller biota (Al-Thawadi, 2020; González-Pleiter et al., 2019). For planktonic crustaceans, like Cladocera and Copepoda, it has been shown that many of them are prone to ingest MP, which can lead to various adverse effects like, e.g., increased oxidative stress, biomass reduction, fitness impairment, higher mortality rates, behavioral alterations, and impacts on their ability to feed and moult (Bosker et al., 2019; Cole et al., 2019; Cole et al., 2015; De Felice et al., 2019; Liu et al., 2022a; Schwarzer et al., 2022). Recent investigations also confirmed size-dependent uptake and toxicity in microzooplankton, like rotifers. For *Brachionus* sp., it was shown that MP ingestion has negative effects on their feeding activity, oxidative status, and gene expression (Drago et al., 2020; Jeong et al., 2016; Zheng et al., 2022). A recent study from Athey and co-authors (Athey et al., 2020) also showed that the marine tintinnid *Favella* spp. quickly ingests MP and that those particles are transferred via predation to estuarine fish larvae (*Menidia beryllina*). Unicellular eukaryotes hold an important role in aquatic ecosystems, as they generally feed on particulate matter, algae, bacteria or other, smaller unicellular eukaryotes (Mansano et al., 2014; Mansano et al., 2016). Furthermore, unicellular eukaryotic organisms also serve as prey for other protists and higher organisms. A decline in their population could, consequently, have an impact on both the bacterial communities that they prey upon, and on primary consumers, potentially altering the food web (Mansano et al., 2016). Nonetheless, only some studies employed these organisms for MP research to investigate, for instance, effects on photosymbiont productivity, food selectivity, population dynamics, and size dependent MP uptake (Bermúdez et al., 2021; Bulannga and Schmidt, 2022; Feng et al., 2021; Geng et al., 2021; Nałęcz-Jawecki et al., 2021; Nugroho and Fyda, 2020; Wang et al., 2024; Makin, 2023; Zhang et al., 2021).

Plenty of unicellular eukaryotes are non-selective feeders. Hence, food choice is dependent on availability (Nisbet, 2012). Given MP abundance in smaller size ranges (<50 μm) in virtually all freshwater compartments (Imhof et al., 2016), these particles are potentially available to a wide range of suspension feeders, including unicellular eukaryotes (Bulannga and Schmidt, 2022). If ingested, MP might be transferred to predators, as unicellular eukaryotes are usually subject to a high predation rate (Mansano et al., 2016). Ultimately, this could lead to a transfer of MP along the food web. Despite the constantly increasing effort spent by the scientific community on the investigation of the possible impact of MP exposure on different organisms, the uptake of MP by eukaryotic unicellular organisms of freshwater ecosystems as well as trophic transfer in these lower trophic levels are still scarcely researched.

In this study, two freshwater Ciliophora *Tetrahymena pyriformis* and *Paramecium caudatum*, that normally feed on suspended particulate matter, small bacteria and small eukaryotes, were employed as model organisms to investigate MP ingestion. Both organisms are likely to unintentionally ingest MP via phagocytosis, since they cannot differentiate between food (e.g., suspended particulate matter, bacteria and algae) and inorganic particulate matter (Scherer et al., 2018). We used *Amoeba proteus* as a generalist predator that feeds on other unicellular eukaryotes. Amoebae also feed on particulate matter via phagocytosis, but, unlike *T. pyriformis* and *P. caudatum,* they are known to be able to discern between organic and inorganic matter (Mast and Hahnert, 1935). Moreover, *A. proteus* shows a permanent, but minor pinocytosis-driven ingestion. As pinocytosis channels in *A. proteus* reach a diameter of up to 6 μm (Klein et al., 1988), they might be large enough for a passive uptake of MP smaller than this size.

Hence, the main objective of this study is to investigate if three distinct unicellular freshwater eukaryotes *P. caudatum*, *T. pyriformis*, and *A. proteus* are able to ingest MP particles of different sizes. To achieve this, we employed fluorescent polystyrene (PS) beads in two sizes (0.5 and 6 μm) as well as larger PS fragments (<30 μm). The selection of different size categories is essential for discerning potential size-dependent uptake patterns. We further analyzed in *A.proteus*, if the uptake of the different sized particles takes place via phagocytosis or pinocytosis. Finally, we investigated the potential for trophic transfer of MP by introducing *A. proteus* to its potential prey, *P. caudatum* and *T. pyriformis*, after these prey organisms have been exposed to MP.

2. Material and methods

2.1. Materials

If not otherwise indicated, materials and chemicals were obtained from Carl Roth GmbH (Karlsruhe, Germany) or Thermo Fisher Scientific (Schwerte, Germany). Fetal calf serum and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (Taufkirchen, Germany), cell culture material from Greiner Bio-One (Frickenhausen, Germany). Dulbecco's phosphate-buffered saline (DPBS) was obtained from Lonza Group AG (Visp, Switzerland). Non-functionalized 0.5 and 6 μm polystyrene (PS) beads (fluorescent, Fluoresbrite® PC Red Microspheres, Cat. # 19507–5 and Cat. # 19111–2, Ex_{Max} 491 nm, Em_{Max} 565 nm, 3.64×10^{11} beads/mL ($PS_{0.5\mu m}$) and 2.10×10^{8} beads/mL ($PS_{6\mu m}$), size CV $\leq$ 5) were purchased from Polyscience (Polyscience Europe GmbH, Eppenheim, Germany). PS beads were delivered in a sterile aqueous suspension at a

concentration of 2.5 % (*w/v*). According to the supplier, all MP particles therein are plain beads with a slight residual anionic surface charge from sulphate ester. Larger PS fragments (Magic Pyramid, Frechen, Germany) were prepared in house by milling and sieving (detailed information in the SI, Figs. S1 and S2) to fractions of <30 μm (produced within the CRC 1357, department Macromolecular Chemistry I, University of Bayreuth; https://www.sfb-mikroplastik.uni-bayreuth.de/en/index.html, Fig. S1). The obtained fractions were then suspended in bi-distilled water, supplemented with 0.1 % (*w/v*) Tween20® to avoid aggregation. Afterwards, the suspension was centrifuged at 13,000 x*g* for 5 min at room temperature (RT), the supernatant was discarded, and the MP were resuspended in bi-distilled water ($PS_{<30\mu m}$, 5×10^5 fragments/mL). In the following, the respective MP particles will be referred to as $PS_{0.5\mu m}$, $PS_{6\mu m}$ (beads), and $PS_{<30\mu m}$ (fragments). The different particle sizes were chosen due to the different advantages of the respective size ranges. For $PS_{0.5\mu m}$ and $PS_{6\mu m,}$ narrow size ranges with a well-defined characterization were possible due to the manufacturing (suspension polymerization). Larger fragments ($PS_{<30\mu m}$) are more comparable to environmental particles but were not available in narrow size ranges due to their manufacturing (milling and sieving of larger fractions). Before usage, the respective MP stock suspensions were diluted to the desired concentration in the suitable growth media.

2.2. Methods

2.2.1. Particle characterization

The ζ-potentials of the particle fractions were measured using the LiteSizer 500 (Anton Paar Germany GmbH, Ostfildern-Scharnhausen, Germany) and Omega cuvettes (Anton Paar Germany GmbH, Ostfildern-Scharnhausen, Germany). For the measurements, 2.5 μL of the particle suspension were directly diluted in the respective media (i.e., $Prescott_{ref}$ (pH 6.4), $Prescott_{NaCl}$ (pH 4.3), see below for compostion) and incubated overnight. Thereafter, the particles were collected by centrifugation (13,000 ×*g* for 10 min at room temperature (RT)) and resuspended in 1 mL of a 1 mM KCl (pH 6.0) solution for measurement. Three measurements with at least 100 runs each were performed at 21 °C with an adjusted voltage of 200 V. The ζ-potential was calculated using the Helmholtz-Smoluchowski equation (Smoluchowski, 1906).

Particle size distributions were measured by laser diffraction (three lasers and two detectors) coupled with dynamic image analysis, using a Microtrac FlowSync Particle Size Analyzer (Microtrac Retsch GmbH, Haan, Germany, Fig. S1). Wet analysis was performed using deionized water as the mobile phase with 1 mg of particles added to 1 mL of 5 % (*w/v*) $Na_4P_2O_7$ and 2 % (*w/v*) Tween® 20 detergent to avoid aggregation. This mixture was poured into the water-filled measuring cell and diluted with 200 mL ultrapure water. A 180 s ultrasound treatment (40 kHz) was carried out to ensure complete suspension of the particles.

2.2.2. Cultivation of Tetrahymena pyriformis, Paramecium caudatum and Amoeba proteus

T. pyriformis (Icon biotech, Teltow, Germany) was cultivated at room temperature (20–25 °C) in 2 % (*w/w*) proteose-peptone broth. The freshwater ciliate *P. caudatum* originated from a well-established continuous in-house freshwater cultivation (Carolina Science GmbH, Berlin, Germany). The organisms were kept in 800 mL Erlenmeyer Duran flasks (DURAN group, Wertheim, Germany) filled with diluted Z medium ($Ca(NO_3)_2$ 16.66 mM, $NaNO_3$ 549.45 mM, K_2HPO_4 48.24 mM, $MgSO_4$ 10.14 mM, $NaHCO_3$ 199.98 mM, Na_2EDTA 199.98 mM, $FeSO_4$ 3.41 mM, H_3BO_3 0.23 mM, $MnSO_4$ 2.48 mM, $(NH_4)_6Mo_7O_{24}$ 0.02 mM, $ZnSO_4$ 0.07 mM, $Co(NO_3)_2$ 0.24 mM, $CuSO_4$ 0.01 mM; pH ~ 7.6) (Prusch and Minck, 1985). *P.caudatum* culture was maintained in static conditions in dark cabinets and fed ad libitum with an infusion of dried celeriac root (*Apium graveolens*) prepared in-house.

A. proteus (Carolina Science GmbH, Berlin, Germany) was cultivated as proposed by Prescott and James (1955) in the following referred to as $Prescott_{ref}$ method and medium. Briefly, amoebae were cultured in a 10 cm glass Petri dish (VWR, Germany) at room temperature in $Prescott_{ref}$ medium (KCl 12.4 mM, $CaHPO_4$ 2.9 mM, $MgSO_4$ 1.7 mM, pH 6.4) and were fed twice a week with approximately 10–20 *T. pyriformis* (Icon Biotech, Germany) per amoeba. The amount of *T. pyriformis* added was high enough to ensure a close vicinity between prey and predator. For transferring amoebae for experiments, individuals were pipetted with an Eppendorf pipette while observing them under the microscope (Zeiss Primovert trino, 0.5× Camera Adapter, Zeiss, Germany). For documentation purposes, the software Zen 3.0 blue edition 2019 (Carl Zeiss, Jena, Germany) was used.

2.2.3. Uptake of MP

A. proteus: Analogeous to experiments performed by Ahn and Jeon (1982) and Prusch and Minck (1985), initially, 50–100 amoebae per Petri dish were starved for 24 h by transferring them to a 5 mL Petri dish containing 2 mL $Prescott_{ref}$ medium without any food provided, to stimulate the need for food uptake. After 24 h, ~30 amoebae were transferred into single wells of a 12-well glass plate (iBL, Austria) for each treatment, containing 1 mL of $Prescott_{Ref}$ medium. The respective MP suspensions were added to the starved amoebae, corresponding to the following MP end concentrations in a total volume of 1 mL per well: $PS_{0.5\mu m} = 3.6 \times 10^8$ beads/mL (12.5 μg/mL); $PS_{6\mu m} = 2.1 \times 10^5$ beads/mL (12.5 μg/mL); fragments $PS_{<30\mu m} = 5 \times 10^3$ fragments/mL (5 μg/mL). Alongside the MP treatments, a control group was applied in an extra well, with $Prescott_{Ref}$ medium. This process was repeated three times in total, in which the number of particles, and the number of amoebae, containing MP, was recorded. The selection of these concentration ranges was deliberately chosen, aiming at maximizing the likelihood of interaction between particles and *A. proteus*. After the respective incubation time (0.5 h, 2 h, 4 h, 6 h and 24 h), the amoebae were washed by transferring them twice to fresh wells in 1 mL $Prescott_{Ref}$ medium to remove leftover MP. Thereafter, amoebae were observed under the fluorescent microscope and ingested particles were counted manually (CKX41, Olympus-Lifescience, Hamburg, Germany, magnification 400× and 100× for 0.5 μm beads and 6 μm beads / fragments, respectively).

P. caudatum: 200–300 Individuals were exposed to fluorescently labeled $PS_{0.5\mu m}$ (end concentration: 3.6×10^8 beads/mL, 12.5 μg/mL), $PS_{6\mu m}$ (end concentration: 2.1×10^5 beads/mL, 12.5 μg/mL) and $PS_{<30\mu m}$ (end concentration: 5×10^3 fragments/mL, 5 μg/mL) for 24 h in a 5 mL Petri dish containing 2 mL Z medium, respectively.

T. pyriformis were exposed to fluorescently labeled $PS_{0.5\mu m}$ (end concentration: 3.6×10^8 beads/mL), $PS_{6\mu m}$ (end concentration: 2.1×10^5 beads/mL) and $PS_{<30\mu m}$ (end concentration: 5×10^3 fragments/mL) for 24 h in a 5 mL Petri dish, containing 2 mL $Prescott_{Ref}$ medium, respectively.

Both Ciliophora species were not starved or washed before MP exposure. Ingestion of large PS beads ($PS_{6\mu m}$) and fragments ($PS_{<30\mu m}$) by Ciliophora was quantified microscopically for 15 randomly chosen individuals from each Petri dish at different time points (0.5 h, 2 h, 4 h, 6 h and 24 h). For observations, MP exposed Ciliophora were transferred onto a microscopy slide and covered with a cover slip. This way, the Ciliophora got gently squeezed while the water was evaporating, rendering them unable to move around freely, while they were still completely intact. For particle quantification, the samples (MP exposed Ciliophora) were sealed with nail polish (trend it up nail polish, dm-drogerie markt GmbH + Co. KG, Germany), which prevented the water from evaporating. The ingested particles were then quantified under a fluorescence microscope (Zeiss Imager.Z2m, 1× Camera Adapter, Carl Zeiss, Jena, Germany). For the smaller $PS_{0.5\mu m}$ beads ingested by the Ciliophora, an exact quantification of ingested particles was not possible. This difficulty arose from particle agglomeration inside the food vacuola. For that reason, individuals were divided into two groups ("particle uptake" or "no uptake") by estimating the presence of visible intracellular MP under the microscope.

Ciliophora length to particle ratio was measured and calculated

using ZEN 2 core v2.5 / ZEN 2 starter v2.5 (Carl Zeiss Microscopy GmbH, Jena, Germany).

2.2.4. Induction of pinocytosis in A. proteus

Induction of pinocytosis was conducted to analyze if MP particles are unintentionally internalized together with the medium. To induce pinocytosis, the pH of the $Prescott_{ref}$ medium was adjusted to pH 4.3 with HCl. In addition, the medium was supplemented with 125 mM NaCl (referred to as "$Prescott_{NaCl}$ medium"). For the experiments, the amoebae were incubated for 1 h in the $Prescott_{NaCl}$ medium and were then transferred back to the standard $Prescott_{ref}$ medium and observed for 14 days.

2.2.5. Induction of phagocytosis in A. proteus

To explore if phagocytosis of larger PS-MP is dependent on an eco-corona (Ramsperger et al., 2020) covering the MP surface, protein-coated PS-MP ($PS_{<30\mu m}$) were compared to non-coated ones. Particles were pre-coated with a protein corona as described by Jasinski et al. (2022), where also the characterization of the protein coating is described. Briefly, PS-MP ($PS_{<30\mu m}$) (1 μL of the 2.5 % (*w/v*) aqueous suspension) were incubated overnight at 37 °C in 1 mL of a 2 % (*w/v*) bovine serum albumin (BSA) solution or 1 mL of a 10 % fetal calf serum (FCS) solution in $Prescott_{ref}$ medium. Afterwards, the particles were centrifuged at 13,000 x*g* for 5 min at room temperature. The resulting pellet was once washed with bi-distilled water (13,000 x *g*, 5 min), resuspended in $Prescott_{ref}$ and used immediately for the experiments.

To evaluate whether the introduction of a known chemical trigger in the medium, such as chemoattractants produced by prey organisms, could stimulate the phagocytosis of larger PS-MP fragments ($PS_{<30\mu m}$), a second attempt to induce phagocytosis was undertaken by co-cultivating 100 individuals of *A. proteus* with *T. pyriformis* at a concentration of 5×10^4 Ciliophora/mL (prey: predator = 500:1), in the presence of $PS_{<30\mu m}$ particles at a concentration of 5×10^3 fragments/mL. This concentration was chosen to increase uptake probability of prey and particles, and to enable a possible enrichment of chemoattractants released by *T. pyriformis*. Subsequent analysis of the uptake of $PS_{<30\mu m}$ was conducted following the same methodology as previously described.

2.2.6. Trophic transfer of MP particles

T. pyriformis and *P. caudatum* were incubated with the respective amount of MP (end concentrations: $PS_{0.5\mu m} = 3.6 \times 10^8$ beads/mL; $PS_{6\mu m} = 2.1 \times 10^5$ beads/mL; $PS_{<30\mu m} = 5 \times 10^3$ fragments/mL) for 24 h. Afterwards, both Ciliophora were washed by centrifugation (400 x*g*, 5 min) to remove leftover MP, and resuspended in $Prescott_{ref}$ medium. The organisms were then offered to pre-starved amoebae in a prey: predator ratio of approximately 10:1. After the indicated incubation time (up to 24 h), the amoebae were washed (as described above for the uptake experiments), transferred to a microscopy slide and observed under a fluorescence microscope (Zeiss Imager.Z2m, 1× Camera Adapter, Carl Zeiss, Jena, Germany) to obtain images of amoeba feeding on MP burdened *T. pyriformis* and *P. caudatum*.

The remaining amoebae were observed for the respective time (up to 24 h) to record a possible excretion of particles.

2.2.7. Data analysis

Data analysis and visualization was performed using Origin software 2019b (Origin, Northampton, MA, USA). Figures obtained via fluorescence microscopy were prepared using ZEN 2 (blue edition) / ZEN 2 core v2.5 / ZEN 2 starter v2.5 (Carl Zeiss Microscopy GmbH, Jena, Germany) and Inkscape v1.1 (Open Source Scalable Vector Graphics Editor).

3. Results

3.1. Particle characterization

The $PS_{<30\mu m}$ used in this study were first characterized by their size (Figs. S1 and S2) and the ζ-potential of $PS_{0.5\mu m}$ and $PS_{6\mu m}$ was analyzed (Table 1). Since the zeta sizer is only applicable for particles of up to 10 μm in size, accurate ζ-potential measurements were only possible for the smaller particles ($PS_{0.5\mu m}$ and $PS_{6\mu m}$). The surface charge of the measured beads was always negative, but highly dependent on the surrounding matrix. While no major differences of the ζ-potential were detected after incubation in FCS compared to $Prescott_{ref}$, for $Prescott_{NaCl}$ and BSA incubated particles, a trend towards a decrease of the ζ-potential was detectable in beads of both sizes.

3.2. Microplastic uptake by unicellular eukaryotes

Our results show that the uptake of MP in Ciliphora is size-dependent. High uptake of $PS_{0.5\mu m}$ and $PS_{6\mu m}$ beads was observed in both organisms (>95 % of individuals) (Figs. 1, 2 and S2). *P. caudatum* took up larger particles in its vacuola ($PS_{<30\mu m}$, 75 % of the individuals, Figs. 1 and 2), with the largest particle found being 27.7 μm in diameter (Fig. S3). $PS_{0.5\mu m}$ beads were incorporated by both organisms, which can be identified by the accumulation of MP in food vacuoles (Fig. 2). An exact determination of the number of ingested $PS_{0.5\mu m}$ beads was not possible, due to the pronounced tendency for agglomeration inside the food vacuoles of both Ciliophora. Particle counting could not be improved by immobilizing or fixating the cells, as the high number of beads inside the vacuola and their small size did not allow an accurate quantification. Six μm beads were easier to identify, as they were individually found in *T. pyriformis* and, even if more than one was detected in the vacuola of *P. caudatum*, counting was still possible (on average 16.0 ± 13.8 MP per individual). Larger MP fragments ($PS_{<30\mu m}$) were detected in separate food vacuola in *P. caudatum,* so it was possible to perform an accurate count of the particles. In *T. pyriformis*, only a few of the observed cells, out of all the observed individuals were found to have ingested $PS_{<30\mu m}$ (Fig. 1). Particle size to cell length ratio showed a possible particle uptake of up to 20 % ($PS_{6\mu m}$, Fig. S4) of the total size of *T. pyriformis* (average size: 39.6 μm, Fig. S5) and up to 10 % ($PS_{<30\mu m}$, Fig. S3) of the total size of *P. caudatum* (average size: 195.5 μm, Fig. S6).

For *T. pyriformis*, a high initial rate of uptake (95 % of individuals with particles after 0.5 h of incubation) could be observed for $PS_{0.5\mu m}$ (Fig. 1A). The number of individuals without particles increased over time and was highest after 24 h at 75 % (Fig. 1A). For $PS_{6\mu m}$, no uptake was observable after 24 h (Fig. 1C). For $PS_{<30\mu m}$, low uptake rates were only observed at 2, 4, and 6 h (Fig. 1E).

In case of *P. caudatum*, $PS_{0.5\mu m}$ could be detected in all observed individuals over the whole observation time (0.5 h–24 h, Fig. 1B). Also, particle release could be observed for $PS_{0.5\mu m}$ (Fig. S7A/B). For $PS_{6\mu m}$, a time-dependent uptake could be observed. While the ingestion rate rose until 6 h (100 % of individuals with >5 particles), after 24 h, increased clearance rates decreased the number of ingested MP (Fig. 1D). Similar to $PS_{6\mu m}$, uptake decreased with increasing time for $PS_{<30\mu m}$, whereas only 25 % of individuals with ingested MP were detected after 24 h (Fig. 1F).

For *A. proteus* (500 μm in average, Marshall et al., 2012), a size-

Table 1
ζ-potential analysis and size distribution of the used MP.

	ζ-potential [mV]				Size [μm]
	$Prescott_{ref}$	$Prescott_{NaCl}$	BSA	FCS	
$PS_{0.5\mu m}$	−53.4 ± 0.2	−30.7 ± 0.2	−24.8 ± 0.4	−49.8 ± 0.3	0.5 ± 0.02
$PS_{6\mu m}$	−44.7 ± 0.5	−12.2 ± 1.2	−14.2 ± 1.1	−49.3 ± 5.7	6.0 ± 0.6
$PS_{<30\mu m}$	n.a.	n.a.	n.a.	n.a.	16.2 ± 7.4

n.a.: not applicable. BSA: bovine serum albumin. FCS: Fetal calf serum. $Prescott_{ref}$: standard Prescott medium, pH 6.4. $Prescott_{NaCl}$: standard Prescott medium supplemented with 125 mM NaCl, pH 4.3. Data represent mean values ± SD, ζ-potential measurement $n = 3$, for $PS_{<30\mu m}$ see Fig. S1.

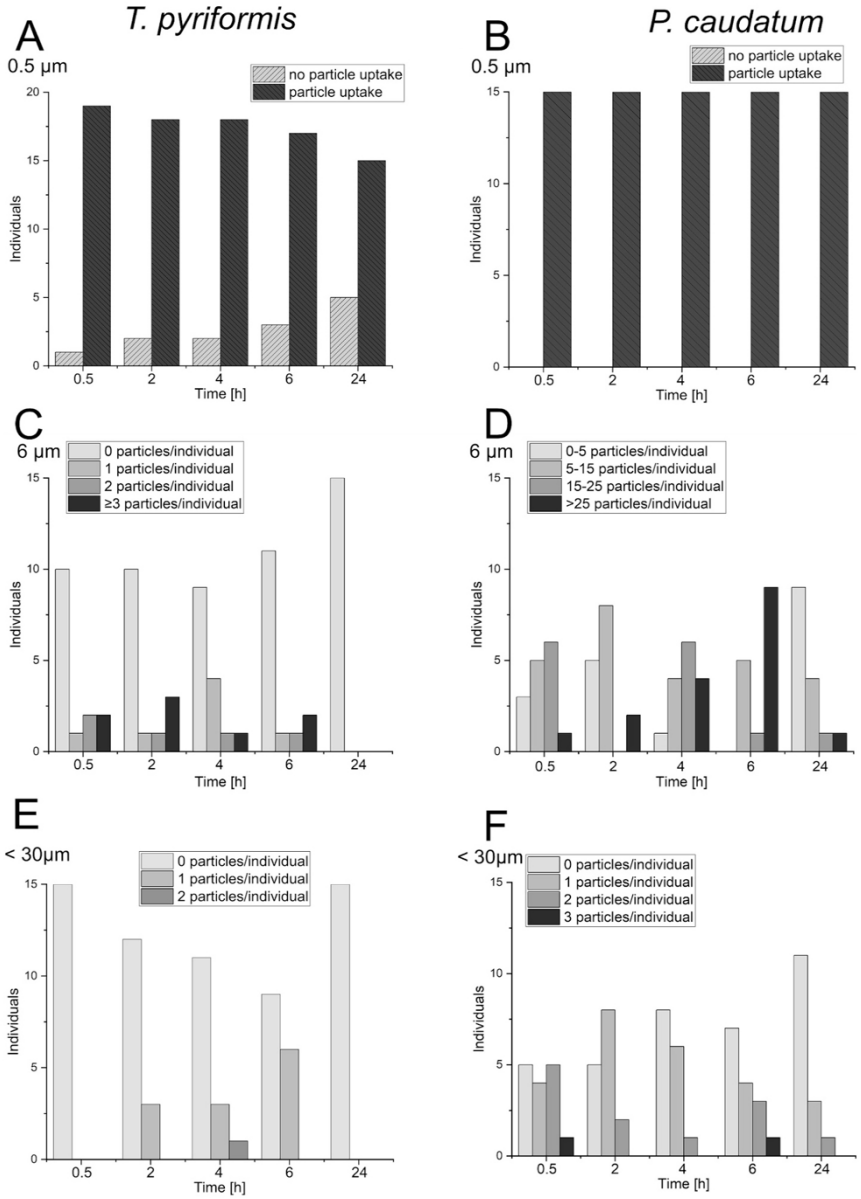


Fig. 1. Uptake of particles after ingestion in *T. pyriformis* (A, C, E) and *P. caudatum* (B, D, F). Both Ciliophora were exposed to $PS_{0.5\mu m}$, $PS_{6\mu m}$ and $PS_{<30\mu m}$. *T. pyriformis* exposed (A) to $PS_{0.5\mu m}$ (number of *T. pyriformis* = 20), (C) to $PS_{6\mu m}$ (number of *T. pyriformis* = 15) and (E) $PS_{<30\mu m}$ (number of *T. pyriformis* = 15). *P. caudatum* exposed to (B) $PS_{0.5\mu m}$ (number of *P. caudatum* = 15), (D) $PS_{6\mu m}$ (number of *P. caudatum* = 15) and (F) $PS_{<30\mu m}$ (number of *P. caudatum* = 15).

dependent uptake could be observed (Fig. 3). While beads with a size of ≤6 µm were taken up (Fig. 3A–F), larger fragments ($PS_{<30\mu m}$) were not observed inside any amoeba.

Quantification of the ingested beads per amoeba, by performing manual live-counting under the microscope, confirmed this size dependency (Fig. 3G). The number of MP, ingested by an individual *A. proteus*, exhibited substantial variation, ranging from as few as one to exceeding 50 beads for $PS_{0.5\mu m}$ (with an average of 18.8 ± 18.1 beads per amoeba). In contrast, *A. proteus* took up a maximum of 15 $PS_{6\mu m}$ particles with an average of 5.7 ± 3.4 beads per amoeba. For $PS_{<30\mu m}$, no uptake was observed in *A. proteus*.

3.3. Analysis of the MP uptake route in A. proteus

To unravel the size-dependent MP uptake route by *A. proteus*, pinocytosis and phagocytosis were investigated separately, by stimulating the respective uptake mechanism.

3.3.1. Induction of pinocytosis

Pinocytosis was successfully induced by using $Prescott_{NaCl}$ medium as shown by the high amount of pinocytosis channels (Fig. 4A/B) and the amount of $PS_{0.5\mu m}$ inside the amoebae (Fig. 4C).

Initially, amoebae ingested under pinocytic conditions about 3.8-fold more $PS_{0.5\mu m}$ beads ($Prescott_{NaCl}$ medium, 68.0 ± 44.0 beads per amoeba) than under non-inductive conditions ($Prescott_{ref}$ medium, average: 18.8 ± 18.1 beads per amoeba) (Fig. 5). Independently of the amount of $PS_{0.5\mu m}$ engulfed on day 0, no decrease of the number of particles per amoeba was detectable within the first three days of incubation. During this period, morphology and locomotion, representatives for cellular fitness, did not differ from the corresponding control

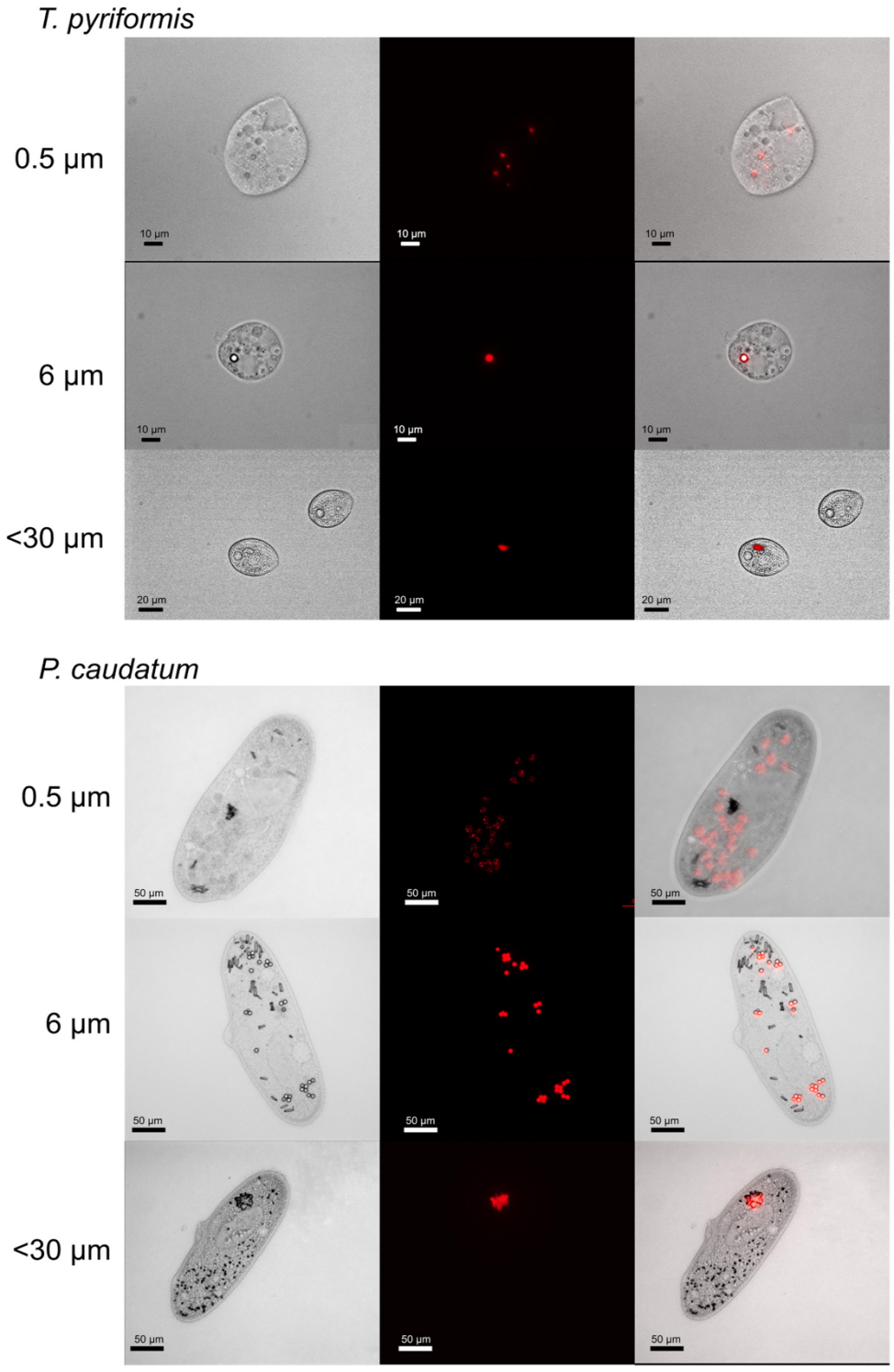


Fig. 2. Ingestion of $PS_{0.5\mu m}$ and $PS_{6\mu m}$ beads, and $PS_{<30\mu m}$ fragments by *T. pyriformis* and *P. caudatum* after 2 h of incubation. Fluorescently labeled MP beads and fragments taken up by Ciliophora, visualized by epifluorescence microscopy. Left column: bright field fluorescence, middle column: fluorescence microscopy, right column: merged pictures. Scale bar *T. pyriformis* $PS_{0.5\mu m}$ and $PS_{6\mu m}$ = 10 µm (63× magnification); Scale bar *T. pyriformis* $PS_{<30\mu m}$ = 20 µm (40× magnification). Scale bar *P. caudatum* = 50 µm (20× magnification).

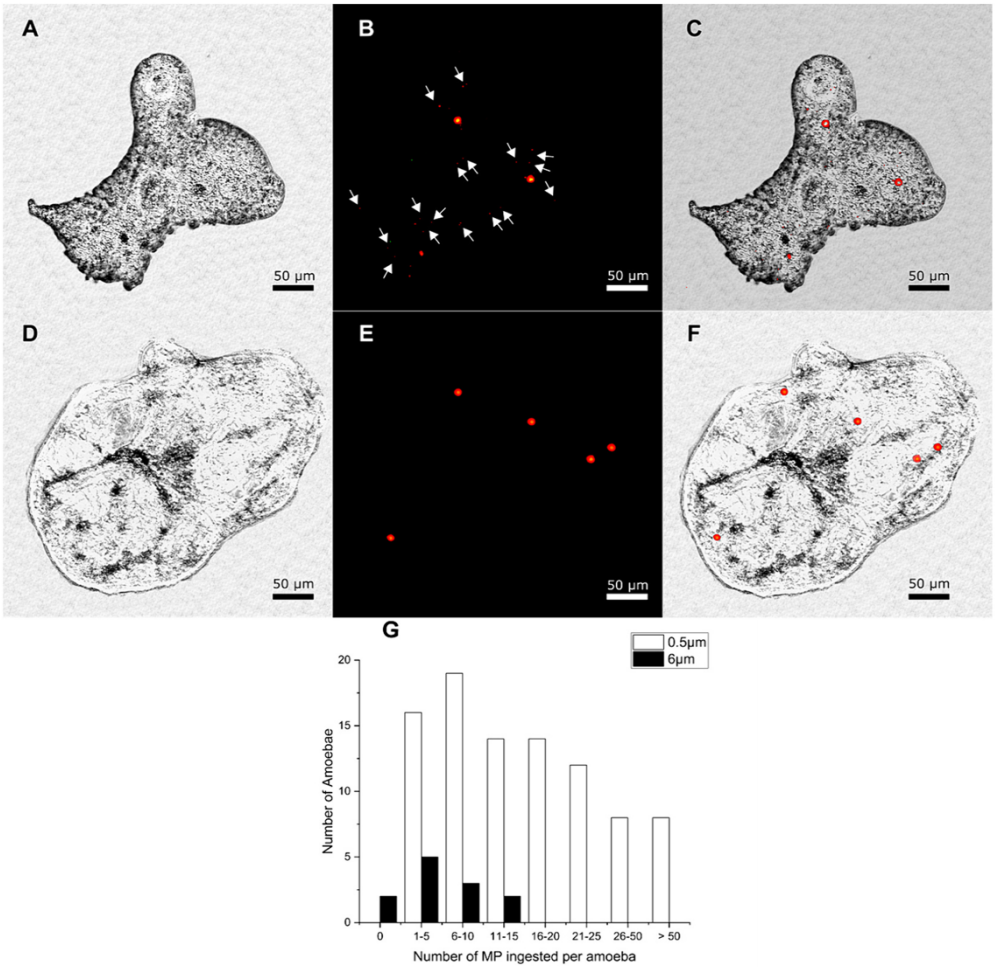


Fig. 3. Ingestion of $PS_{0.5\mu m}$ and $PS_{6\mu m}$ in *A. proteus* after 1 h of incubation. A–F: Fluorescently labeled MP beads engulfed by *A. proteus,* visualized by epifluorescence microscopy. A–C: *A. proteus* with $PS_{0.5\mu m}$ red fluorescent beads, white arrows indicate single beads, larger red dots represent agglomerations of various particles inside a vacuole. D–F: *A. proteus* with $PS_{6\mu m}$ red fluorescent beads. A, D: bright field; B, E: epifluorescence; C, F: merged photos. Scale bar = 50 μm, magnification 10×. G: Bar plot representing the number of amoebae that ingested $PS_{0.5\mu m}$ (white bars, number of *A. proteus* = 91) and $PS_{6\mu m}$ (black bars, number of *A. proteus* = 12).

group. After six days of cultivation, a 1.7-fold reduction of MP count was only recorded for amoebae cultured in $Prescott_{NaCl}$, indicating that some egestion of MP occurred. After 14 days, the remaining $PS_{0.5\mu m}$ per amoeba was low for both populations ($Prescott_{NaCl}$, $Prescott_{ref}$).

3.3.2. Induction of phagocytosis with pre-coated particles

In an attempt to trigger the receptor pathway for induced phagocytosis, $PS_{<30\mu m}$ particles were pre-coated with either BSA (a well-characterized single protein type) or FCS (a mixture of non-characterized proteins) to form a protein corona on their surfaces. However, despite this deliberate modification of the fragment surfaces, none of the coated microplastic particles were internalized by the organisms.

3.3.3. Induction of phagocytosis by co-incubation with prey organisms

To evaluate, whether the introduction of a known chemical trigger in the medium, such as chemoattractants produced by prey organisms, could stimulate the phagocytosis of larger PS-MP fragments, we conducted a co-incubation experiment involving a mixture of fragments ($PS_{<30\mu m}$) and *T. pyriformis* in the presence of *A. proteus*. However, here also, we observed no uptake of the larger MP fragments.

3.4. Trophic transfer of MP particles

To investigate the potential for trophic transfer of MP, we employed two specific prey organisms for *A. proteus*, namely, *T. pyriformis* and *P. caudatum*. These prey organisms were fed with $PS_{0.5\mu m}$ and $PS_{<30\mu m}$ particles for a period of 24 h. Subsequently, they were exposed to *A. proteus* in our experimental setup to verify the occurrence of trophic transfer from these to the latter. Independently of the species, shortly after co-incubation, the amoebae predated on the Ciliophora and took up the MP they carried (Figs. 6 and 7, S8 and S9). Especially in case of $PS_{0.5\mu m}$ beads, food vacuoles with clumped beads could still be observed 24 h post-ingestion inside the amoebae (Fig. S10).

24 h ($PS_{<30\mu m}$ fragments) and 72 h ($MP_{0.5\mu m}$ beads) post-ingestion of the prey, <10 % of amoebae still contained detectable amounts of MP (Fig. S9A–C). Our observations further showed that after digestion of the Ciliophora, a release of the remaining undigestible MP occurred (Fig. S9D–F).

4. Discussion

The uptake and transfer of MP in aquatic environments has been one of the major concerns of the ecological hazard and risk assessment in the

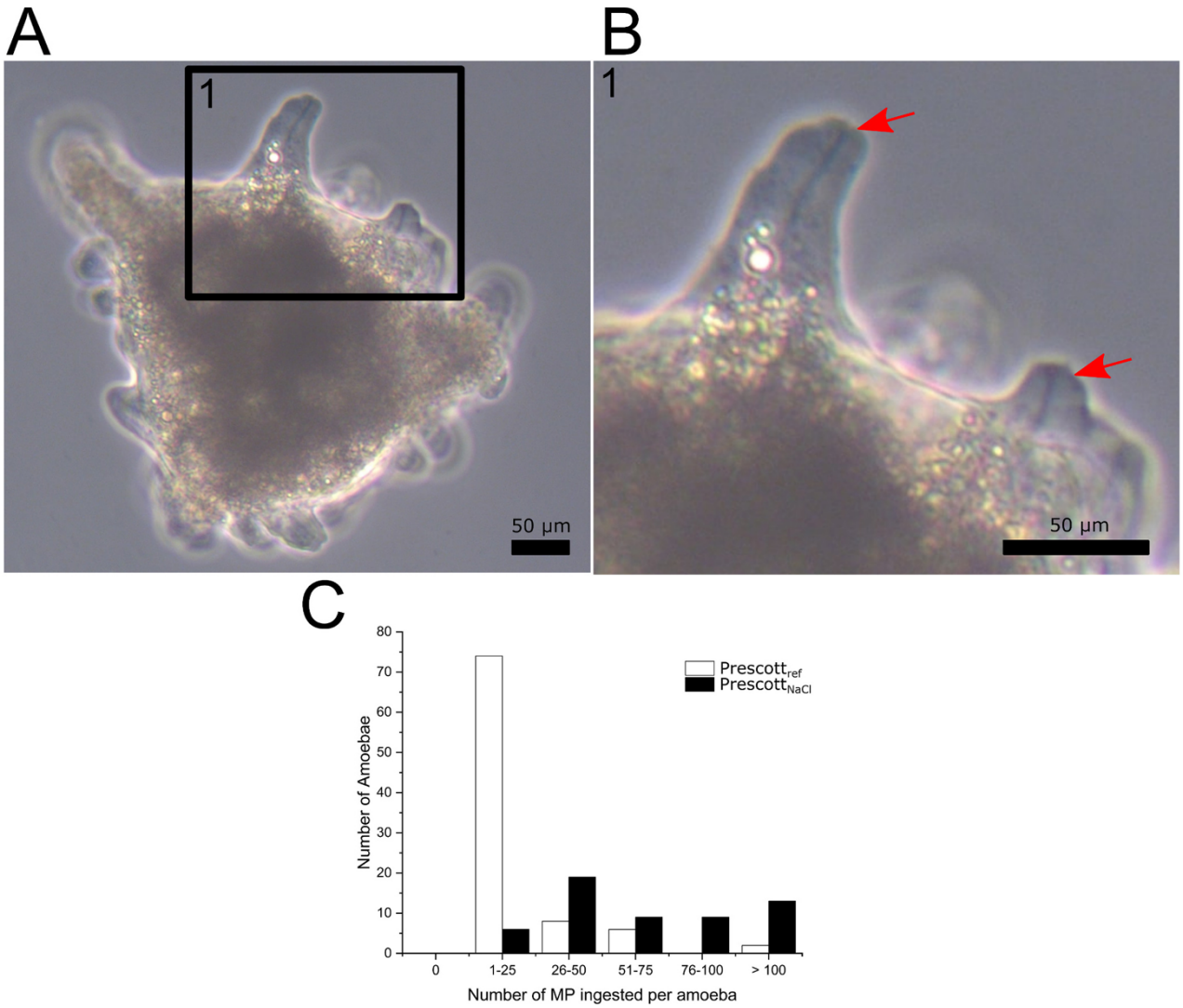


Fig. 4. A: Induction of the formation of pinocytosis channels in *A. proteus* after 20 min incubation in Prescott$_{NaCl}$ medium. B: Zoomed in section of the pinocytosis channels for better visibility. 10× magnification. Scale bar = 50 µm; C: Numerical distribution of $PS_{0.5µm}$ beads uptake in amoebae for Prescott$_{ref}$ and Prescott$_{NaCl}$ media. Quantitative analysis of the MP ingestion per amoeba after 60 min incubation with $PS_{0.5µm}$ beads in Prescott$_{ref}$ (white bars, number of *A. proteus* = 91) and Prescott$_{NaCl}$ (black bars number of *A. proteus* = 55) media.

last years (Koelmans et al., 2016). However, only a few studies exist which analyze MP transfer in the lower trophic levels, with them mainly focusing on the marine environment (Wagner et al., 2014). Regarding the uptake of MP in freshwater unicellular eukaryotes, which represent an important fraction of the lower trophic levels, only very little data is available. Therefore, this study analyzed the uptake of MP in a large size range (0.5 µm - 30 µm) in three freshwater unicellular eukaryotic organisms: *T. pyriformis, P. caudatum* and *A. proteus*. In addition, a possible trophic transfer of MP was examined.

4.1. Size dependent uptake

The uptake of MP in all three unicellular eukaryotes proved to be size-dependent and varied between the organisms. *T. pyriformis* ingested 0.5 and 6 µm MP, which is in line with Lavin et al. (1990), who also found an uptake of 6.17 µm PS beads. Additionally, *T. pyriformis* was found to ingest PS fragments of up to 10 µm in size (Fig. S12). It is noteworthy, however, that the $PS_{<30µm}$ particles of this size (~10 µm) found inside *T. pyriformis* individuals possessed an elongated shape. Therefore, it can be assumed that these particles were taken up via the oral cavity in a lengthwise manner. Consequently, this uptake may not be possible with spheroid particles in the same size (~10 µm). *P. caudatum*, on the other hand, incorporated fragments up to 27.7 µm in size (Fig. S3). For *Paramecium* sp., past studies only showed an ingestion of particles (spherical) up to 10 µm in size (Bulannga and Schmidt, 2022; Nałęcz-Jawecki et al., 2021). Both unicellular organisms feed by creating a water current with their cilia that channels the potential food into their oral groove, where a food vacuole is then formed (Mast, 1947). Therefore, the variation in particle uptake between *T. pyriformis* and *P. caudatum* is likely attributed to the distinct sizes of these organisms (*T. pyriformis*: average size 39.6 µm, Fig. S5; *P. caudatum*: average size 194.5 µm, Fig. S6) and that of their oral grooves. However, since relatively small *T. pyriformis* cells were found with incorporated 6 µm MP, the particle-to-cell length ratio was higher for *T. pyriformis*. For *T. pyriformis* and *P. caudatum*, high initial uptake rates were observed (95 % and 75 %, respectively). Presumably driven by the organisms'

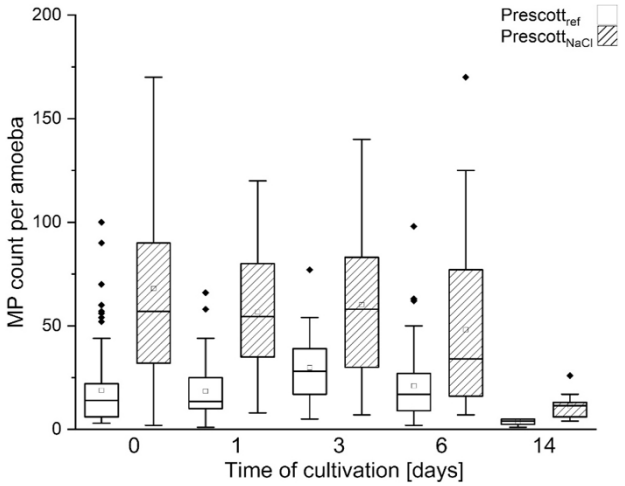


Fig. 5. $PS_{0.5\mu m}$ retention after pinocytic ingestion in *A. proteus*.
Amoebae were incubated with 3.6×10^8 $PS_{0.5\mu m}$ beads/mL in $Prescott_{ref}$ and $Prescott_{NaCl}$ media for 60 min and were thereafter placed into $Prescott_{ref}$ medium for the remaining cultivation time.

rapid doubling time (in this study approximately 2 h for *T. pyriformis* and 24 h for *P. caudatum*, in line with prior research (Ruehle et al., 2016; Takahashi, 2016)), the number of organisms harboring MP started to decline noticeably after 24 h, as illustrated in Fig. 1. In case of *P. caudatum*, a release of $PS_{0.5\mu m}$ could be observed (Fig. S7). Since only individuals with incorporated particles were found, released particles were probably re-ingested, probably due to the high concentrations of $PS_{0.5\mu m}$ particles provided. In *T. pyriformis*, particle release was also observed for $PS_{6\mu m}$ and $PS_{<30\mu m}$.

A. proteus (500 μm) directly took up beads up to 6 μm in size (Fig. 3), but none of the larger analyzed fragments ($PS_{<30\mu m}$). This is most likely associated with the uptake mechanism of the organism. *A. proteus* takes up extracellular matter either via pinocytosis or via phagocytosis. Both uptake pathways are initiated by the interaction between a solute/prey with membrane receptors on the surface of the amoeba. Depending on the targeted receptor, channels (pinocytosis) or pseudopods (phagocytosis) are formed with subsequent uptake of particulates (Prusch and Minck, 1985). For phagocytosis in *A. proteus*, an active food selection can be presumed, as it is also reported for, e.g., heterotrophic flagellates (Boenigk and Arndt, 2002; Xinyao et al., 2006). Hence, chemical and mechanical stimuli enable the amoeba to avoid ingesting inorganic matter through phagocytosis, such as MP. However, *A. proteus* generally also shows a permanent, but minor pinocytosis-driven ingestion in the uroid region (Wohlfarth-Bottermann and Stockem, 1966), with pinocytic channels reaching diameters of up to 6 μm (Klein et al., 1988), which might be large enough for a passive uptake of small MP (≤6 μm). The observed high increase of pinocytosis channels after incubation of Amoebae in $Prescott_{NaCl}$ is further accompanied by an increase in MP internalization for 0.5 μm particles (Fig. 4). It is, therefore, plausible to conclude that the passive pinocytotic pathway is responsible for the internalization of small MP in *A. proteus*. This increased uptake might further be correlated with the alteration of the surface charge of the particles in the different employed media ($Prescott_{Ref} = -53.4$ mV; $Prescott_{NaCl} = -30.7$ mV), that results in a pKa being closer to a typical organic one. Surprisingly, the ingested MP by pinocytosis remained inside the cytoplasm for up to 14 days (Fig. 5). As previously reported, organic (e.g., amino acids and proteins) and inorganic (e.g., cations) substances, that are taken up by the amoebae via pinocytosis, can then be excreted within minutes, to restore the standard concentration gradient of the medium to "normal" cultivation concentration (Klein and Stockem, 1995). However, it is important to consider that the MP utilized in our study may not disrupt the internal osmotic balance of the amoeba. In such a scenario, the amoeba may not recognize the unintentionally ingested particles, leading to the retention of these foreign matter particles within their cytoplasm.

While we could detect MP in the amoebae up to 14 days after the ingestion, it became evident that a decline in the number of retained MP particles started from day 3 onwards (Fig. 5). This phenomenon is most likely attributable to an asymmetrical cellular division process, which occurs at intervals ranging from 20 to 54 h (Ord, 1968; Prescott, 1955). This division process results in a gradual dilution of the MP content within the amoeba, rather than an active egestion of the particles. Due to the maximum diameter of *A. proteus'* pinocytic channels being limited to 6 μm, it becomes evident that larger particles can only be internalized through the process of phagocytosis.

In our study, phagocytosis could not be observed with fragments ($PS_{<30\mu m}$), despite the attempts of induction (particle coating with BSA and FCS proteins). In contrast, ingestion upon induction of phagocytosis was already recorded in other studies that observed the uptake of protein coated agarose beads (10–40 μm, coated with e.g., α-lactalbumin, RNase A) by *A. proteus* (Ahn and Jeon, 1982; Prusch and Britton, 1987; Prusch and Minck, 1985). Uptake efficiency was found to be highly dependent on the employed protein. The reason for a lack of phagocytosis of the protein-coated MP in our study can only be speculated upon and might be linked to a non-adequate surface charge. Since surface charge and size of MP was shown to be a critical parameter determining the ingestion of MP by ciliates as well as for the phagocytic ingestion by murine macrophages (Bulannga and Schmidt, 2022; Ramsperger et al., 2022; Rudolph et al., 2021), the same could be hypothesized for *A. proteus*.

Another important parameter in inducing phagocytosis might be the presence of an eco-corona or a biofilm for larger particles, which can alter the physicochemical characteristics of particles and make them more palatable for uptake (Liu et al., 2022b; Witzmann et al., 2021).

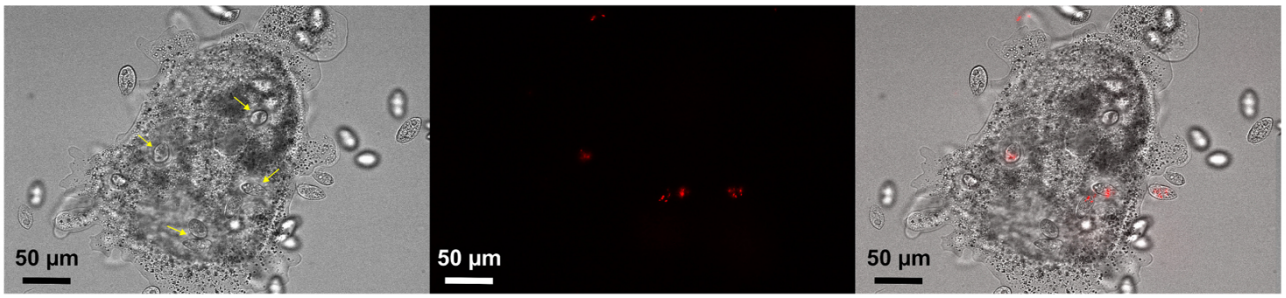


Fig. 6. *A. proteus* feeding on MP ($PS_{0.5\mu m}$) burdened *T. pyriformis*. At $t = 10$ min; multiple *T. pyriformis* have been taken up via phagocytosis, of which three cells contained MP. Left panel: bright field (arrows indicate *T. pyrifomis* inside *A. proteus*); middle panel: epifluorescence; right panel: merged (see Fig. S11 for larger image). 20× magnification. Scale bar = 50 μm.

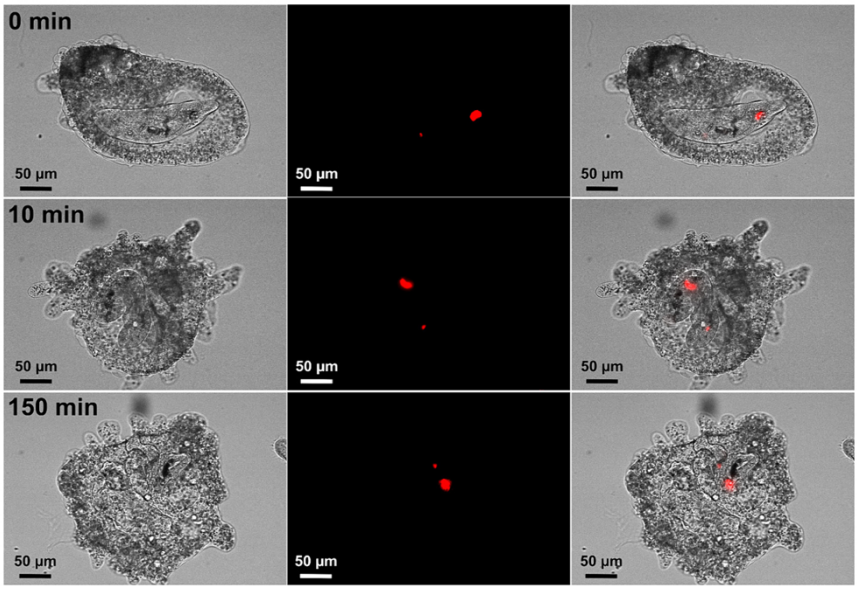


Fig. 7. Ingestion of $PS_{<30\mu m}$ loaded *P. caudatum* (two particles) by *A. proteus*. At $t = 0$ min one individual of *A. proteus* initiates the process of phagocytosis of one paramecium. The individuals are observed at t = 10 min in which *P. caudatum* is completely internalized, and at $t = 150$ min, when the digestion process made the *P. caudatum* cell almost unrecognizable. Left panel: bright field; middle panel: epifluorescence; right panel: merged. 20× magnification. Scale bar = 50 μm.

While a biofilm might not be relevant when considering small MP, the formation of an eco-corona is possible when particles are exposed to biological macromolecules which can adhere to their surface. As described in the pertinent literature, such natural MP coating significantly increases their uptake and, in some cases, biological effects in different organisms (Fabra et al., 2021; Ramsperger et al., 2022). In fact, while many studies report an increase in uptake and effects of coated MP (e.g. Fabra et al., 2021), contrasting findings have been recently reported (e.g. Amariei et al., 2022; Schür et al., 2021), which indicate a diminished influence (e.g., reduced uptake and toxicity) of coated MP.

In our experiments, a co-incubation of a prey organism (*T. pyriformis*) and MP fragments in a comparable size range ($PS_{<30\mu m}$) did not induce phagocytosis of MP in *A. proteus* either. *T. pyriformis* was chosen because it is preferably ingested by *A. proteus* via phagocytosis (Klein and Stockem, 1995) and secretes chemotactic substances, to which the amoebae are actively attracted (Jeon and Bell, 1962). It would also be possible that amoebae were exposed to fecal pellets potentially containing MP, released by the Ciliophora, as they are known to sometimes excrete inedible particles or pathogens this way (Denoncourt et al., 2017; Denoncourt et al., 2020). Nevertheless, the lack of particle ingestion despite the co-exposure with a known prey further confirms that amoebae can discriminate between prey and MP fragments and that soluble substances released by *T. pyriformis*, if any, are not sufficient to induce an inadvertent uptake of large MP fragments. In this study, not all the different employed particle sizes were coated or co-incubated with a known prey organisms. Therefore, even though a co-exposure with prey organisms did not alter the uptake in this study, it cannot be excluded that the size of the particles plays a role in this process. Further studies may consider this as an aspect to include.

Furthermore, next to chemical stimuli, physical cues conveyed by the movement of the prey are also known to be important for the induction of phagocytosis (De la Fuente et al., 2019). Mast and Hahnert (1935) already showed that *A. proteus* mainly feeds on living organisms, indicating that the motility of the prey might be an important co-stimulus for ingestion. This could explain the absence of phagocytic uptake of MP. Overall, phagocytosis and the recognition of the prey is a complex process whose characteristics could not only vary for different prey organisms but for different amoeba strains as well (Shi et al., 2021). A phagocytic uptake of MP by amoeba in the natural environment, therefore, cannot be excluded.

Although we did not investigate whether any negative impact on the unicellular eukaryotes' survival occurred after the uptake of the offered MP, a low clearance rate within their cytoplasm could still pose a threat, as a leaching of plastic associated chemicals and residual monomers (Brehm et al., 2022; Setälä et al., 2014) into the organism could be possible. The release of plastic leachates in the environment potentially exposes the biota to a complex mixture of chemicals, of which only a fraction is known and regulated (Gunaalan et al., 2020). The released chemicals can include compounds that act as a potential threat to the ecosystem. However, even though evidence of the environmental risks of these chemicals already exists (e.g. Delaeter et al., 2022), research on the topic is still lacking.

However, even though release of MP was observable in *T. pyriformis* and *P. caudatum*, the MP particles stayed inside some cells for an, yet unknown, period of time. This uptake and retention could influence the metabolism and uptake rate of other nutritional particles, which is an important determining factor in ciliate physiology (Lavin et al., 1990; Sherr and Sherr, 1987). Additionally, increased metabolic costs, caused by the formation of food vacuoles and production and transport of digestive enzymes, could arise for MP-fed eukaryotes, as they try to digest the particles (Mueller et al., 1965). In this study, we used primary PS-MP, while in the environment, the organisms are exposed to a highly diverse composition of MP, differing substantially in their physico-chemical properties (Rochman et al., 2019). This could in turn cause diverse effects on these organisms in the environment, for which no data is available at present. Hence, our study on the uptake of different particle sizes may support research on the effects of this diverse array of contaminants on unicellular organisms. Moreover, a prolonged retention of MP inside the cytoplasm, while having no immediate negative influence on their survival, might increase the likelihood of a transfer of MP to higher trophic levels.

4.2. Trophic transfer of microplastic particles in a simplified food web

MP bioaccumulation in the lower trophic levels could be particularly concerning, as not only could it alter population dynamics, as it was

shown for marine ciliates (Zhang et al., 2021), but it could also cascade over time to higher trophic levels (Bulannga and Schmidt, 2024). Trophic transfer of MP was reported for different organisms, belonging to various genera in the marine (Carbery et al., 2018) and freshwater (Nair and Perumal, 2022) environment, e.g., from aquatic plants to gammarids (Iannilli et al., 2023), from copepods to jellyfish (Costa et al., 2020), from mussels to gastropods (Xu et al., 2022) and crabs (Farrell and Nelson, 2013), or from daphnids to fish (Mattsson et al., 2017). Additionally, biomagnification seems evident, as MP abundancies increase with an increase of the trophic level (Sarker et al., 2022). Given the ubiquitous presence of MP in the environment, trophic transfer in most ecosystems, therefore, seems inevitable.

When amoebae were offered MP-fed prey, we could observe trophic transfer of MP. *T. pyriformis* and *P. caudatum* cells, which had taken up $PS_{0.5\mu m}$ and $PS_{<30\mu m}$ were promptly ingested via phagocytosis by *A. proteus* (Figs. 6 and 7). This way, *A. proteus* ingested larger particles via prey, which would not have been taken up otherwise. Interestingly, MP, small and large alike taken up via prey organisms, were observed to be released by the amoebae within 24 h of observation (Fig. S9). On the contrary, 0.5 μm beads, that were taken up via pinocytosis, were detected in the cytoplasm up to 14 days after their internalization. It is known that inedible matter like glass, carbon beads, or indigestible remnants of prey organisms, such as the cuticle of small cladocerans, are excreted shortly after digestion (Mast and Hahnert, 1935). These observations could suggest that this process might take place also in the case of MP taken up via trophic transfer. Therefore, if this was the case, this process could reduce bioaccumulation, thanks to the shorter retention time of MP within the cytoplasm. A possible transfer through trophic levels depends hereby in part also on the retention time inside the organisms and the uptake of MP-fed prey by their predators. Further studies would be required to test this hypothesis. However, in favorable conditions, *A. proteus* can ingest up to 70 cells per day, which in turn are able to take up hundreds of MP of various sizes per cell. If the rate of ingestion exceeds the excretion rate, possible biomagnification of inedible, non-nutritive material at the low trophic levels can be proposed, posing a potential hazard to these organisms and their predators.

Unicellular predators are not the only organisms that could take up contaminated Ciliophora. For example, Stienbarger and coauthors showed that fish larvae of *Centropristis striata* ingest exponentially more MP while feeding on contaminated prey (*Favella* spp., Ciliophora) than when exposed to the particles alone (Stienbarger et al., 2021). Predators belonging to higher trophic levels could, furthermore, be impaired not only by feeding upon contaminated prey, but also by a decrease of their biomass. For example, a study conducted by Geng et al. (2021) showed a concentration dependent collapse of *Strombidium sulcatum's* population exposed to different sizes of PS fluorescent beads within 72 h from the beginning of the exposure, together with a reduction of their size (Geng et al., 2021). Hence, a subsequent death of *S. sulcatum* could directly affect the predators' population by the reduction of the number of prey and, indirectly, by the increase of MP available in the water. While we did not study population dynamics of the selected organisms exposed to PS-MP, we cannot exclude that a decrease of the population may happen over longer periods of time or with environmentally relevant MP.

5. Conclusion

This study suggests that an accumulation of MP in unicellular eukaryotes is likely and should be considered when assessing the hazard of MP contamination in aquatic environments. MP bioaccumulation may have detrimental effects, given the crucial role that these organisms have within the food web. Overall, while the potential impact on the survival of the exposed unicellular eukaryotes was not investigated, neither long term effects, nor the possibility of cascading effects over higher trophic levels can be excluded. In this context, further investigations are necessary to assess the potential effects of different MP on the life history parameters of unicellular organisms and their potential transfer along the food web. In future experimental designs, it would be appropriate to consider a more environmentally relevant scenario. In particular, it would be valuable to apply diverse protein or eco-corona coatings to all utilized particles and to investigate potential effects on the survival of the studied organisms depending on the physico-chemical properties of the particles.

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CRediT authorship contribution statement

Simona Mondellini: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Michael Schwarzer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Matthias Völkl:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Julia Jasinski:** Investigation. **Valérie Jérôme:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Thomas Scheibel:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Christian Laforsch:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Ruth Freitag:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no competing interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.172470.

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Supplementary material pertaining to Article II

Size dependent uptake and trophic transfer of polystyrene microplastics in unicellular freshwater eukaryotes

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Supplementary Materials and Methods

Preparation of PS fragments

PS doped with Rhodamin B, was purchased by Magic Pyramid (Frechen, Germany). The number- and weight average molecular weights (M_n and M_w) as well as the dispersity ($Đ = M_w/M_n$) was determined by gel permeation chromatography (GPC; M_n = 72000 g/mol, M_w = 166000 g/mol, $Đ$ = 2.3). Pellets were grounded with an Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany). The pellets were precooled with dry ice and milled with a 12 teeth rotor and a 120 µm sieve at a speed of 18000 rpm. The separation into several fractions was done with an Alpine Air Jet Sieve E200 LS (Hosokawa Alpine AG, Augsburg, Germany) by using the 125 µm, 75 µm and 20 µm sieve.

Supplementary figures

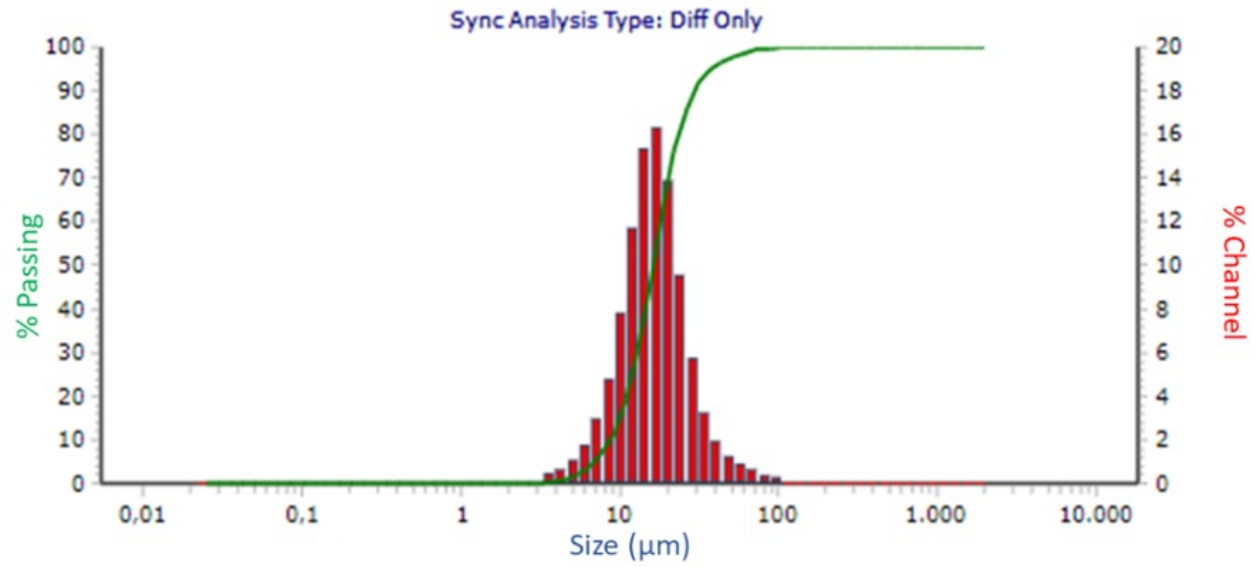


Figure S1 Size distribution of PS fragments <30 µm (d_{90}=29.36 µm, mean particle size 16.2 ± 7.4 µm). Measurements were conducted using LD (laser diffraction) and DIA (dynamic image analysis).

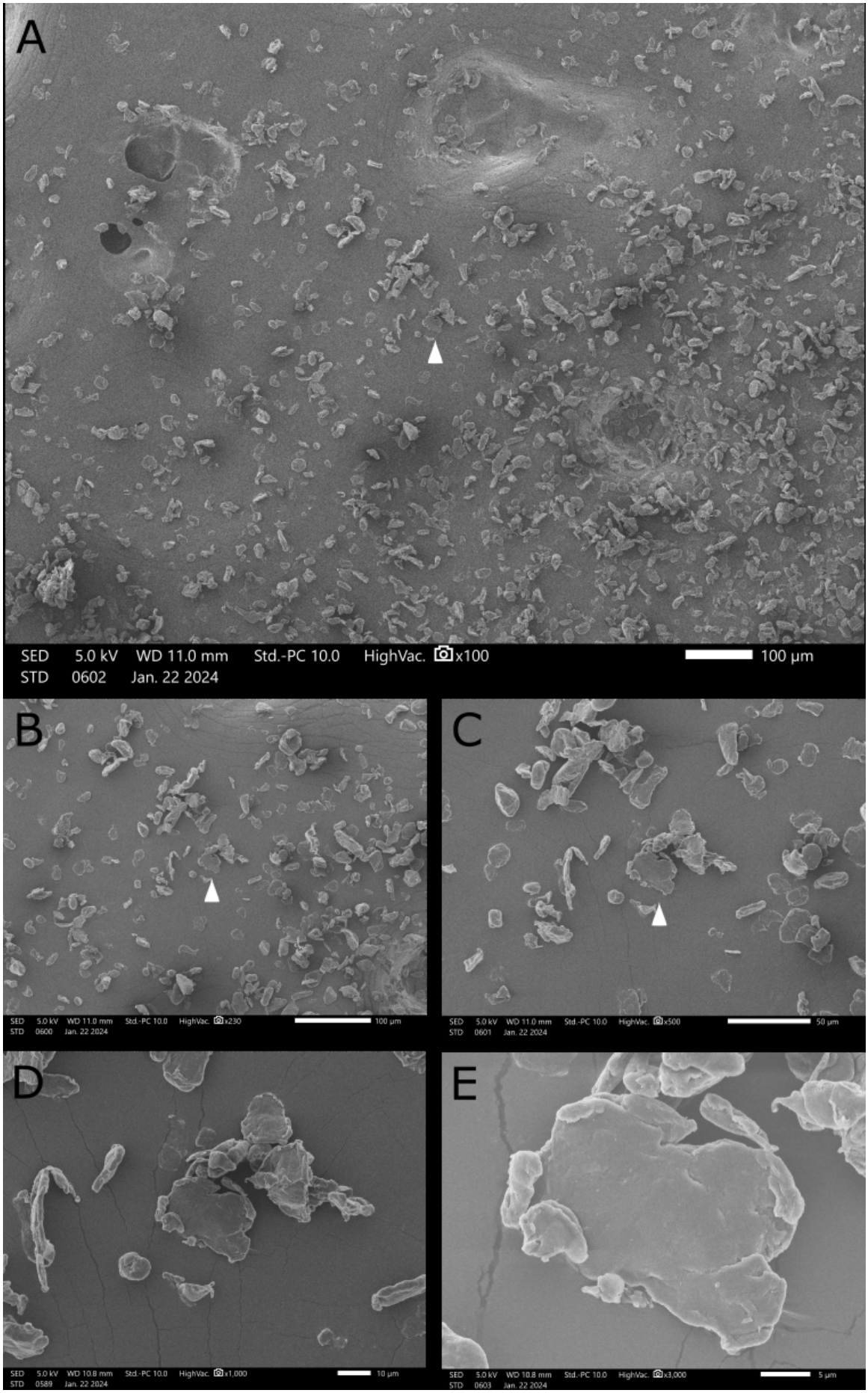
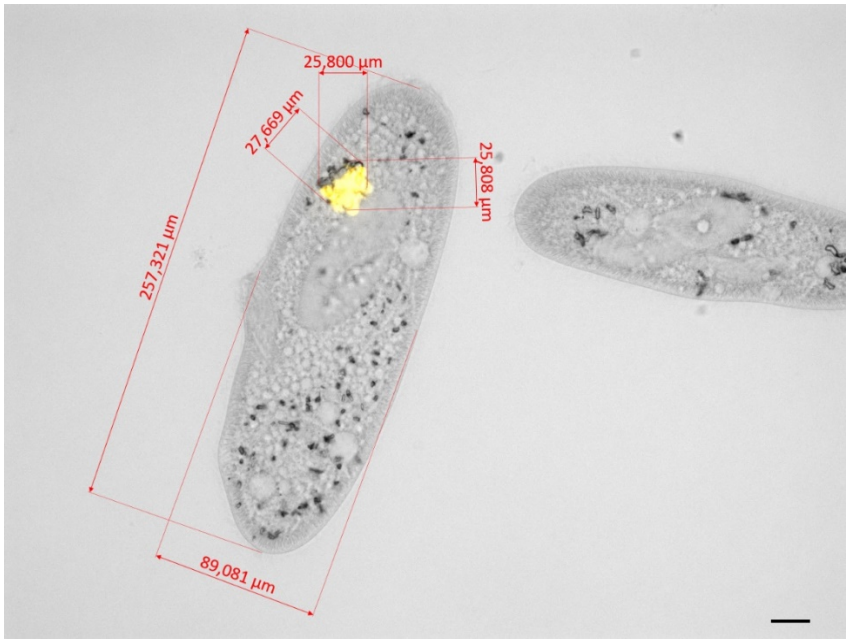

A
SED 5.0 kV WD 11.0 mm Std.-PC 10.0 HighVac. x100 100 µm
STD 0602 Jan. 22 2024
B
SED 5.0 kV WD 11.0 mm Std.-PC 10.0 HighVac. x230 100 µm
STD 0600 Jan. 22 2024
C
SED 5.0 kV WD 11.0 mm Std.-PC 10.0 HighVac. x500 50 µm
STD 0601 Jan. 22 2024
D
SED 5.0 kV WD 10.8 mm Std.-PC 10.0 HighVac. x1,000 10 µm
STD 0589 Jan. 22 2024
E
SED 5.0 kV WD 10.8 mm Std.-PC 10.0 HighVac. x3,000 5 µm
STD 0603 Jan. 22 2024

Figure S2: SEM images of the PS $_{<30\mu m}$ MP fragments (JEOL JSM-IT500 Scanning Electron Microscope, JEOL Ltd., Japan). The particles were coated with 2 nm C and 2 nm Pt (Leica EM ACE600, Leica Microsystems GmbH, Germany). A-E: Gradually increased magnification of the particle centered in the middle (white arrow in A-C). Magnifications: (A) 100x, (B) 230x, (C) 500x, (D) 1,000x, and (E) 3,000x.

A



B

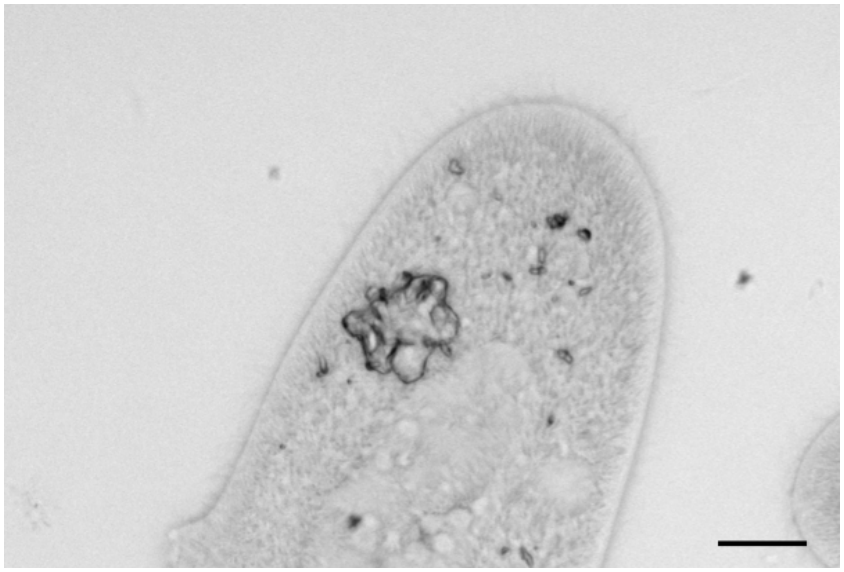


Figure S3 Image of the largest MP particle ($PS_{<30\ \mu m}$) found inside a *P. caudatum* cell. (A) Measurement used for calculation of the particle / cell length ratio. (B) Zooming in (digitally) on the particle indicates a single fragment. Scale bar = 20 µm (20x magnification).

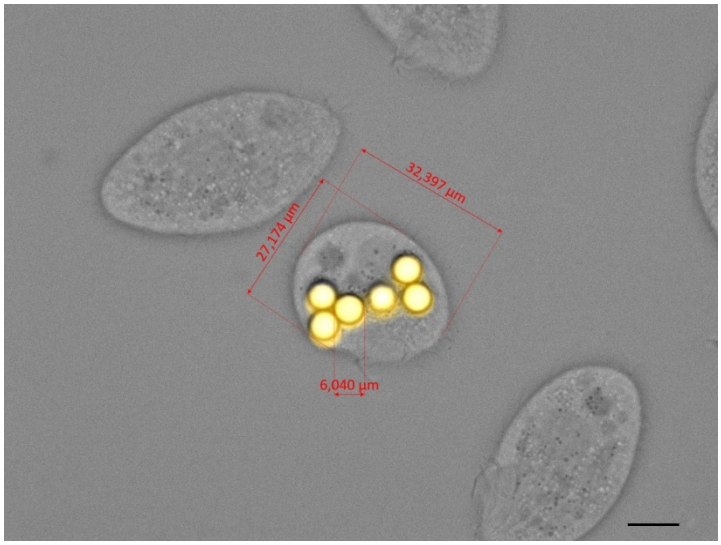


Figure S4 Measurement of a small *T. pyriformis* cell with 6 µm PS beads inside, used for calculation of the particle / cell length ratio. Scale bar = 10 µm (63x magnification).

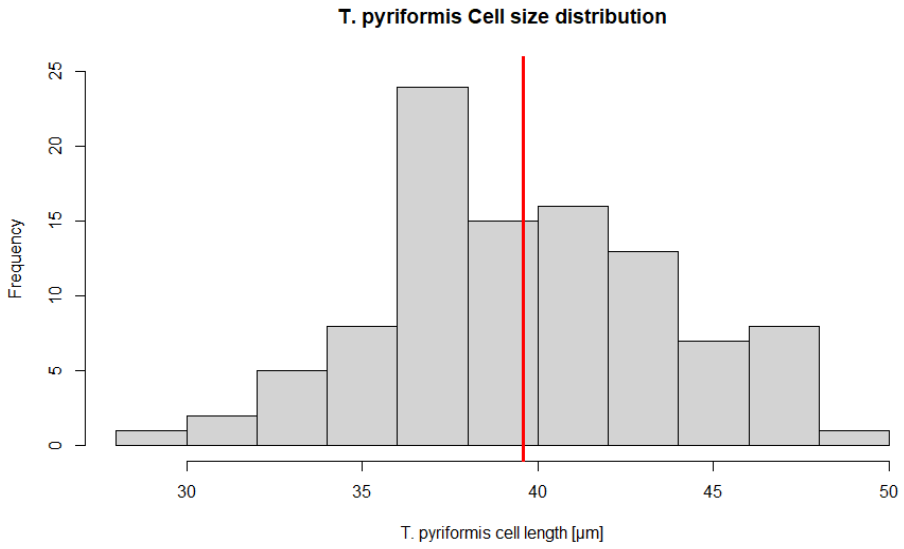


Figure S5: Cell size distribution (cell length) of *T. pyriformis*. Randomly chosen *T. pyriformis* cells were measured using ZEN 2 core v2.5 / ZEN 2 starter v2.5 (Carl Zeiss Microscopy GmbH, Jena, Germany). Histogram was created in RStudio Version 2023.03.0. Red line = mean (39.60µm), n=100.

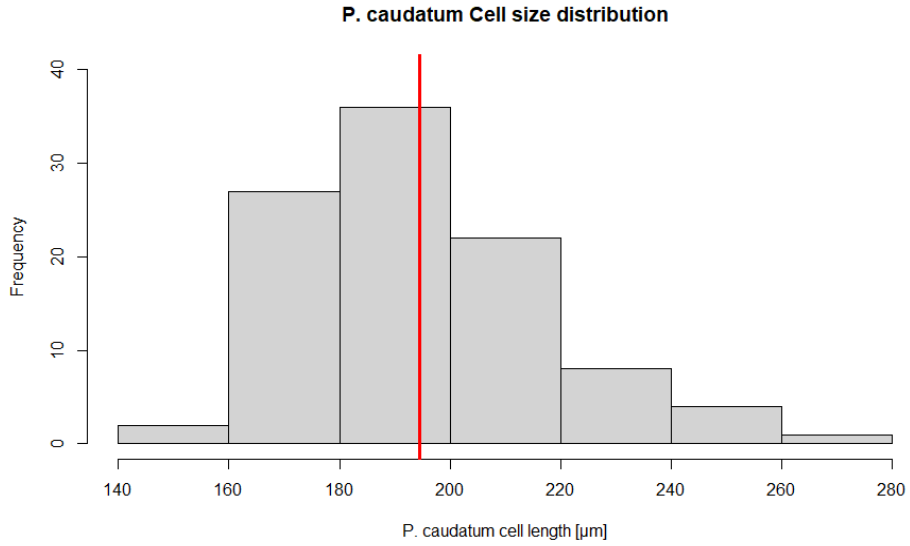
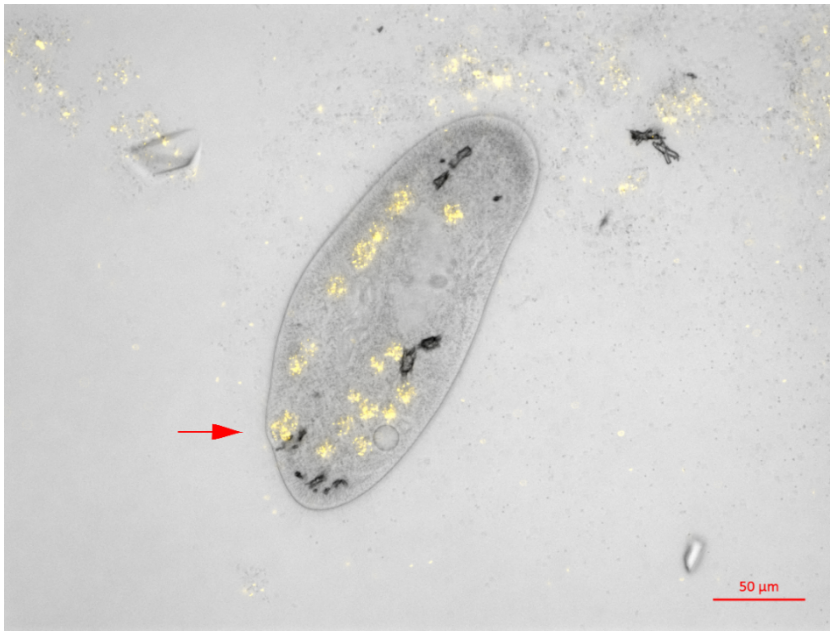


Figure S6: Cell size distribution (cell length) of *P. caudatum*. Randomly chosen *P. caudatum* cells were measured using ZEN 2 core v2.5 / ZEN 2 starter v2.5 (Carl Zeiss Microscopy GmbH, Jena, Germany). Histogram was created in RStudio Version 2023.03.0. Red line = mean (194.46µm), n=100.

A



B

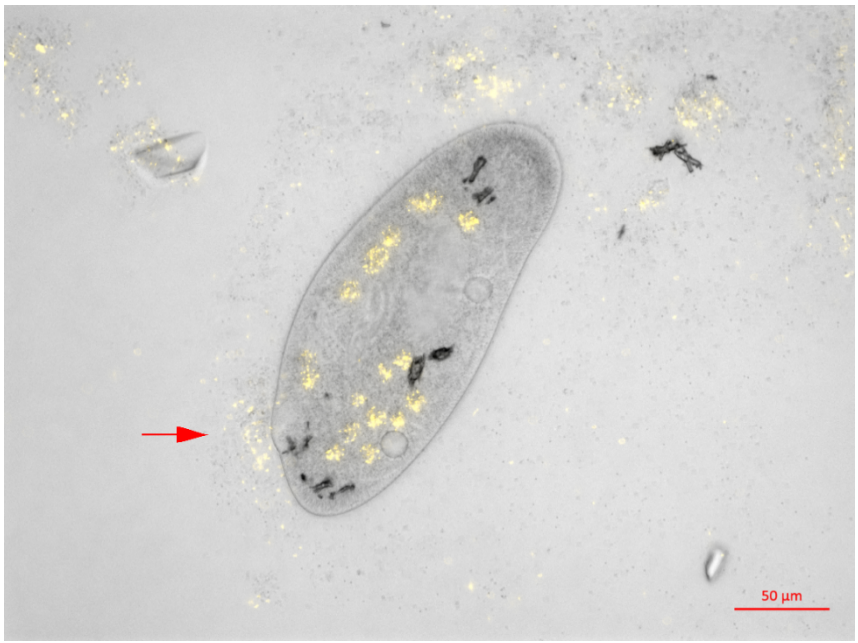


Figure S7: Release of $PS_{0.5\mu m}$ by a *P. caudatum* cell of 0.5 µm PS-MP. (A) Start of the observation under the microscope. Arrow points towards MP inside the cell that are about to be released; (B) Observation made after few seconds incubation. Arrow points towards MP outside the cell after release. Scale bar = 50 µm (20x magnification).

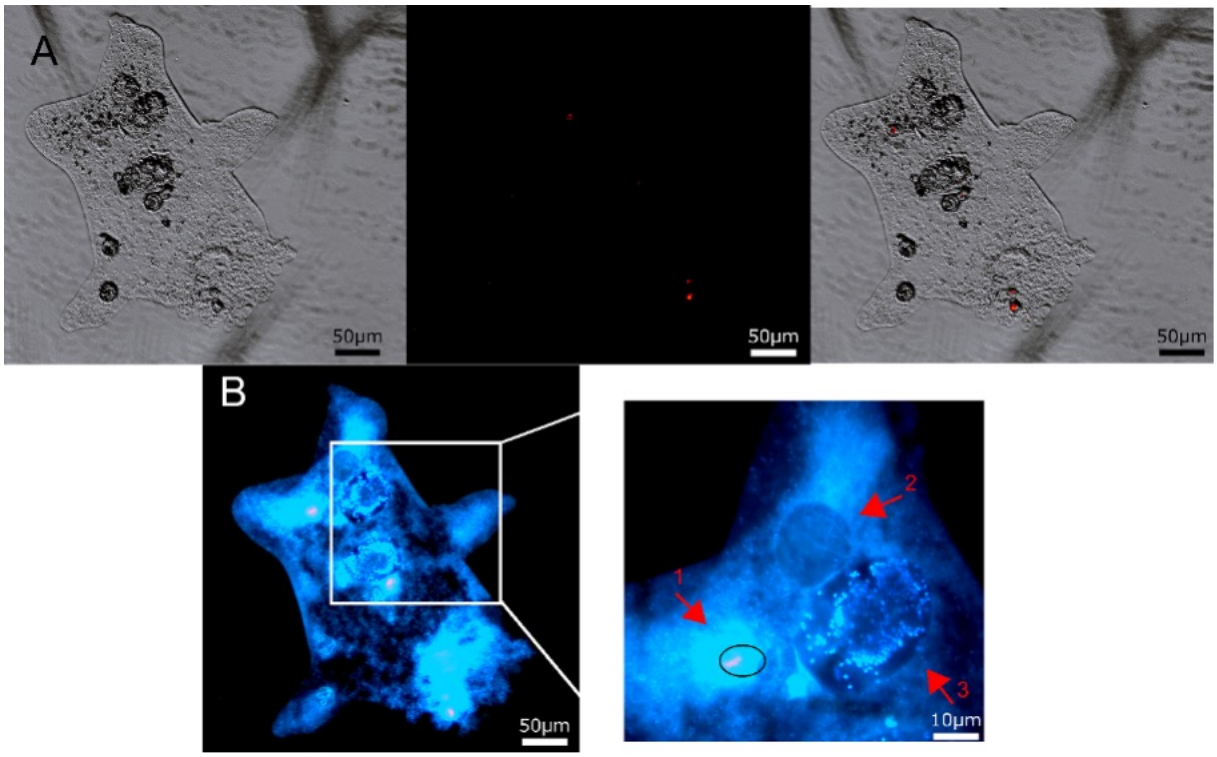


Figure S8: A: *T. pyriformis* loaded with $PS_{0.5\mu m}$ beads, incubated for 6 hours with starving amoebae (Left panel: bright field; middle panel: epifluorescence; right panel: merged). B: *A. proteus* (the same individual as in A), stained with 25 µg/mL Hoechst 33342 dye (Sigma Aldrich, Sigma-Aldrich,Taufkirchen, Germany) to visualize the ingested *T. pyriformis*. Arrow 1: stained *T. pyriformis* with ingested $PS_{0.5\mu m}$ beads (circle). Arrow 2: contractile vacuole, Arrow 3: amoeba nucleus. 10x magnification. Scale bar = 50 µm.

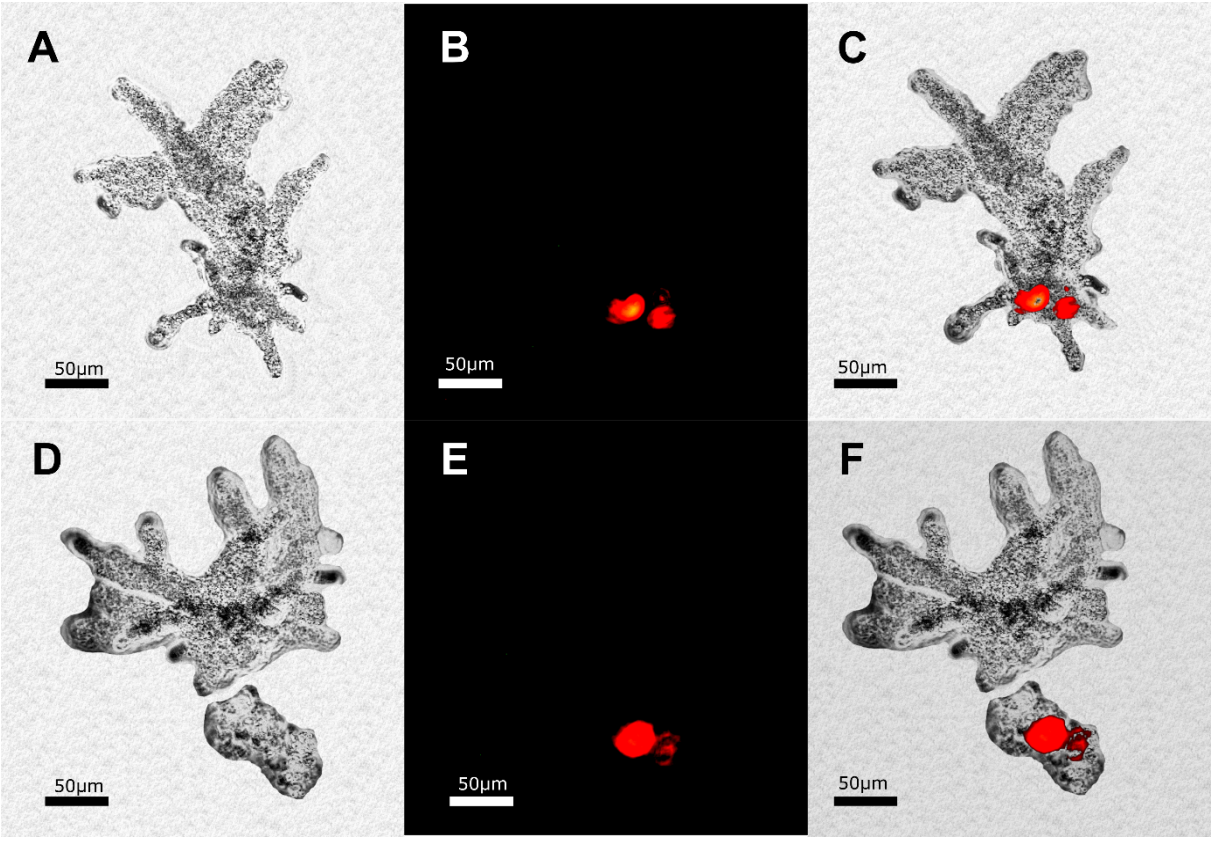


Figure S9: Release of $PS_{<30\mu m}$ and inedible leftovers 24 h after uptake of *P. caudatum*. A-C: Amoeba shortly before the release process of two large MP fragments taken up by an individual *P. caudatum*. D-F: Same amoeba cell 15 min later. Both fragments and inedible leftovers are released. Left panel: bright field; middle panel: epifluorescence; right panel: merged. Size of the fragments seems larger, due to their presence in different z-layers, resulting in a deformation of the visual output. 10x magnification. Scale bar = 50µm.

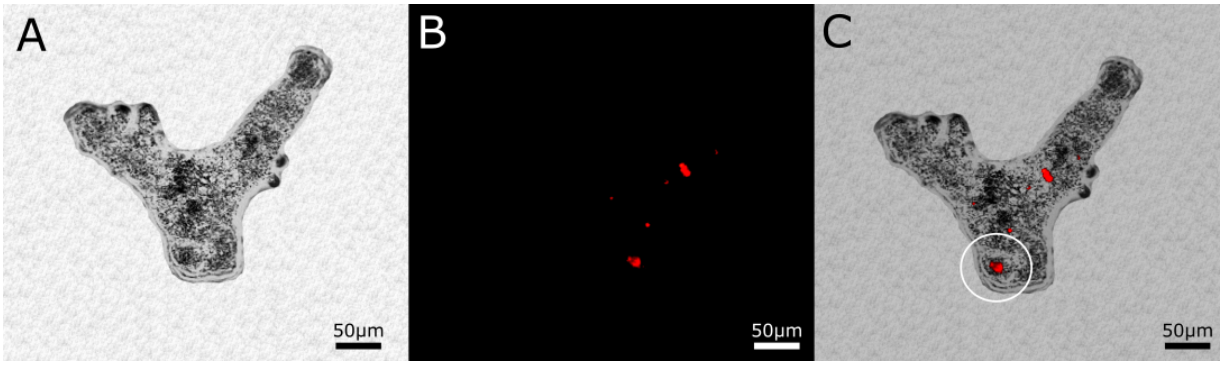


Figure S10: Uptake of $PS_{0.5\mu m}$ loaded *T. pyriformis* by *A. proteus*.

A-C: *A. proteus* with clumped $PS_{0.5\mu m}$ beads 24 h after uptake of *T. pyriformis*. Beads are clumped in a food vacuole and not as distributed as beads taken up by pinocytosis. Left panel: bright field; middle panel: epifluorescence; right panel: merged. White circle identifies a food vacuole. 10x magnification. Scale bar = 50 µm.

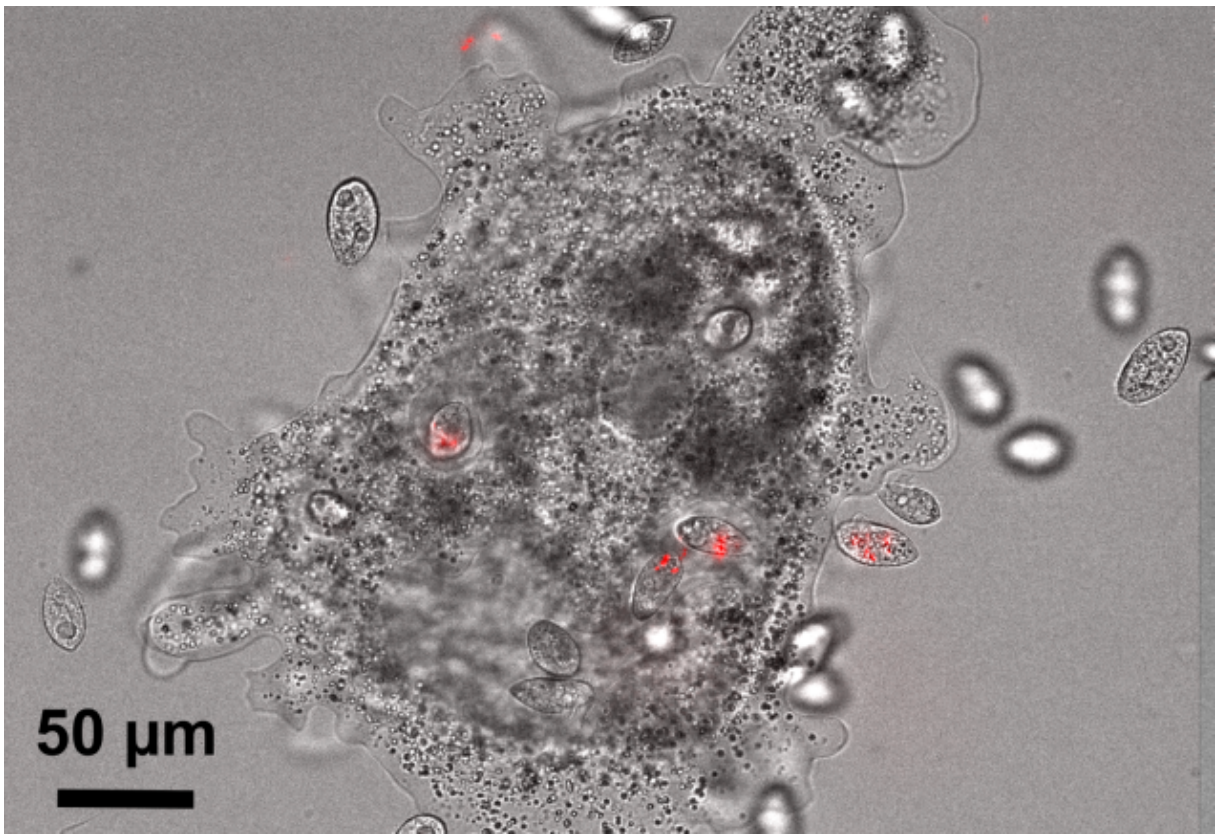


Figure S11: Merged image from Figure 6. Multiple *T. pyrifomis* cells have been taken up by *A. proteus*, of which three cells contained $PS_{0.5\mu m}$ MP. 20x magnification. Scale bar = 50 µm.

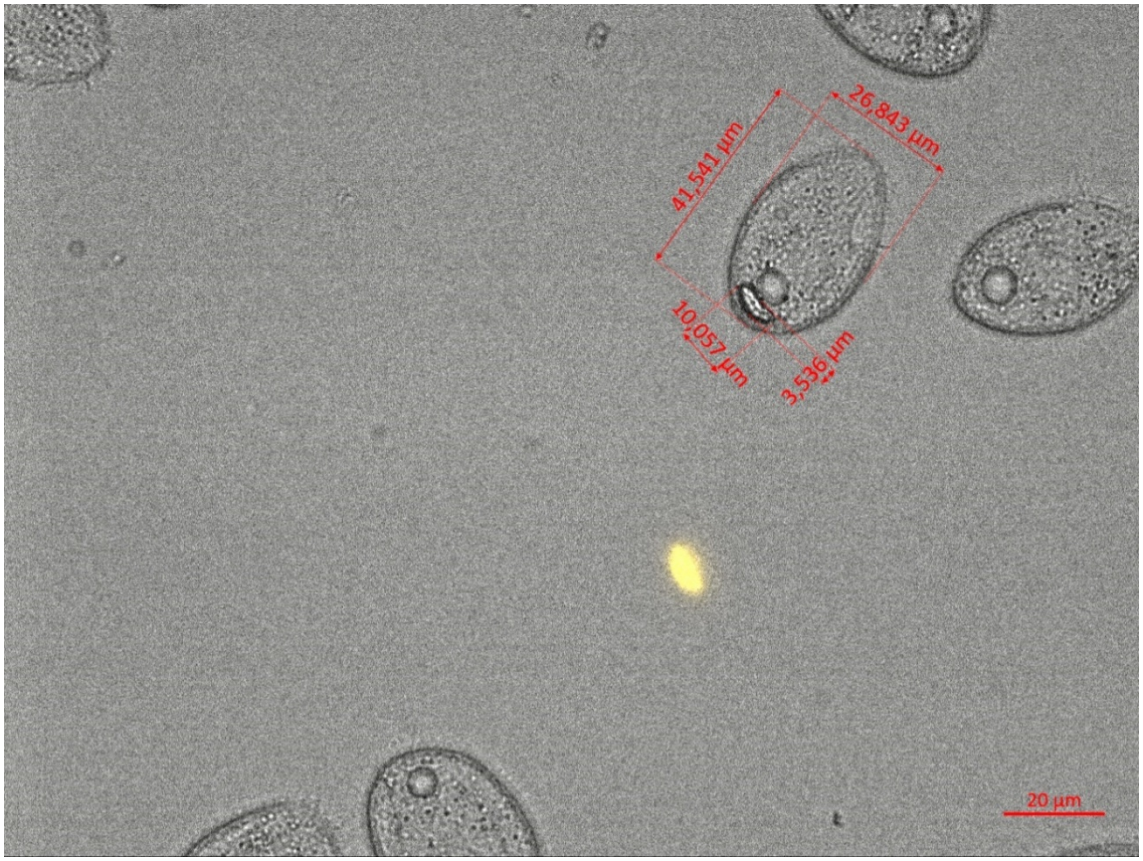


Figure S12: *T. pyriformis* with $PS_{<30\mu m}$ MP fragment taken up by the cell, measurement of particle size inside the living cell, and measurement of cell size (fluorescence signal of the particle is shifted due to the movement of the cell). Cell length = 41.541 µm; particle size (length) = 10.057 µm; particle size (width) = 3.536 µm. Scale bar = 20 µm (40x magnification).

Article III

Same but different: The response to petroleum and biodegradable bio-based microplastics deviates between *Daphnia magna* clones

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(*contributed equally to this work)

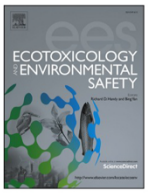
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Same but different: The response to petroleum and biodegradable bio-based microplastics deviates between *Daphnia magna* clones

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ABSTRACT

Microplastics (MP) derived from commodity plastics are considered a threat to ecosystems. Bio-based and biodegradable (BB-) plastics are proposed as eco-friendly alternatives to petroleum-based (PB-) plastics. However, studies on the effects of MP on the model organism *Daphnia magna* often report a wide range of effects for both PB- and BB-plastics. While different physical and chemical MP properties may contribute, other factors, such as differences in clonal sensitivities, are rarely considered. Additionally, only a few studies included a particle control to differentiate between effects caused by particles themselves and those specifically by MP. In this study, these knowledge gaps are addressed by (1) comparing the effects of PB-MP (PET) and two BB-MP (PBS, PLA) on the life-history and morphology of *D. magna*, and (2) examining the responses of two *D. magna* clones to chronic MP exposure (all MP < 20 µm), always including cellulose as a particle control, in a setup with reduced food availability to mimic real environmental scenarios. When comparing PB- to BB-MP, the latter caused similar adverse effects on survival and sublethal life-history traits, while cellulose had no effect or positive effects. We observed a similar concentration-dependent increase in mortality for both clones while data on sublethal parameters were significantly different between the clones. We conclude that particle controls and genetic variability are crucial parameters that should be considered in *D. magna* experiments and that MP effects need to be investigated under environmentally relevant conditions.

1. Introduction

Due to extensive press coverage over the past 20 years, the public perception of microplastics (MP, 1–1000 µm; (ISO 24187:2023, 2023) is that they present a significant threat to ecosystems. Meanwhile, scientific interest in the occurrence and ecotoxicological effects of MP has

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grown. MP have been found ubiquitously in the environment; they contaminate the oceans, lakes, rivers, ponds, glaciers, sediments, and soils (Akdogan and Guven, 2019). MP not only interact with and alter different environmental matrices but also have various impacts on organisms across different trophic levels (e.g., Bhatt and Chauhan, 2023).

Given the accumulation of and impacts on the environment from conventional petroleum-based (PB-) plastics such as polypropylene (PP), polyethylene (PE), polystyrene (PS), polyethylene terephthalate (PET), and polyvinyl chloride (PVC) (PlasticsEurope, 2024), the demand for alternatives such as biodegradable and/or bio-based (BB) plastics has increased (Shruti and Kutralam-Muniasamy, 2019). Biodegradable plastics are introduced as a more environmentally friendly alternative to traditional non-degradable plastics. These materials can be petroleum-based or entirely or partly made from renewable resources (bio-based). Due to their presumed lower environmental impacts compared to conventional plastics, market demand for them has grown (Shruti and Kutralam-Muniasamy, 2019). Whether bio-based or not, biodegradable plastics are designed to decompose under suitable environmental conditions (Savva et al., 2023). However, recent research shows that these materials may not fully degrade in nature (Lara-Topete et al., 2024) and can also form persistent MP (Steiner et al., 2022). Among the most produced biodegradable and/or bio-based polymers are polylactic acid (PLA) and polybutylene succinate (PBS) (Haider et al., 2019). OECD (2022) reports that 0.5 % (2.3 Mt) of produced plastics are labeled as "bioplastics". Global "bioplastic" production is projected to rise from 2.47 Mt in 2024–5.73 Mt within five years (European Bioplastics, 2024). Meanwhile, OECD estimates that overall plastic production, including bioplastics, will triple by 2060, reaching 1230 Mt worldwide (OECD, 2023).

Although plastics are mainly produced and used on land, freshwater environments are among the most seriously impacted ecosystems by plastic pollution (Cuenca Cambronero et al., 2018), including MP. These particles primarily originate from mismanaged plastic waste and littering, urban and agricultural runoff, atmospheric deposition, wastewater, and industrial discharges (Ding et al., 2021). According to the OECD, 109 Mt of plastic accumulated in rivers worldwide in 2019 (OECD, 2022), and MP has been found in lakes and reservoirs worldwide (OECD, 2022). The behavior and transport processes of MP in freshwater systems have been extensively studied in recent years and are highly dependent on hydrodynamics, the particles' physicochemical properties (such as size, shape, surface charge), and the possible interactions with the organic matter present (Guo et al., 2024). Therefore, freshwater ecosystems and their organisms are the focus of numerous studies on MP pollution (e.g., O'Connor et al., 2020; Brehm et al., 2022). When examining the effects of contaminants on freshwater biota, Cladocera, especially *Daphnia* species, are key organisms in most lentic freshwater systems and are well-established model organisms in ecotoxicology and risk assessment (Sarma and Nandini, 2006). Their advantages include sensitivity to various toxicants, short generation times, and rapid population growth through amictic parthenogenesis (Sarma and Nandini, 2006). Parthenogenetic reproduction is often highly valued because it allows researchers to exclude genetic variation when interpreting results (Guan and Wang, 2006). *Daphnia magna* is a preferred species within the genus *Daphnia* for ecotoxicological studies, especially those focused on MP (Brehm et al., 2023). Despite using this clonal model organism, studies on MP toxicity in *D. magna* often produce inconsistent results (e.g., Brehm et al., 2023). A major reason for this variability is the wide range of MP used across studies, which have different physicochemical properties such as plastic-associated chemicals, surface roughness, shape, or surface charge (Wagner et al., 2024; Ali et al., 2024). A recent study on mammalian cell lines emphasized the importance of considering physicochemical properties when interpreting microplastic toxicity studies. Exposure to seemingly identical MP from different manufacturers, all made of the same polymer, within the same size class and with the same shape, elicited different effects (Wieland et al., 2024). Additionally, observed effects could be solely due to the presence of particles in the gastrointestinal tract of exposed organisms, highlighting the need for appropriate particle controls in MP ecotoxicological research (Rozman and Kalčíková 2022).

In addition to the various physicochemical properties of MP, different clonal sensitivities of *D. magna* strains to environmental stressors may hinder the overall comparability of studies. Important ecotoxicological endpoints include life-history traits (Barata et al., 2000), which vary between *Daphnia* clones due to genetic differences. Typically, researchers conduct experiments using in-house *Daphnia* cultures, consisting of a single clone obtained either from wild populations or other husbandries (e.g., Baird et al., 1991). As a result, studies on *Daphnia* can yield significantly different outcomes, even when using standardized methods, due to the use of different clones. Only a few studies have directly compared the effects of MP on multiple *Daphnia* clones of the same species (Imhof et al., 2017; Sadler et al., 2019; de Souza Machado et al., 2023; Brehm et al., 2023), highlighting the importance of considering genetic variation in MP toxicity experiments.

To fill these knowledge gaps, two *D. magna* clones (Aig and BL2.2) originating from different habitats were chronically exposed to MP in comparison to a particle control. The selected clones are known to respond with different life-history strategies to predation stress, as they originate from different locations. We exposed the two *D. magna* clones to irregular MP (< 20 μm), which was made of PBS, PLA (both biodegradable and bio-based, BB-MP), or PET (petroleum-based, PB-MP). Additionally, microcrystalline cellulose fragments were included as particle control (alongside the blank control) to ensure that a mere particle effect did not cause the observed results. Cellulose was chosen as a control particle because it is a naturally occurring polymer with a density similar to that of the MP used.

This study is the first to directly examine the potential toxicity of the BB-MP PBS on *D. magna*, and compare it with another BB-MP, PLA, as well as with a PB-MP, PET. It is also the first to explore this aspect in the context of clonal sensitivity and an environmentally relevant experimental setup. Such a setup with a lower food supply was selected because feeding *ad libitum* might reduce MP effects (Ogonowski et al., 2016; Schür et al., 2020). However, the chosen food concentration still allows the organisms to grow and reproduce (Lampert, 1978; Schür et al., 2020).

2. Materials and Methods

2.1. Daphnia magna cultivation

Two *D. magna* clones from a well-established husbandry were selected for the experiments. The first clone (BL2.2) originated from a pond (Oud Meren) in Leuven (Belgium) and has been maintained in husbandry since 1997 (Imhof et al., 2017). BL2.2 was chosen to enable comparability across studies using the same clone (e.g., Imhof et al., 2017; Schwarzer et al., 2022). The second clone (Aig) was collected in October 2021 from a fishpond of a riverine area called "Aischgrund", located north-west of Nuremberg (49.657554 N, 10.848952 E), Germany, and has been in culture in our lab since then. The Aig clone was selected because of its different geographical origin, which might translate into different responses to the same environmental stressor (i.e., MP) (Soares et al., 1992). Test organisms used were randomly selected from the second brood of age-synchronized mothers held in 1.5 L glass beakers (Weck, Wehr-Öflingen, Germany). All animals were maintained in M4 medium (Elendt and Bias 1990) with the addition of 4.48 μg L^{-1} of selenium dioxide (SeO_2) and kept at 20 ± 0.5 °C in a climate chamber with a photoperiod of 14:9 h light: darkness with 30 min of dusk and dawn. The *D. magna* culture was fed *ad libitum* (2 mg C L^{-1}) with the green algae *Acutodesmus obliquus* (SAG 276–3a, Sammlung von Algenkulturen, Göttingen, Germany). The mothers were separated from the offspring (< 24 h) to ensure the use of similarly aged daphnids for the experiments.

2.2. Acutodesmus obliquus culture

The green algae *A. obliquus* were cultivated in-house. Algae cultures were maintained in 2.5 L Erlenmeyer flasks, equipped with a filtered aeration system. Each flask was filled with 1.5 L of Z medium (Prusch and Minck, 1985) and 500 mL of *A. obliquus* batch. The glassware, aeration tubes, and Z medium were sterilized before setting up a new culture. The so-prepared cultivation was maintained in a climate chamber at a temperature of 21 ± 0.5 °C, with a photoperiod of 16: 8 h light: darkness.

2.3. Microplastics and cellulose particle production and characterization

Three types of MP and one type of cellulose fragments (< 20 μm) were used in this study. Two plastics were biodegradable and bio-based (BB-): PBS (BioPBS FZ91PM, Mitsubishi Chemicals, Japan; molecular weight (Mw) = 2.19 × 10^5 g mol^{-1}) and PLA (PDLLA, NatureWorks, USA; Mw = 1.95 ×10^5 g mol^{-1}), and one was a petroleum-based (PB-): PET (NEOGROUP, Lithuania; Mw = 9.77 ×10^4 g mol^{-1}). We used microcrystalline cellulose fragments as non-plastic control particles (CAS: 9004–34–6, Sigma Aldrich, Germany).

While the plastic fragments were produced in-house by milling and sieving, the purchased cellulose fragments were used as received to prepare the particle stock suspensions, which are described below. The following outlines the required steps for producing the employed MP fragments: As a base material, 1 kg of polymer granulate was mixed with liquid N_2 for 10 min and then milled using an ultra-centrifugal mill (ZM 200, Retsch, Germany, 18000 min^{-1}, 1000 μm spacer sieve, 24 tooth rotor). The ground polymer was then dried under a high vacuum at 40 °C for 24 h. The 1000 μm polymer fragments were first mixed with shredded dry ice (1:1 V/V) and then with liquid N_2 for 20 min. This mixture was poured into the ultra-centrifugal mill (approx. 10 min, 18000 min^{-1}, 200 μm spacer sieve, 24 tooth rotor). Subsequently, the particles were dried as described above and sieved using an ALPINE Air Jet sieve e200LS machine (Fritsch, Germany) with a 75 μm sieve (20 min, 2000 Pa). The fraction < 75 μm was again sieved using a 20 μm sieve (20 min, 2000 Pa), and the obtained particles (< 20 μm) were used for experiments. The particle size distributions were determined via a combination of laser diffraction and dynamic image analysis (Figure SI 1, FLOWSYNC, Microtrac MRB, Montgomeryville and York, USA).

For particle characterization, gel permeation chromatography (GPC) and nuclear magnetic resonance spectroscopy (NMR) were performed. GPC was used to measure molecular weight (MW) on a 1260 Infinity system (Agilent Technologies). The instrument was fitted with a pre-column SDV 5 μm, the main column SDV linear XL 5 μm (PSS, Mainz, Germany), and a refractive index detector (RI, Agilent Technologies, Figure SI 2). These measurements were conducted on 2–3 mg of each polymer. ^1H (300 MHz) NMR spectra were recorded on an Ultrashield-300 spectrometer (Bruker) in $CDCl_3$, and the residual peak from the $CDCl_3$ was used as an internal standard. These measurements were conducted on 15–20 mg of plastics.

Scanning electron micrographs of the MP and cellulose particles were obtained using a JEOL JSM-IT500 Scanning Electron Microscope (SEM, JEOL Ltd., Japan). Before imaging, particles were placed on conductive carbon pads (G3347, Plano GmbH, Wetzlar, Germany) and attached to aluminum stubs (∅diameter 12 mm, Plano GmbH, Wetzlar, Germany). The samples were then coated with 2 nm of carbon and 2 nm of platinum (Leica EM ACE600, Leica Microsystems GmbH, Germany).

Before the start of the experiment, particle suspensions were prepared by adding 100 mg of each particle type (MP and cellulose) to 100 mL of autoclaved M4 medium. Before use, stock suspensions were shaken for 48 h at 100 rpm. Particle counting was conducted at an optical microscope (Zeiss Axio Lab.A1, Carl Zeiss Microscopy GmbH, Jena, Germany) and repeated three times using a hemocytometer (Neubauer Improved, Brand, Germany) to determine the average particle concentration in the stock suspensions.

To assess the potential digestion of cellulose particles, fecal pellets from *D. magna* individuals were collected in a separate experimental setup (one animal in a 160 mL Weck beaker, replicated three times, 5000 particles mL^{-1} concentration) and measured using μFT-IR spectroscopy. To collect the fecal pellets, the animals were transferred onto a microscopic slide after 24 h of incubation (without food), and observed under a stereo microscope (Leica MS5, Leica Microsystems, Wetzlar, Germany). The fecal pellets were collected immediately after being released by the individual daphnid and then promptly transferred onto an Anodisc 25 (0.2 μm pore size, 25 mm diameter; Product number: 6809–6022, Whatman, Germany). The collected samples were dried overnight at room temperature inside a petri dish. Spectra were obtained using a Lumos II μFT-IR microscope (Bruker Corporation, USA).

2.4. Chronic toxicity test

A 21-day chronic toxicity experiment was conducted following OECD Guideline 211 (Test No. 211: Daphnia magna Reproduction Test, 2008) with a few modifications (Fig. 1).

The animals were exposed under semi-static conditions to two particle concentrations, selected based on Schwarzer et al. (2022): 500 and 5000 particles mL^{-1}. These concentrations were selected to allow the detection of potential concentration-dependent effects, and are therefore higher than those found in freshwater environments (Gupta et al., 2023). As a control, we exposed the organisms to cellulose particles at the same concentrations. Each treatment included 15 replicates, meaning 160 mL Weck beakers containing 50 mL of modified M4 medium and one individual daphnid. A blank control without added particles was included for both clones. However, for analysis, it was decided to use the cellulose exposure group as a control for comparison, to distinguish between effects attributable to particles in general and those attributable to MP. Therefore, in this manuscript, the blank control will be referred to as "concentration 0" (0 particles mL^{-1}) and will be considered a "particle concentration."

To ensure consistent exposure conditions, the medium, including the particles, was renewed every other day. Daphnids were fed 0.5 mg C L^{-1} (0.025 mg C per individual) of *A. obliquus*, provided to the animals with the renewed medium. Most recent studies offer food *ad libitum* (i.e., 1.5–2 mg C L^{-1}), a scenario that rarely occurs in nature. In this work, the term "reduced food availability" refers to a food supply lower than the amount recommended by the OECD guidelines for testing on *D. magna* (OECD No. 211), aiming to better reflect actual environmental conditions.

Every other day, pH and O_2 levels were measured, showing only minor differences between treatments (mean values and respective standard deviations: BL2.2: pH: 7.59 ± 0.49, O_2: 8.11 ± 0.14 mg L^{-1}; Aig: pH: 7.57 ± 0.51, O_2: 8.18 ± 0.17 mg L^{-1}).

Different parameters were recorded at primiparity (when the first clutch of eggs appears in the *Daphnia* brood chamber) and at the end of the exposure. Specifically, during the 21-day exposure, we recorded mortality on a daily basis, body length at primiparity and at the end of exposure, age at primiparity, the daily number of hatched neonates, and the sex of the neonates. For analysis, the body length at primiparity was normalized based on the number of days needed for each individual to reach that stage. Images for body length analysis were taken using a dissecting microscope equipped with a digital camera (Leica M50, Germany; camera: Olympus DP26, Germany; light: Leica KL 300 LED, Germany). Body length was measured from the top of the compound eye to the base of the spine (Figure SI 3) with the software cellSens Dimension (v1.11, Olympus, Germany). The newly hatched neonates were collected and reared separately for five days, allowing for visual sex determination.

2.5. Statistical analysis

The statistical analyses were conducted using R version 4.2.2 (R Core

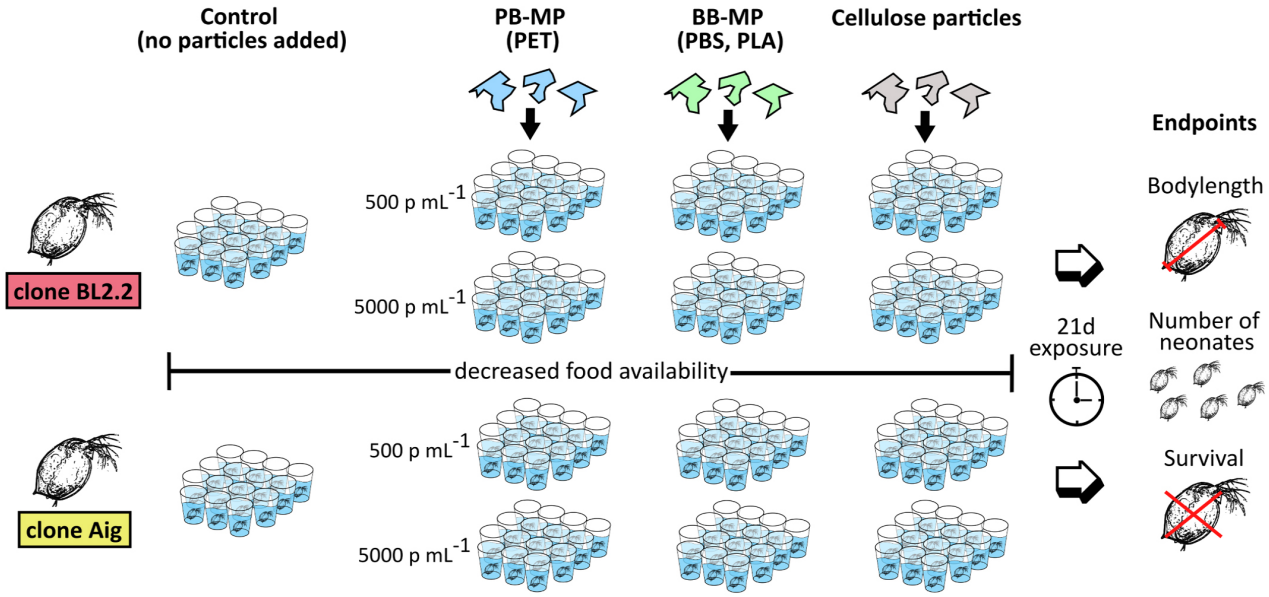


Fig. 1. Schematic depiction of the experimental setup (n = 15 per treatment at t = 0).

Team, 2022). Regression models (ANOVA) were applied to analyze the data. A linear regression model was employed for morphological parameters and reproduction data, while a logistic regression model, using a logarithmic scale for concentration, was used to analyze survival. Normality and homogeneity of variance of residuals were tested as prerequisites for ANOVA. Each response parameter was analyzed with a three-way interaction between treatment, particle concentration, and clone. Significant models were subsequently analyzed with pairwise comparisons using Tukey HSD post hoc tests and Holm's correction method for multiple comparisons. Furthermore, Cohen's D effect size was calculated. The package car version 3.1.1 (Fox and Weisberg, 2018) was used for ANOVA, emmeans version 1.8.2 (Lenth et al., 2020) for post hoc tests, and DHARMa version 0.4.6 (Hartig, 2022) to validate the logistic regression models used in the survival analyses. Dead individuals were excluded from analyses of sublethal endpoints. Juvenile body length growth rate was included as a parameter to account for the number of days each individual took to reach sexual maturity, improving data comparability.

The two clones may respond differently to the treatments, but they also have an inherent difference (i.e., their size already varies under control conditions). To distinguish the inherent clone difference from the variation in response to the treatment, we additionally centered the values of all individuals for each considered sublethal parameter (juvenile body length growth rate, body length, number of neonates) to the respective test concentration 0 (further details and results are in the SI).

3. Results

3.1. Particle characterization

The identity of the employed polymers was confirmed via H NMR (Fig. 2). The respective MW analyses conducted via GPC are reported in

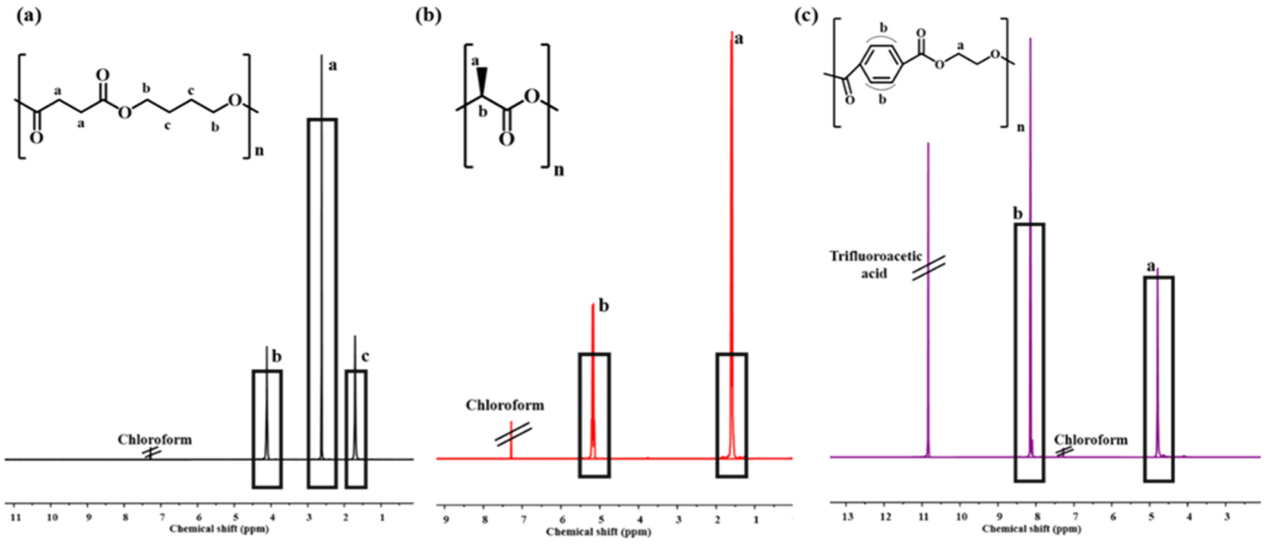


Fig. 2. ^{1}H NMR (300 MHz) spectra of a) PBS, b) PLA, and c) PET in CDCl3.

the SI (Figure SI 2).

Size characterization was conducted for each particle type, including those milled in-house and the purchased cellulose fragments. We selected particles with similar d_{50} values: cellulose $d_{50} = 25.65$ µm, PBS $d_{50} = 22.45$ µm, PLA $d_{50} = 21.25$ µm, and PET $d_{50} = 21.82$ µm. Size distribution charts are shown in the SI (Figure SI 1).

SEM analysis revealed that all MP particles (PBS, PLA, PET) and the particle control (cellulose) were similar in shape and size (Fig. 3).

3.2. Survival

No mortality was observed at concentration 0 (no particles added) and the cellulose treatment at any of the applied concentrations (Table SI 1). On the other hand, all MP treatments (BB- and PB-) showed a negative effect on survival ($\chi^2_{(3,\ 341)} = 56.3$, $p < 0.001$) that increased at the higher MP concentration of 5000 p mL^{-1} ($\chi^2_{(3,\ 341)} = 85.2$, $p < 0.001$) (Fig. 4 and Figure SI 4). PBS had the highest mortality in both clones, followed by PET and PLA. At the highest PBS concentration (5000 p mL^{-1}), only one out of 15 individuals (6.67 %) survived in the case of the Aig clone, and four (26.67 %) survived in the BL2.2 clone after 21 days of exposure. The lower PBS concentration (500 p mL^{-1}) also induced mortality in BL2.2 clones, with only five (33.33 %) animals surviving at the end of the exposure (Table SI 1). At the highest PET concentration (5000 p mL^{-1}), only five (33.33 %) individuals survived across both clones. The highest concentration of PLA (5000 p mL^{-1}) also caused mortality, albeit to a lesser extent, with ten (66.67 %) and nine (60 %) individuals surviving for the Aig and BL2.2 clones, respectively. Analysis of the survival data revealed no clonal dependency (Table 1 & 2), as mortality rates were similar between the two clones.

3.3. Reproduction

The analyses conducted on the average number of neonates indicated a significant effect of the clone ($F_{(3263)} = 172.5$, $p < 0.001$) and an interaction between the treatment and the particle concentration ($F_{(3263)} = 3.50$, $p = 0.015$). The cellulose particles showed neutral to positive effects (not significant), while the MP showed significant adverse effects. The interaction between the treatment and the clone ($F_{(3263)} = 2.96$, $p = 0.032$) and the interaction between particle concentration and clone ($F_{(3263)} = 9.17$, $p = 0.002$) were also significant.

Exposure to MP (both BB- and PB-) in the BL2.2 clone caused a concentration-dependent reduction in the reproductive output from the concentration 0 compared to the organisms exposed to cellulose, which produced a steady number of offspring regardless of the concentration. In contrast, the Aig clone had a generally low reproductive output across the selected treatments, including the concentration 0 and the cellulose control (Table SI 2).

In the BL2.2 clone, a moderate effect caused by all MP treatments (BB- and PB-) was observed (effect size in Table 1). This effect decreased with concentration. For the Aig clone (also confirmed by the effect size analyses, Table 1), the number of neonates produced was generally lower. As a result, no differences between the treatments were observed. In the BL2.2 clone, PBS and PET caused significant decreases in neonate numbers (Fig. 5 and Figure SI 5). All hatched neonates from both clones were female. Clonal comparison revealed significant differences between the two clones for each treatment (Table 2).

3.4. Juvenile growth rate

Analysis of juvenile body length growth rate showed that the exposed organisms were significantly affected by the treatment ($F_{(3299)}$

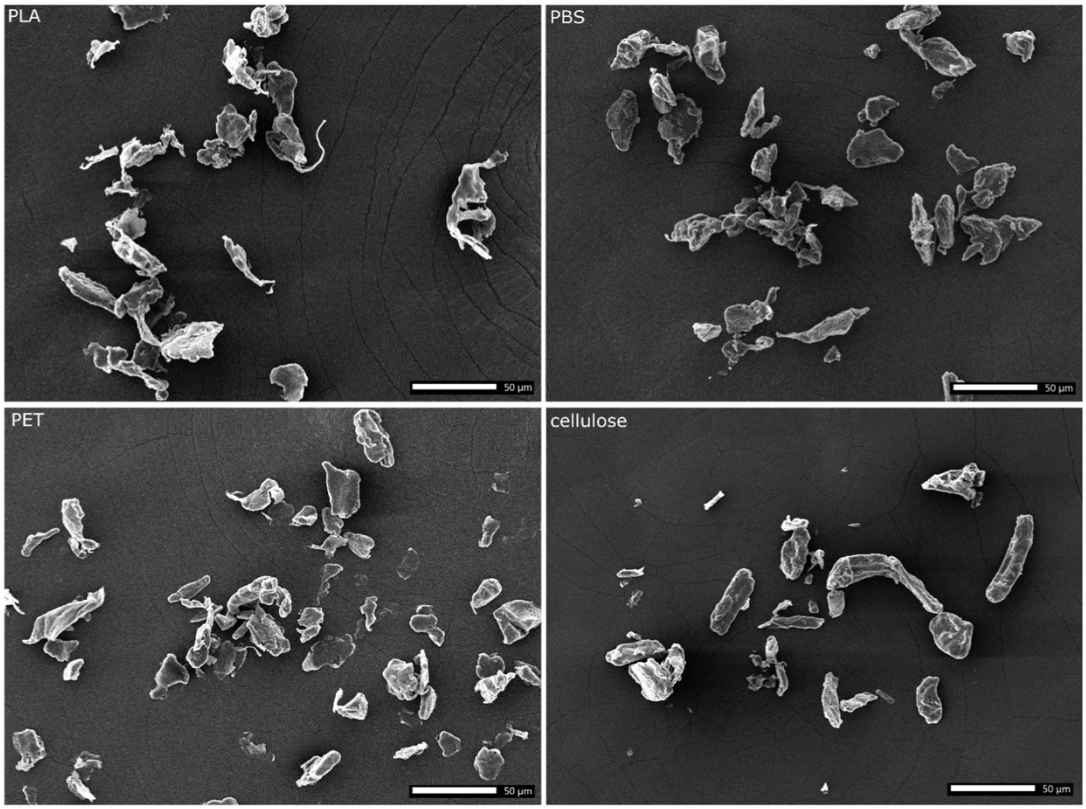


Fig. 3. SEM micrographs of MP and cellulose particles. 400x magnification, scale bars = 50 µm.

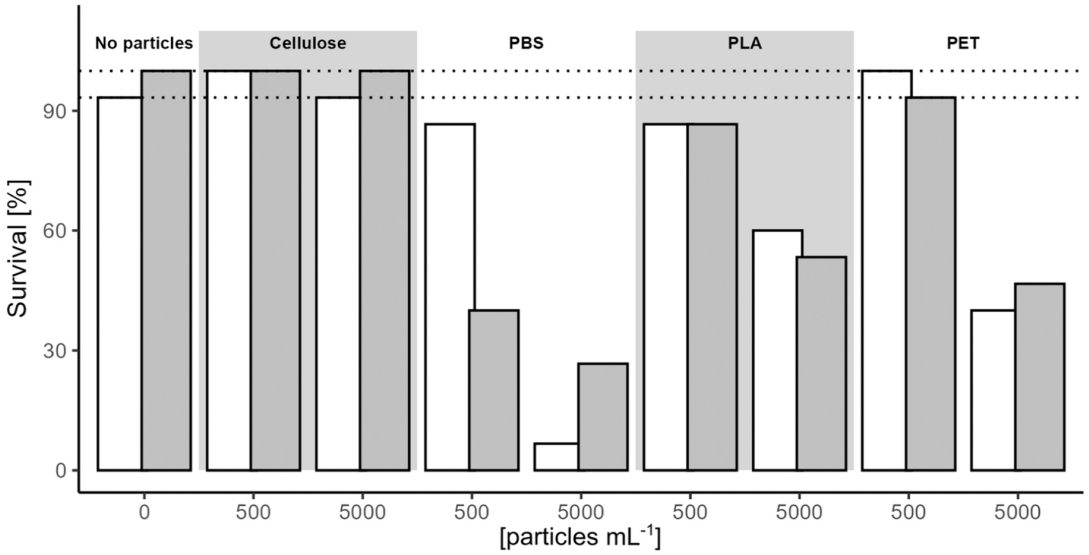


Fig. 4. Percentage of surviving *D. magna* individuals at the end of exposure (n = 15 per treatment at t = 0) for the BL2.2 clone (grey bars) and the Aig clone (white bars), exposed to PET, PBS, and PLA MP, or cellulose fragments. The dashed lines show the value of the blank control (0 particles mL^{-1}).

Table 1
Pairwise comparison of the treatments between both clones for life history parameters of *D. magna* exposed to PBS, PLA, and PET MP or cellulose fragments. To indicate effect size, we present z-values for survival and t-values for the other traits. Bold values represent significance and a detectable effect ($p < 0.05$ and $D > 0.3$).

	Aig vs BL2.2			
Response	**Treatment**	**t/z value**	**P value**	**D**
Survival	cellulose	-0.01	0.98	**-18.8**
	PBS	0.98	0.32	**1.12**
	PLA	-1.15	0.25	**-1.76**
	PET	-1.36	0.17	**-3.01**
Num. of neonates	cellulose	-9.06	**< 0.001**	**-1.99**
	PBS	-3.25	**0.001**	**-1.09**
	PLA	-7.04	**< 0.001**	**-1.67**
	PET	-4.74	**< 0.001**	**-1.17**
Juvenile growth rate	cellulose	-2.81	**< 0.01**	**-2.08**
	PBS	-2.06	**0.03**	**-0.49**
	PLA	-4.28	**< 0.001**	**-0.96**
	PET	-2.23	**0.02**	**-0.50**
Body length (21 d)	cellulose	-6.90	**< 0.001**	**-1.52**
	PBS	-3.64	**< 0.001**	**-1.22**
	PLA	-5.60	**< 0.001**	**-1.33**
	PET	-4.09	**< 0.001**	**-1.01**

= 3.75, p = 0.011), particle concentration ($F_{(3299)} = 64.49$, $p < 0.001$), clone ($F_{(3299)} = 41.48$, $p < 0.001$), and the interaction between treatment and particle concentration ($F_{(3299)} = 4.15$, $p < 0.001$).

The individuals in all MP treatments (BB- and PB-) grew slightly slower compared to the respective cellulose control treatment when exposed to 5000 particles mL^{-1}, while those exposed to 500 particles mL^{-1} had a growth rate comparable to both the cellulose control and the concentration 0. Surprisingly, regarding the BL2.2 clone, the animals grew faster when exposed to cellulose, especially at the higher concentration. Here, significant differences between animals exposed to cellulose were found. A concentration-dependent decrease was also observed for the Aig clone, in this case also in the organisms exposed to cellulose. Pairwise comparisons of the treatments within the separate clones indicate significant differences only between PBS and PLA ($t = 2.74$, $p = 0.038$) for the Aig clone, with the organisms exposed to PBS being larger than those exposed to PLA (Fig. 6, Figure SI 6, and Table SI 2). The clonal comparison indicates significant differences across all treatments, also supported by the corresponding effect sizes (Table 2). Further data regarding the age of primiparity and body length at primiparity are reported in the SI (Figure SI 7 and 8, Table SI 2–4).

3.5. Body length after 21 days of exposure

Analysis of the body length measurements of the test animals recorded on day 21 (Fig. 7 and Figure SI 9) indicated a significant effect of the treatment ($F_{(3263)} = 5.8$, $p < 0.001$), the particle concentration ($F_{(3263)} = 77.4$, $p < 0.001$), the clone ($F_{(3263)} = 122.7$, $p < 0.001$), and the interaction between treatment and particle concentration ($F_{(3263)} = 11.2$, $p < 0.001$).

Individuals from the BL2.2 clone generally grew larger than those from the Aig clone, which was already clear at concentration 0 (Table SI 2). The two clones were confirmed to be significantly different through clonal comparison analyses (Table 2). When examining the clones individually, the only significant difference was found for the treatment with cellulose and PLA for the BL2.2 clone (Table 1). We did not observe concentration-dependent differences in organisms exposed to cellulose, as their sizes remained steady. However, a decrease in size at higher MP concentrations was observed. Concerning the Aig clone, we observed an overall size decrease at high concentrations, regardless of the treatment, including cellulose. Analysis of effect sizes showed a low effect for the Aig clone exposed to both BB-MP (PBS and PLA), while a medium effect was seen in the BL2.2 clone across all MP treatments (Table 1).

4. Discussion

In this study, we examined the effects of PB-MP (PET) and two BB-MP (PBS and PLA) compared to cellulose fragments as a particle control on two *D. magna* clones to assess interspecific sensitivity. Polymer identity was confirmed via H NMR spectroscopy (Fig. 1), and size analyses confirmed the use of similar-sized particles with a $d_{50} \sim 20$ µm (Figure SI 1). BB-MP had significant effects on the life history of *D. magna* that were similar to, if not stronger than, those of conventional PET-MP, while cellulose control particles had no or slightly positive effects. This suggests that the observed effects cannot be attributed solely to particle exposure. In cellulose-exposed animals, mortality was nearly absent. In the MP treatments, on the other hand, increased mortality rates with higher MP concentrations were found, being similar

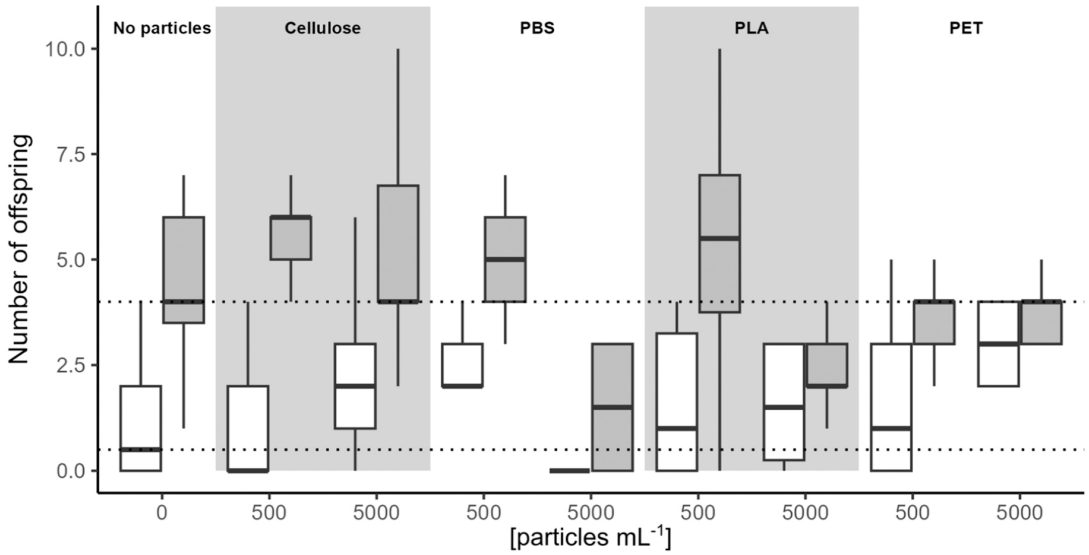


Fig. 5. Cumulative number of neonates from *D. magna* individuals of the BL2.2 clone (grey boxes) and the Aig clone (white boxes) hatched during chronic exposure to PET, PBS, and PLA MP or cellulose fragments. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. The whiskers represent the 1.5-fold of the reported quartiles. The dashed lines report the median value of the blank control (0 particles mL^{-1}).

Table 2
Pairwise comparison between the separate clones of the life history parameters of *D. magna* exposed to PBS, PLA, and PET MP or cellulose fragments. To indicate effect size, we present z-values for survival and t-values for the other traits. Bold values represent significance and a detectable effect ($p < 0.05$ and $D > 0.3$).

		BL2.2			***Aig***		
Response	**Comparison**	**t/z value**	**P value**	**D**	**t/z value**	**P value**	**D**
Survival	Cellulose-PBS	0.02	1.000	**21.75**	1.41	0.791	**1.82**
	Cellulose-PLA	0.02	1.000	**19.04**	1.88	0.354	**1.99**
	Cellulose-PET	0.01	1.000	**17.81**	1.00	1.000	**1.27**
	PBS-PLA	-1.74	0.410	**-2.70**	0.15	1.000	0.17
	PBS-PET	-2.20	0.165	**-4.66**	-0.41	1.000	**-0.54**
	PLA-PET	-1.61	1.000	**-1.96**	-0.66	1.000	**-0.71**
Num. of neonates	Cellulose-PBS	2.65	**0.041**	**0.68**	-0.70	1.000	-0.21
	Cellulose-PLA	1.62	0.418	**0.37**	0.20	1.000	0.04
	Cellulose-PET	2.78	**0.034**	**0.65**	-0.71	1.000	-0.17
	PBS-PLA	-1.19	0.703	-0.31	0.84	1.000	0.26
	PBS-PET	-0.14	0.887	-0.03	0.15	1.000	0.04
	PLA-PET	1.15	0.703	0.27	-0.89	1.000	-0.21
Juvenile growth rate	Cellulose-PBS	2.21	0.108	**0.50**	-0.54	1.000	-0.12
	Cellulose-PLA	2.96	**0.016**	**0.65**	2.33	0.100	**0.52**
	Cellulose-PET	3.16	**0.010**	**0.70**	0.53	1.000	0.11
	PBS-PLA	0.68	1.000	0.15	2.74	**0.038**	**1.11**
	PBS-PET	0.88	1.000	0.20	1.04	0.899	**0.70**
	PLA-PET	0.21	1.000	0.05	-1.80	0.290	0.03
Body length	Cellulose-PBS	2.32	0.084	**0.60**	0.99	1.000	**0.3**
	Cellulose-PLA	2.70	**0.044**	**0.61**	1.85	0.387	**0.42**
(21 d)	Cellulose-PET	2.59	**0.050**	**0.60**	0.43	0.436	0.1
	PBS-PLA	0.05	1.000	0.01	0.39	1.000	0.12
	PBS-PET	0.01	1.000	0.003	-0.64	1.000	-0.20
	PLA-PET	-0.04	1.000	-0.009	-1.33	0.915	**-0.32**

across both clones. Life-history analyses and morphological parameters showed different sensitivities between clones.

4.1. Effects of MP on D. magna's life history

BB-MP generally were at least as toxic to the *D. magna* clones (PBS > PLA) as PET-MP. In this study, we included cellulose as a non-plastic control particle to rule out a mere particle effect on the daphnids. Our results showed that cellulose particles had no effect or slightly positive effects on the selected sublethal parameters, which was also previously reported by Schwarzer and colleagues (2022). Therefore, the effects observed from MP are likely caused by the properties of the different MP tested rather than by general particle exposure. In this context, other studies suggest that the effects of MP may be exacerbated by reduced food intake resulting from the presence of MP in the gastrointestinal system (e.g., Höss et al., 2022). The use of microcrystalline cellulose as a control particle demonstrates that, despite providing less food, the mere presence of particles does not explain the adverse effects observed in this study. The experimental setup, which involved reduced food availability and MP exposure, also significantly affected the Aig clone, making it impossible to isolate the specific effects of MP on reproduction for this clone.

Several recent studies also found adverse effects of BB-MP on aquatic organisms, especially of MP made of PLA and PHB (Zimmermann et al.,

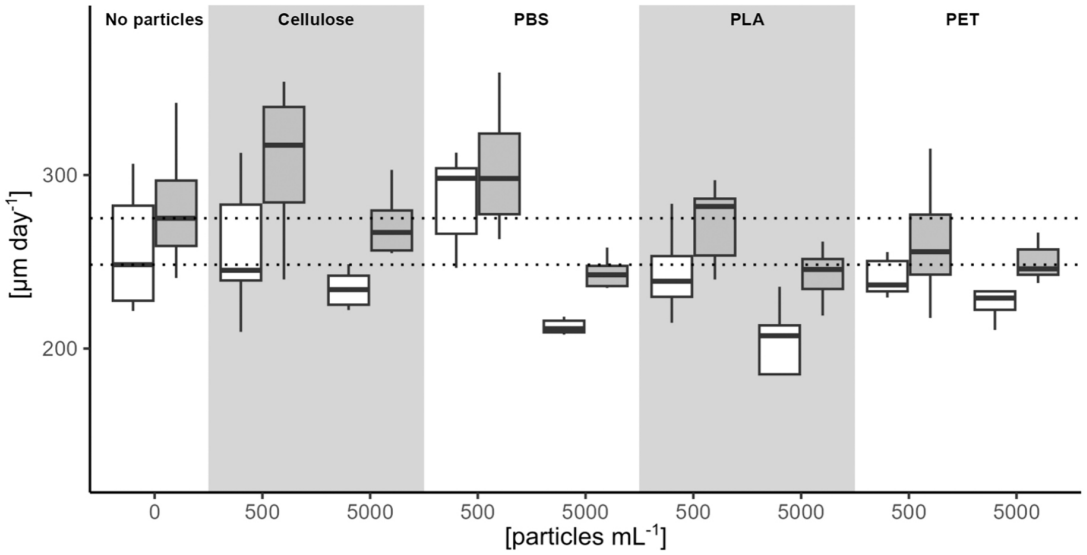


Fig. 6. Juvenile growth rate (µm day^{-1}) of *D. magna* from the BL2.2 clone (grey boxes) and the Aig clone (white boxes) exposed to PET, PBS, and PLA MP or cellulose fragments. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. The whiskers represent the 1.5-fold of the reported quartiles. The dashed lines report the median value of the blank control (0 particles mL^{-1}).

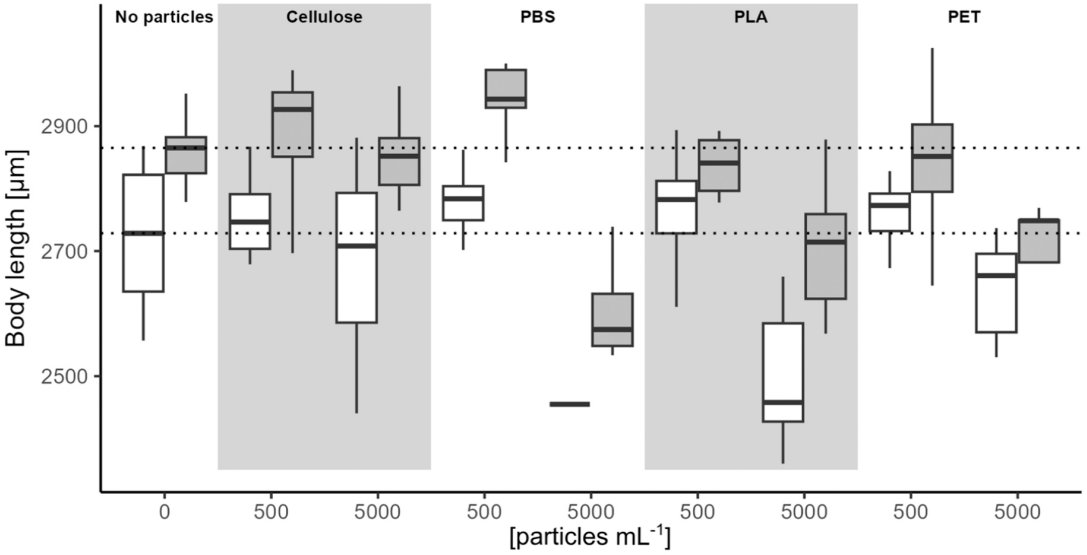


Fig. 7. Body length at the end of chronic exposure of *D. magna* individuals from the BL2.2 clone (grey boxes) and the Aig clone (white boxes) exposed to PET, PBS, and PLA MP or cellulose fragments. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. The whiskers represent the 1.5-fold of the reported quartiles. The dashed lines report the median value of the blank control (0 particles mL^{-1}).

2020; An et al., 2024; De Felice et al., 2024). For instance, Zimmermann and co-authors (2020) demonstrated, in a similar experimental setup, that PLA-MP exerted effects comparable to those of conventional MP (PVC and PUR) when compared to kaolin as a particle control. The experiment, conducted across a wide concentration range (10–500 mg L^{-1}), showed a concentration-dependent decrease in both the number of offspring produced and the mean body length for all MP types. Notably, PLA exposure was associated with a significant increase in mortality. Similarly, a study by An et al. (2024) found that PLA exhibited greater toxicity than PET-MP in a 21-day chronic exposure test. Compared to PET, PLA resulted in a reduction in survival rate and fitness, accompanied by the downregulation of genes related to reproduction, oxidative stress defense, and cellular energy production. Likewise, De Felice et al. (2024) reported that PLA altered biomarkers associated with oxidative stress and energy metabolism, as well as the swimming activity of *Daphnia* after 21 days of exposure.

These studies collectively highlight that PLA, despite being marketed as an environmentally friendly alternative, is capable of inducing significant adverse effects in *Daphnia*, comparable to or even exceeding those caused by PB-MP.

Concerning PBS, recent scientific literature has not yet provided studies on its impact on *Daphnia*. Thus, the present study represents the first report on PBS toxicity on *Daphnia*. In our study, we found that PBS was more toxic than PLA or PET. Despite these findings, other research reports very different outcomes when comparing PB- and BB-MP. For example, a study by Brehm et al. (2022) on *Dreissena bugensis* reported

that MP from recycled PET had stronger adverse effects than PLA-MP. These differences in assessing the toxicity of the same MP may be due to the diverse physicochemical properties of the particles used, mainly related to leaching chemicals (Zimmermann et al., 2020; Brehm et al., 2022; An et al., 2024), which, in the case of Brehm et al. (2022), likely accumulated in the recycled material. Similarly, a more recent study reported stronger adverse effects of PET compared to BB-MP derived from PHB (Serra et al., 2025). Since the chemical composition of the same plastic type varies based on their final properties, products made from the same polymer can have very different chemical compositions (Laforsch et al., 2020). The chemicals used in plastic manufacturing are largely unknown because producers are not required to disclose the compounds they use (Wagner et al., 2024). To date, over 16,000 plastic chemicals have been identified; about 4200 of these are of concern for environmental and human health because they are classified as persistent, bioaccumulative, mobile, and/or toxic (Wagner et al., 2024). MP toxicity is, in addition to their potential hazardous chemical compositions, also influenced by their shape and size (Schwarzer et al., 2022). In this study, however, these factors can largely be excluded, as the morphology of the particles used appeared to be very similar (Fig. 2).

While most chronic MP toxicity studies using *Daphnia* as a model organism are conducted with *ad libitum* food (e.g., Brehm et al., 2023), we performed our experiment using a more environmentally relevant setup. A high amount of added food in an experiment could reduce the potentially harmful effects of MP particles, as excessive food intake may lower particle uptake and limit contact with the gastrointestinal tract, making MP effects harder to detect (Schür et al., 2020; Lyu et al., 2022). This method of conducting experiments with *ad libitum* food is recommended by OECD guidelines for *D. magna* experiments (OECD No 211). However, this protocol was originally designed for testing soluble chemicals that interact with the entire body surface, regardless of food levels. Particulate contaminants, such as MP, which are mixed with food particles potentially compete with each other. Furthermore, feeding *ad libitum* does not accurately mimic natural environments where nutrient levels are lower and fluctuate seasonally (Giebelhausen and Lampert, 2001). Effects on life-history traits are often influenced by high food intake. However, food scarcity is common in natural environments, which means that the potential threat posed by MP may be significantly underestimated in experiments that feed animals *ad libitum* (Lyu et al., 2022). Therefore, in our study, animals were fed 0.5 mg C L^{-1}, a realistic food amount for mesotrophic lakes that still supports growth (Giebelhausen and Lampert, 2001) and egg production in *Daphnia* (> 0.2 mg C L^{-1}; Lampert, 1978). This more natural scenario could have influenced the measured endpoints, as the available food concentration is a key factor in *Daphnia*'s growth and reproduction (Lampert and Trubetskova, 1996). As a result, both clones probably responded to the reduced food availability, as indicated by their generally low neonate production. Both clones showed a significantly lower survival rate across all three MP treatments compared to cellulose, suggesting a combined adverse impact of limited food availability and MP exposure. We recognize that confirming this hypothesis requires future studies to explore how food scarcity influences MP effects. Nonetheless, prior research supports this combined effect (Jemec et al., 2016), and similar findings have been observed in mussels, where food particles mitigated MP's adverse effects (Wang et al., 2021). Conversely, Aljaibachi and Callaghan (2018) reported that *D. magna* mortality from chronic MP exposure depended more on food availability than MP concentration. However, in our study, mortality increased with higher MP levels, indicating that MP toxicity is also dose-dependent. To rule out effects from general particle exposure, we used a cellulose control group. An advantage of using cellulose as a control is its similar density to that of most MP (Schwarzer et al., 2022). Nonetheless, *Daphnia* can produce cellulase enzymes and potentially use cellulose as a carbon source (Schoenberg et al., 1984). Therefore, we cannot exclude the possibility that cellulose fragments in the particle control treatment may have partially offset the reduced food availability, especially for the BL2.2 clone, particularly regarding juvenile growth. To examine this, we analyzed *D. magna* fecal pellets (Figure SI 10) containing the cellulose particles using μFT-IR spectroscopy. The spectra (Figure SI 11) showed no significant difference between cellulose in fecal pellets and pristine cellulose. This suggests that, if digestion occurs, it is limited to the outer layers of the particles and does not change their chemistry, size, or appearance. Another aspect to consider is the potential positive effect of nutritious biofilm formation on the surface of the particles, which could provide nutrients to the daphnids (Amariei et al., 2022) and thus counteract the negative effect of reduced food availability in our experimental setup, explaining the neutral to positive outcomes observed in the cellulose-exposed animals. However, further studies are needed to better explore this aspect.

In general, the mechanisms underlying increased MP toxicity when combined with a limited food supply remain not fully understood. However, in environments where food availability is a limiting factor, MP concentrations are usually lower than those used in this study (Yang et al., 2022). Nonetheless, the harmful effects of MP on *Daphnia* in the environment may depend not only on MP concentration but also on MP properties (Zimmermann et al., 2020; Schwarzer et al., 2022). This is evidenced by the fact that all MP used in this study caused adverse sublethal effects compared to cellulose fragments, even at the lower concentration. These sublethal effects of MP might have long-term consequences for aquatic ecosystems (Haney and Rochman, 2025). However, for risk assessment of these factors, lower particle concentrations should be used, as the ones used in this study are substantially higher than those found in the environment (Gupta et al., 2023).

4.2. Effects of clonal variation

The results of our study showed that the chosen *D. magna* clones significantly differed in all the examined sublethal parameters (Table 1). These findings align with previous research (de Souza Machado et al., 2023), which also observed clonal differences across various stressors, such as chemicals (Barata et al., 2002), predation (Weber and Declerck, 1997), food availability (Glazier and Calow, 1992), and temperature (Sadler et al., 2019).

In the present study, the Aig clone was more negatively affected by MP exposure than the BL2.2 clone. The observed differences could be due to inherent genetic differences between the *D. magna* clones, with the Aig clone being more vulnerable to the introduced stressors than BL2.2. For example, the Aig clone may be more sensitive to reduced food availability, which could have contributed to the stronger effects of MP. Additionally, both clones differ in size, with BL2.2 being larger than Aig (Figure SI 12), which may influence their sensitivity to MP-related toxicity. Sensitivity to various contaminants can be affected by the body size of the organisms, a factor that impacts both toxicodynamics and toxicokinetics, determining intra- and interspecific differences (Gergs et al., 2015). Previous research on other contaminants has shown that smaller daphnids tend to be more sensitive in toxicity tests, as they have higher metabolic rates compared to larger organisms (e.g., Vesela and Vijverberg, 2007) and a larger surface-to-volume ratio. The different responses of the clones might be due to adaptations to their respective environments. For instance, the Aig clone originated from a fishpond, where these organisms face greater predation pressure from fish. As a result, they might be smaller to avoid visual-hunting predators (Dodson, 1989). This adaptation to predation risk in their original habitat could have made them more vulnerable to MP and likely other human-made contaminants.

To rule out that the recorded effects could be solely attributed to clone-specific trait differences, it is necessary to consider these traits when comparing the response of the two different clones to MP exposure. Thus, we normalized (centered) the responses of the two clones with their respective controls (more details in the SI, Table SI 5, and Figures SI 12 and 13). This analysis indicated that treatment and MP concentration significantly affected all sublethal endpoints, and that the

clones differed in susceptibility for the endpoints "reproductive output" and "juvenile body length growth rate." In the case of reproduction, the BL2.2 clone was significantly affected by MP exposure, whereas the Aig clone was not. However, such a comparison is somewhat limited by the generally low reproduction of the latter clone. For juvenile body length growth rate, the difference between the clones was significant, indicating a stronger influence during the juvenile stages. Extended developmental periods coupled with smaller body sizes at sexual maturity, as observed in the Aig clone, may have negative consequences for natural populations, potentially leading to their decline and cascading effects on higher trophic levels.

Overall, our results on clonal differences showed that clearly identifying the *Daphnia* clone used in a study is critically important, especially in MP research, where the material complexity already reduces study comparability. While the number of studies using *Daphnia* as a model organism in MP research is steadily increasing, the clone has only been reported in a few cases (Brehm et al., 2023). More precise and transparent reporting of the clones used in various experiments could improve comparability between laboratories, leading to better risk assessments and, ultimately, a more trustworthy tool for policymakers.

5. Conclusion

This study compared the effects of MP from a PB-MP (PET) and two BB-MP (PBS and PLA) with those of the particle control cellulose on two different *D. magna* clones. Our results showed that the selected BB-MP (PBS and PLA) caused even more negative or similar effects on daphnids compared to the chosen PB-plastic (PET), depending on the endpoint or *Daphnia* clone considered. In contrast, cellulose particles exhibited neutral to positive effects. Further studies should incorporate more MP types and a wider range of concentrations to improve risk assessment, including environmentally relevant concentrations. Nevertheless, additional research on the impacts of BB-plastics is urgently needed before they can be regarded as an environmentally friendly alternative to PB-MP. The data discussed in this study, however, add to the growing amount of evidence that these polymers should not be regarded as such. This is especially important because the persistence of BB-MP in the environment has been found to be comparable to that of PB-plastics.

Our study also aimed to assess the sensitivity of two different *D. magna* clones. Despite this study being limited to only two separate clones, the results confirm that clonal differences play a significant role in determining the outcome of exposure studies. While MP-induced mortality did not differ between clones in our study, we found significant differences in sublethal responses. Accordingly, the genetic variability among different clones of *Daphnia* can influence the outcomes of toxicity studies, including those on MP, and should be investigated using molecular approaches in future investigations delving into this topic. Therefore, differences in clonal sensitivities should be considered when comparing the results of MP toxicity studies and understanding the impacts of MP in nature.

Another important aspect to consider in *D. magna* exposure studies is the inclusion of varying food levels to better reflect environmentally relevant conditions. While the present study was conducted under reduced food availability, future research should also examine particle exposure under different nutritional regimes. This would provide a more comprehensive understanding of how food availability modulates the biological responses to MP exposure.

CRediT authorship contribution statement

Christian Laforsch: Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Martin Wagner:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Martin G. J. Löder:** Writing – review & editing, Validation, Supervision, Project administration, Investigation. **Seema Agarwal:** Writing – review & editing, Resources, Project administration, Funding acquisition. **Dipannita Ghosh:** Writing – review & editing, Investigation. **Gabriël Olthof:** Writing – review & editing, Investigation, Conceptualization. **Marvin Kiene:** Writing – review & editing, Visualization, Formal analysis. **Michael Schwarzer:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Simona Mondellini:** Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Declaration of Competing Interest

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

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.119934.

Data availability

Data will be made available on request.

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Supplementary material pertaining to Article III

Same but different: The response to petroleum and biodegradable bio-based microplastics deviates between *Daphnia magna* clones

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(*contributed equally to this work)

Same but different: The response to petroleum and biodegradable bio-based microplastics deviates between *Daphnia magna* clones

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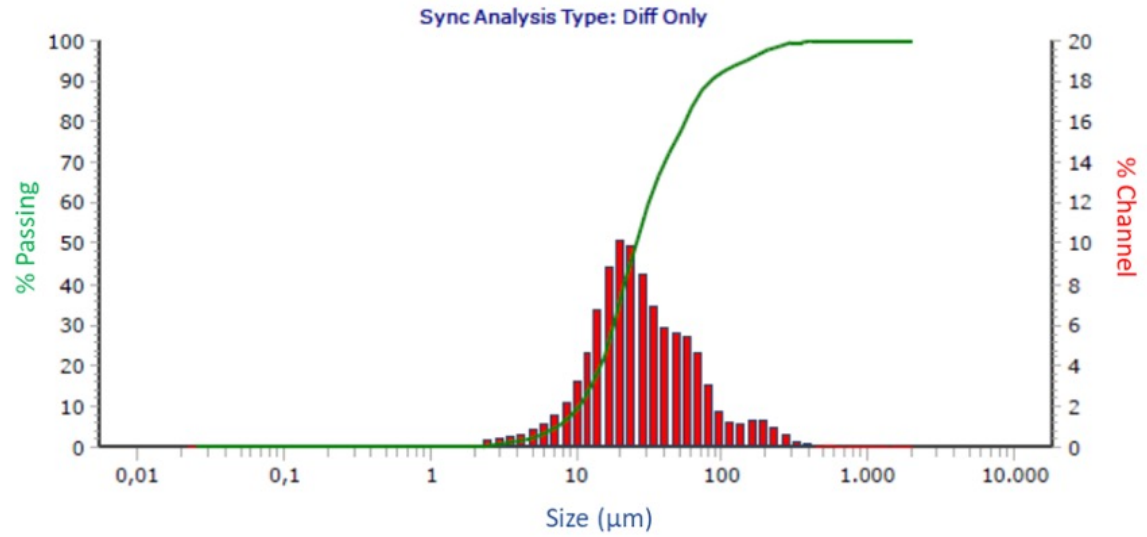
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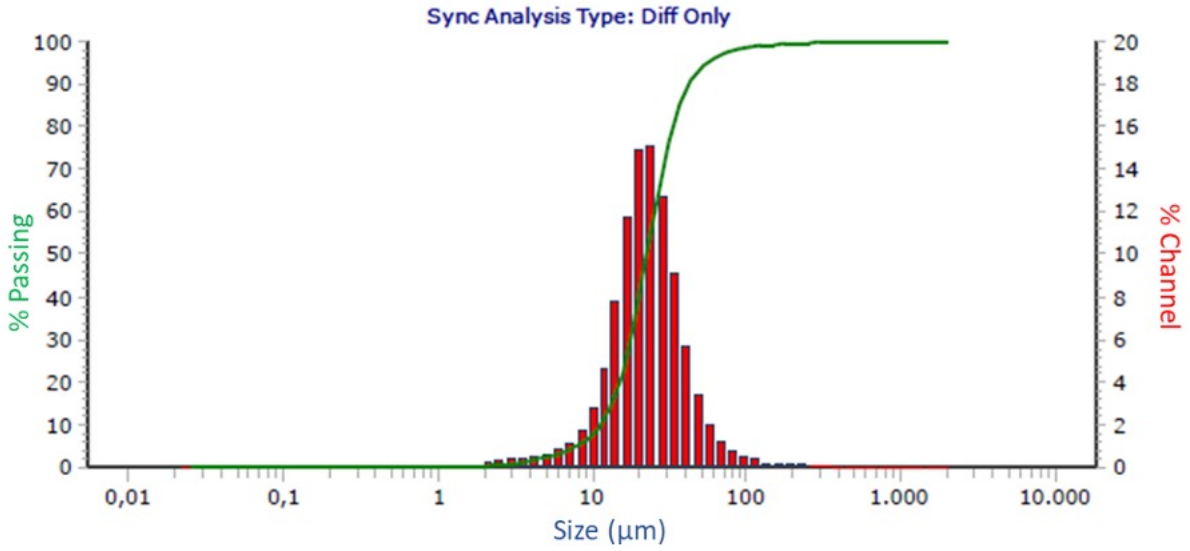
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Supplementary information

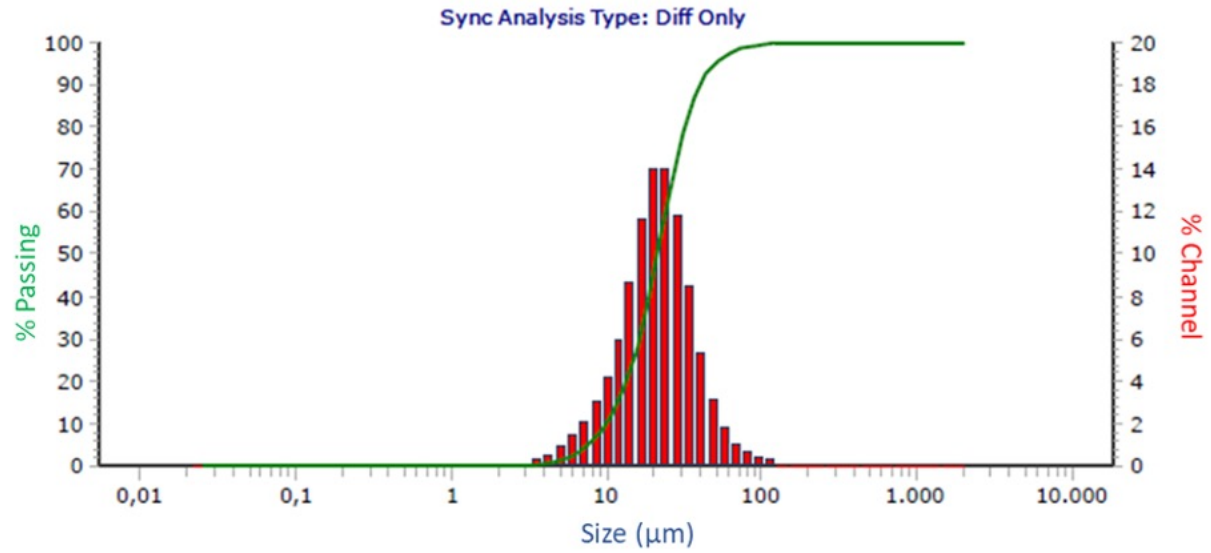
A



B



C



D

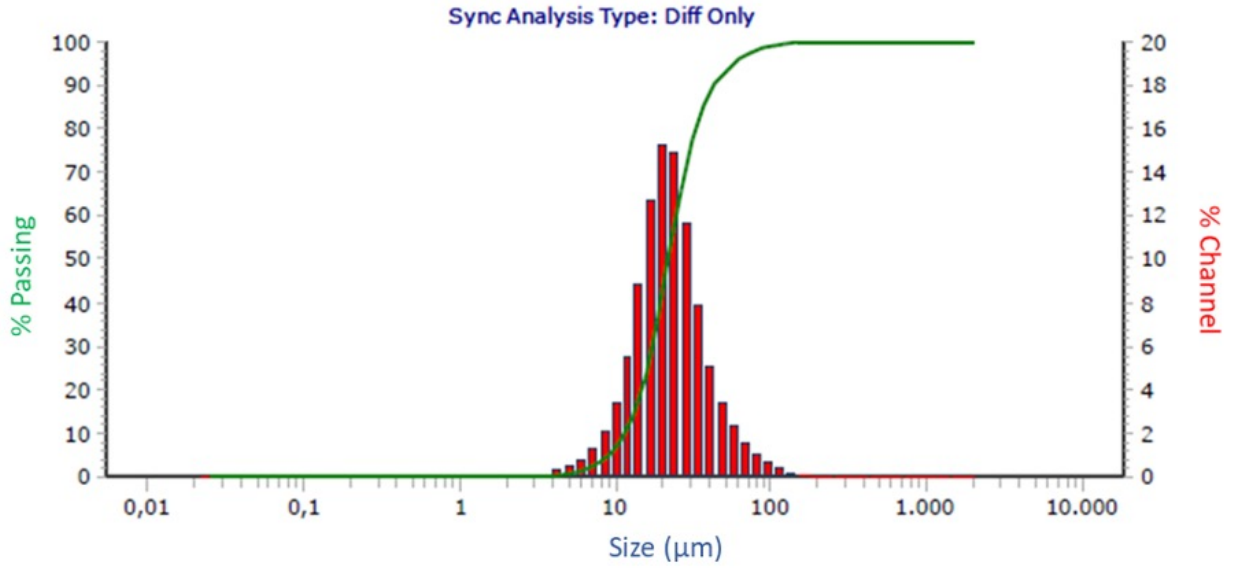


Figure SI 1 Size distribution of the employed particles. A) cellulose, d_{50}= 25.65 µm. B) PBS, d_{50}= 22.45 µm. C) PDLLA, d_{50}= 21.25 µm. D) PET, d_{50}= 21.82 µm. Measurements were conducted using LD (laser diffraction) and DIA (dynamic image analysis).

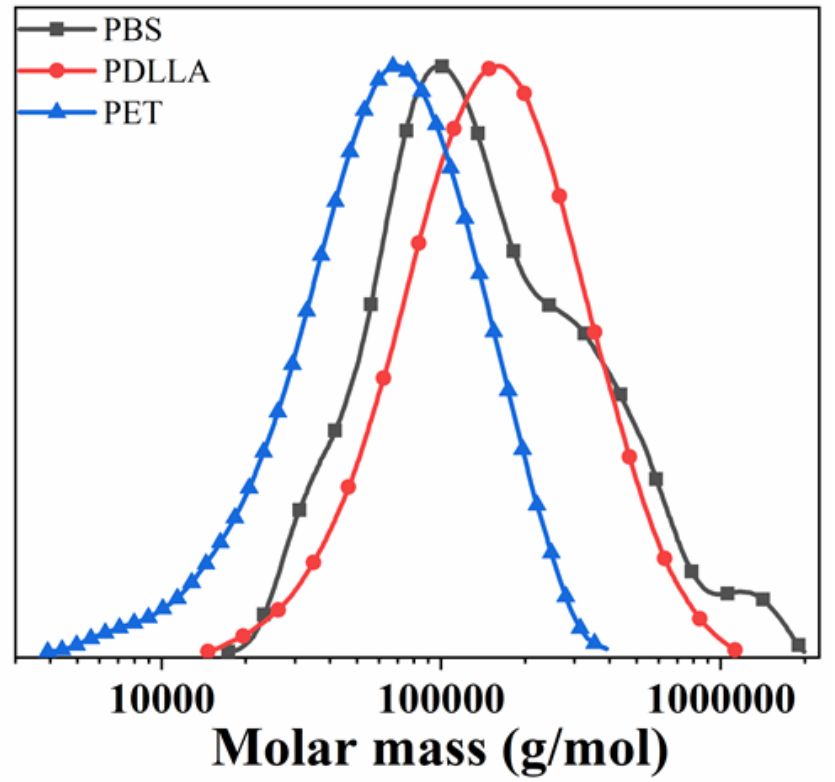


Figure SI 2: GPC of PBS, PLA in $CHCl_3$, and PET in HFTP.



Figure SI 3: Example measurement of body length of *D. magna* (Aig clone). Measured value: 2334 µm; scale bar = 500 µm.

Table SI 1: Number of dead individuals at the end of the 21-day exposure in every treatment

	Number of dead individuals	
Treatment	**Aig**	**BL2.2**
0 p mL^{-1}	1	0
Cellulose500 p mL^{-1}	0	0
Cellulose5000 p mL^{-1}	1	1
PBS 500 p mL^{-1}	2	10
PBS 5000 p mL^{-1}	14	11
PLA 500 p mL^{-1}	3	3
PLA 5000 p mL^{-1}	5	6
PET 500 p mL^{-1}	5	1
PET 5000 p mL^{-1}	10	10

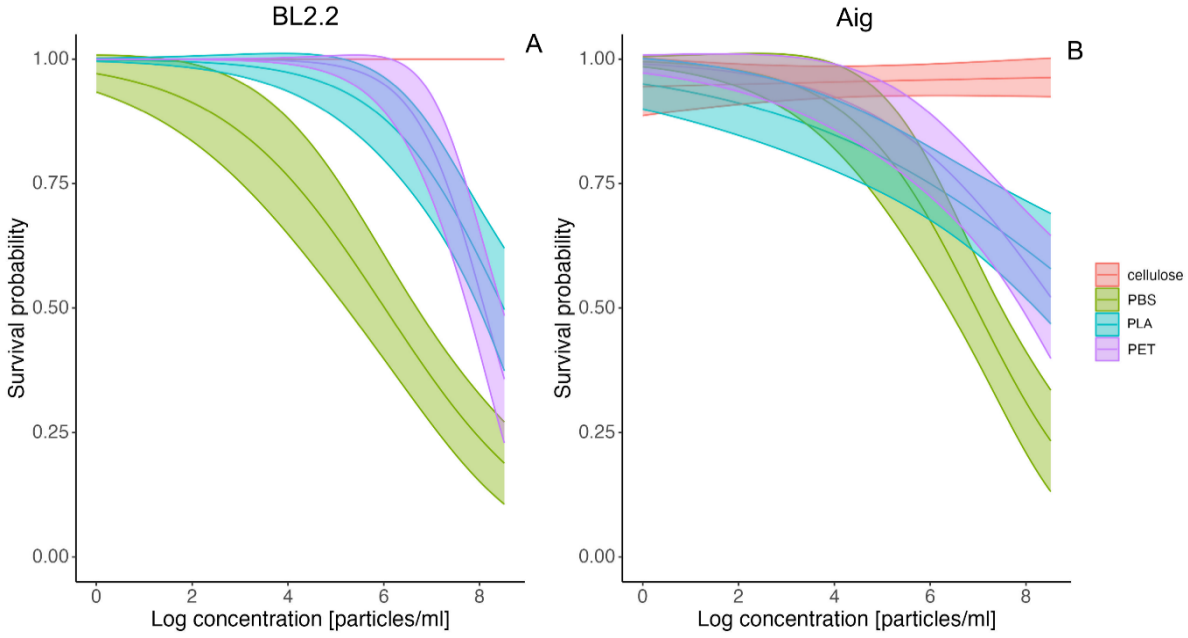


Figure SI 4: Survival probability of *D. magna* individuals of (A) the BL2.2 clone (for cellulose, no SD is shown as no mortality occurred) and (B) the Aig clone exposed to PET, PBS, and PLA MP or cellulose fragments. Data are based on a logistic regression model, with lines showing the mean model predictions and ribbons indicating their standard deviation (concentration on the x-axis is shown on a logarithmic scale). The blank control ("concentration 0", not shown in the graph, values in the text) is considered by the model as the baseline concentration for each treatment.

Table SI 2: mean value and standard deviation (SD) of the analyzed sublethal parameters divided by clone.

	BL 2.2		***Aig***	
Treatment	**Mean value**	**SD**	**Mean value**	**SD**
	Number of neonates			
0 p mL^{-1}	4.4	1.8	1.1	1.4
Cellulose500 p mL^{-1}	5.6	1.3	1.3	1.5
Cellulose5000 p mL^{-1}	5.2	2.5	2.3	1.5
PBS 500 p mL^{-1}	5.0	1.5	3.0	2.0
PBS 5000 p mL^{-1}	1.5	1.7	0	0
PLA 500 p mL^{-1}	5.3	2.9	1.5	1.7
PLA 5000 p mL^{-1}	3.0	2.4	1.6	1.3
PET 500 p mL^{-1}	3.7	1.9	1.6	1.7
PET 5000 p mL^{-1}	3.2	1.9	3.0	1.0
	Age of primiparity (d)			
0 p mL^{-1}	9.0	2.2	9.5	1.2
Cellulose500 p mL^{-1}	8.0	1.1	9.6	1.2
Cellulose5000 p mL^{-1}	9.1	0.9	10.2	0.9
PBS 500 p mL^{-1}	8.3	1.1	8.4	0.6
PBS 5000 p mL^{-1}	11.0	3.2	11.0	0
PLA 500 p mL^{-1}	9.1	0.4	10.4	0.8
PLA 5000 p mL^{-1}	10.1	0.7	12.6	3.2
PET 500 p mL^{-1}	9.4	0.9	10.1	1.4
PET 5000 p mL^{-1}	9.7	0.4	10.6	0.8
	Body length at primiparity (µm)			
0 p mL^{-1}	2420.3	94.6	2385.9	93.7
Cellulose500 p mL^{-1}	2430.6	81.0	2470.5	106.2
Cellulose5000 p mL^{-1}	2506.0	76.9	2446.5	129.8
PBS 500 p mL^{-1}	2472.9	118.3	2410.4	71.3
PBS 5000 p mL^{-1}	2422.6	156.5	2338.4	48.4
PLA 500 p mL^{-1}	2500.1	142.2	2509.9	112.8

PLA 5000 p mL^{-1}	2448.6	92.0	2326.1	68.7
PET 500 p mL^{-1}	2462.8	122.4	2520.2	128.9
PET 5000 p mL^{-1}	2425.8	187.7	2428.5	102.7
	Juvenile growth rate (µm d^{-1})			
0 p mL^{-1}	278.48	44.35	254.73	31.94
Cellulose500 p mL^{-1}	308.6	33.9	255.8	30.6
Cellulose5000 p mL^{-1}	275.8	28.1	235.3	13.3
PBS 500 p mL^{-1}	302.6	33.9	287.4	22.3
PBS 5000 p mL^{-1}	229.8	37.4	212.5	20.3
PLA 500 p mL^{-1}	273.6	20.3	241.0	20.3
PLA 5000 p mL^{-1}	242.1	21.2	242.1	20.4
PET 500 p mL^{-1}	262.7	29.1	255.2	41.4
PET 5000 p mL^{-1}	249.1	9.8	230.0	12.8
	Body length – 21d (µm)			
0 p mL^{-1}	2850.9	64.8	2728.4	64.8
Cellulose500 p mL^{-1}	2886.9	91.6	2750.9	108.7
Cellulose5000 p mL^{-1}	2853.4	59.7	2679.1	138.7
PBS 500 p mL^{-1}	2941.6	91.5	2718.7	44.3
PBS 5000 p mL^{-1}	2605.4	92.7	2454.9	NA
PLA 500 p mL^{-1}	2819.6	86.2	2759.1	105.2
PLA 5000 p mL^{-1}	2711.9	102.2	2496.6	105.9
PET 500 p mL^{-1}	2833.7	113.2	2748.1	66.4
PET 5000 p mL^{-1}	2694.6	100.8	2638.6	86.3

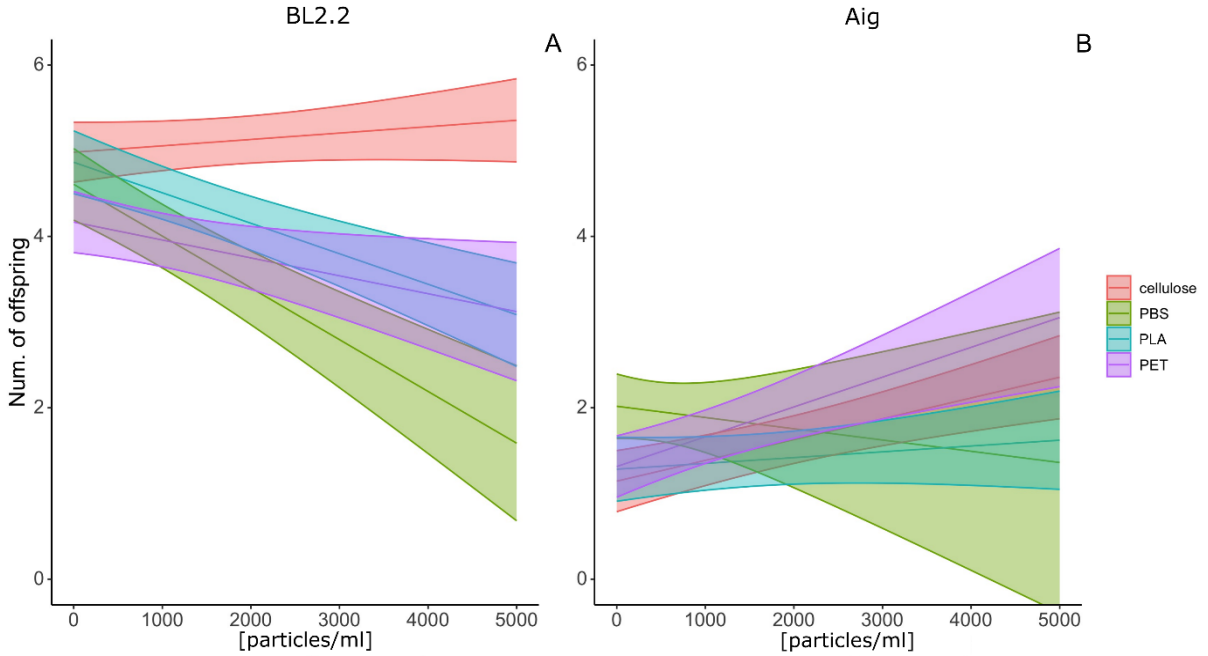


Figure SI 5: Number of neonates of *D. magna* individuals from (A) the BL2.2 clone and (B) the Aig clone hatched during chronic exposure to PET, PBS, and PLA MP or cellulose fragments. Data are based on a linear regression model, with the lines representing the mean model prediction and the ribbons indicating their standard deviation. The blank control ("concentration 0", not shown in the graph, values in the text) is considered by the model as the baseline concentration for each treatment.

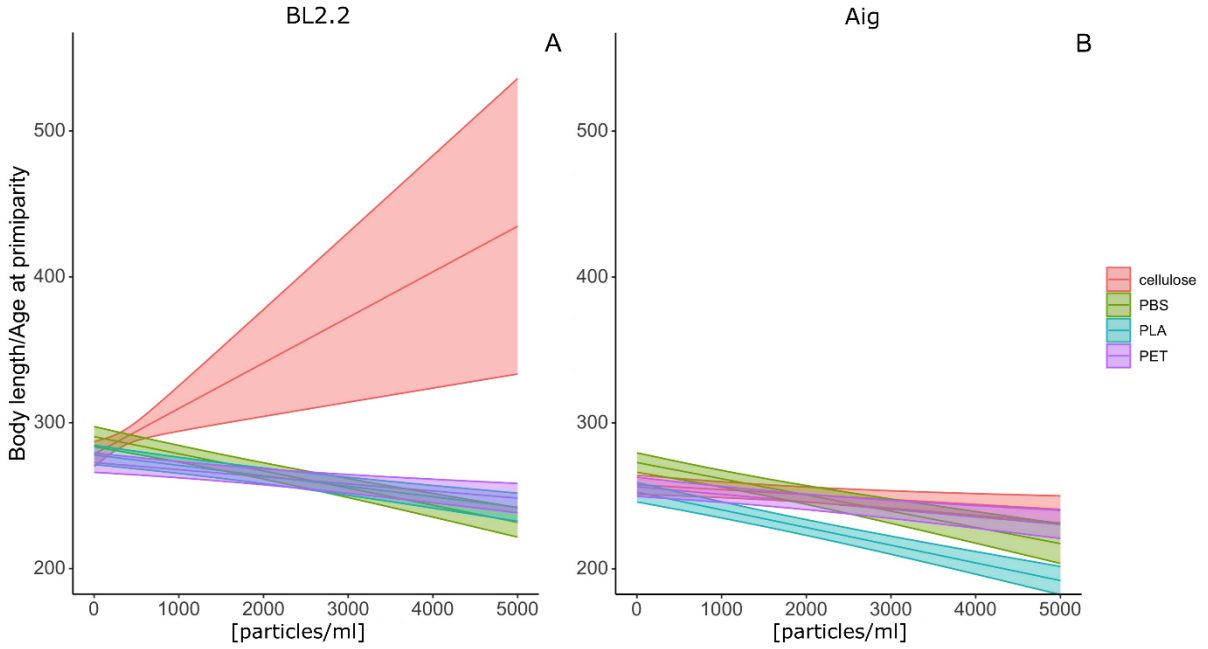


Figure SI 6: Juvenile growth rate (µm d^{-1}) of *D. magna* from (A) the BL2.2 clone and (B) the Aig clone exposed to PET, PBS, and PLA MP or cellulose fragments. Data are based on a

linear regression model, with the lines representing the mean model prediction and the ribbons indicating their standard deviation. The blank control ("concentration 0", not shown in the graph, values in the text) is considered by the model as the baseline concentration for each treatment.

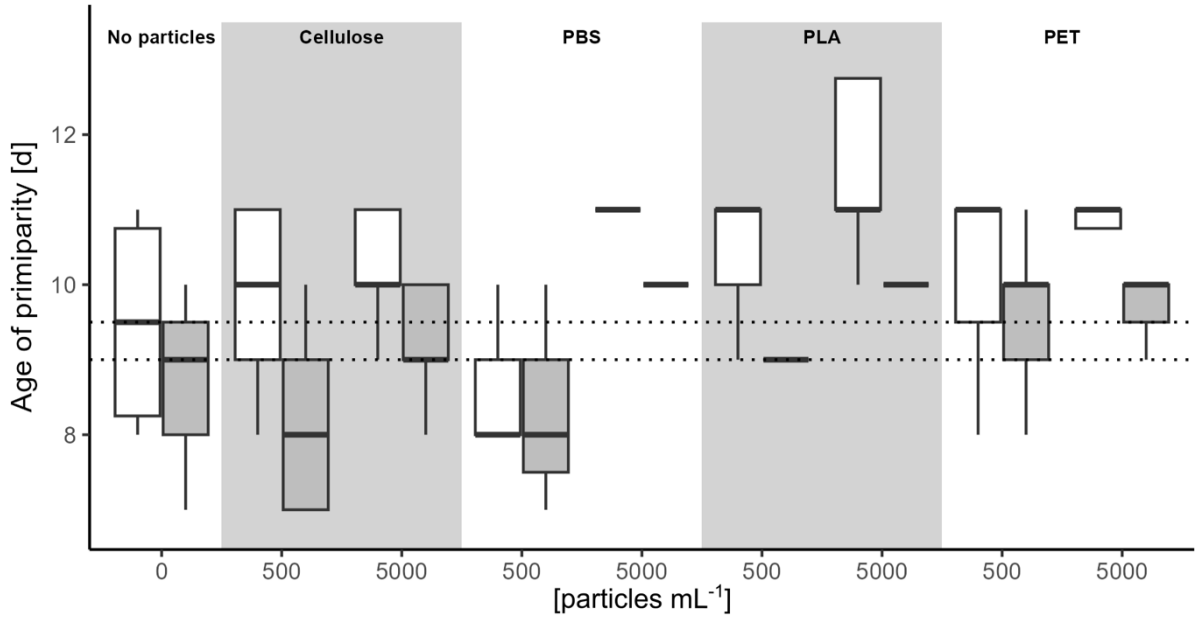


Figure SI 7: Age of primiparity of *D. magna* from the BL2.2 clone (grey boxes) and the Aig clone (white boxes) exposed to PET, PBS, and PLA MP or cellulose fragments. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. The whiskers represent the 1.5-fold of the reported quartiles. The dashed lines report the median value of the blank control (0 particles mL^{-1}).

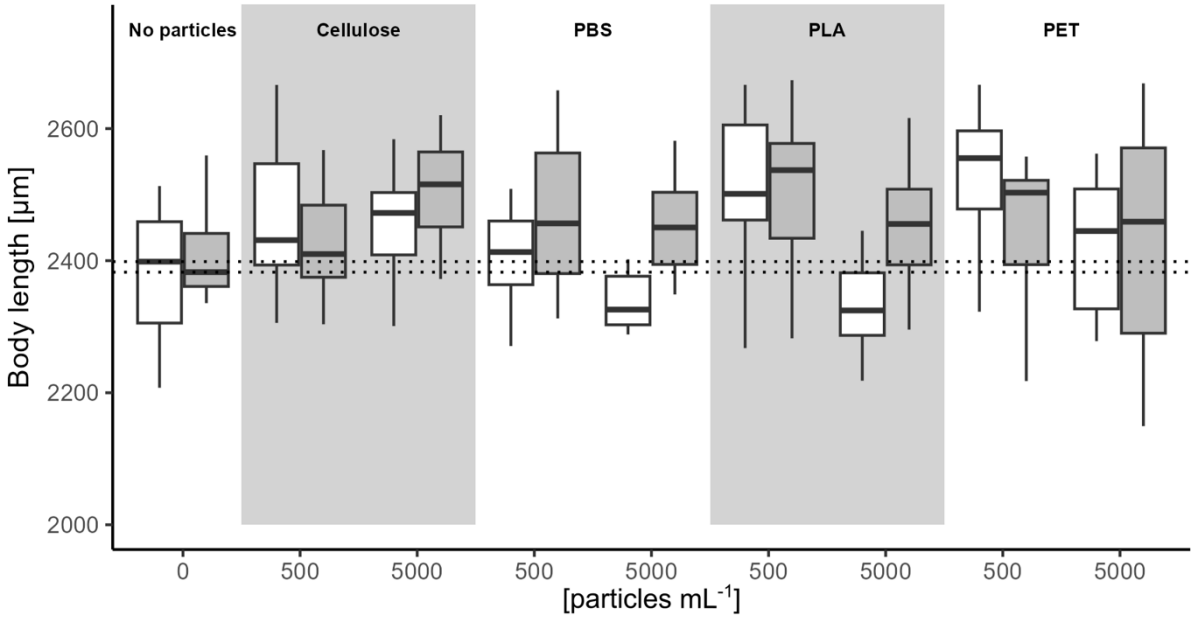


Figure SI 8: Body length at primiparity of *D. magna* from the BL2.2 clone (grey boxes) and the Aig clone (white boxes) exposed to PET, PBS, and PLA MP or cellulose fragments. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. The whiskers represent the 1.5-fold of the reported quartiles. The dashed lines report the median value of the blank control (0 particles mL^{-1}).

Table SI 3: Pairwise comparison of the treatments between both clones for age of primiparity and body length at primiparity of *D. magna* exposed to PBS, PLA, and PET MP or cellulose fragments. To indicate effect size, we present z-values for survival and t-values for the other traits. Bold values represent significance and a detectable effect ($p < 0.05$ and $D > 0.3$).

Aig vs BL2.2

Response	**Treatment**	**t/z value**	**P value**	**D**
Age of primiparity	cellulose	1.81	0.07	**1.33**
	PBS	0.46	0.64	0.11
	PLA	3.54	**<0.001**	**0.79**
	PET	1.82	0.06	**0.40**
Body length at primiparity	cellulose	-0.60	0.548	-0.13
	PBS	-2.02	**0.043**	**-0.49**
	PLA	-1.65	0.099	**-0.37**
	PET	0.467	0.641	0.10

Table SI 4: Pairwise comparison between the separate clones of the age of primiparity and body length at primiparity of *D. magna* exposed to PBS, PLA, and PET MP or cellulose fragments. To indicate effect size, we present z-values for survival and t-values for the other traits. Bold values represent significance and a detectable effect ($p < 0.05$ and $D > 0.3$).

		BL2.2			***Aig***		
Response	**Comparison**	**t/z value**	**P value**	**D**	**t/z value**	**P value**	**D**
Age of primiparity	Cellulose-PBS	-1.40	0.860	**-1.04**	0.79	0.856	0.18
	Cellulose-PLA	-1.40	0.860	**-1.08**	-2.43	0.076	**-0.54**
	Cellulose-PET	-1.40	0.860	**-1.08**	-0.69	0.856	-0.15
	PBS-PLA	-0.185	1.000	-0.04	-3.08	**0.013**	**-0.72**
	PBS-PET	-0.176	1.000	-0.04	-1.44	0.449	**-0.33**
	PLA-PET	0.008	1.000	0.001	1.74	0.328	**0.39**
Body length at primiparity	Cellulose-PBS	0.44	1.000	0.10	1.95	0.258	**0.46**
	Cellulose-PLA	-0.27	1.000	-0.06	0.80	0.842	0.17
	Cellulose-PET	0.48	1.000	0.10	-0.58	0.842	-0.12
	PBS-PLA	-0.70	1.000	-0.16	-1.18	0.711	-0.28
	PBS-PET	0.02	1.000	0.006	-2.48	0.081	**-0.58**
	PLA-PET	0.75	1.000	0.16	-1.37	0.680	**-0.30**

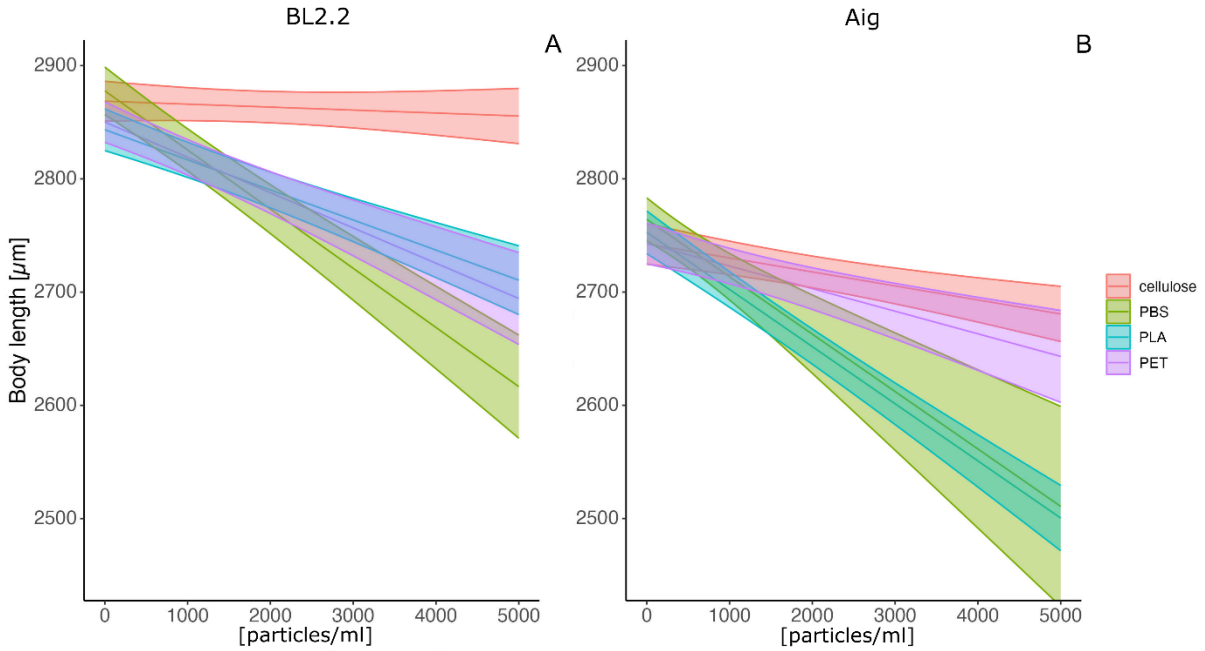


Figure SI 9: Body length at the end of the chronic exposure of *D. magna* individuals from (A) the BL2.2 clone and (B) the Aig clone exposed to PET, PBS, and PLA MP or cellulose fragments. Data are based on a linear regression model, with the lines representing the mean model prediction and the ribbons indicating their standard deviation. The blank control ("concentration 0", not shown in the graph, values in the text) is considered by the model as the baseline concentration for each treatment.

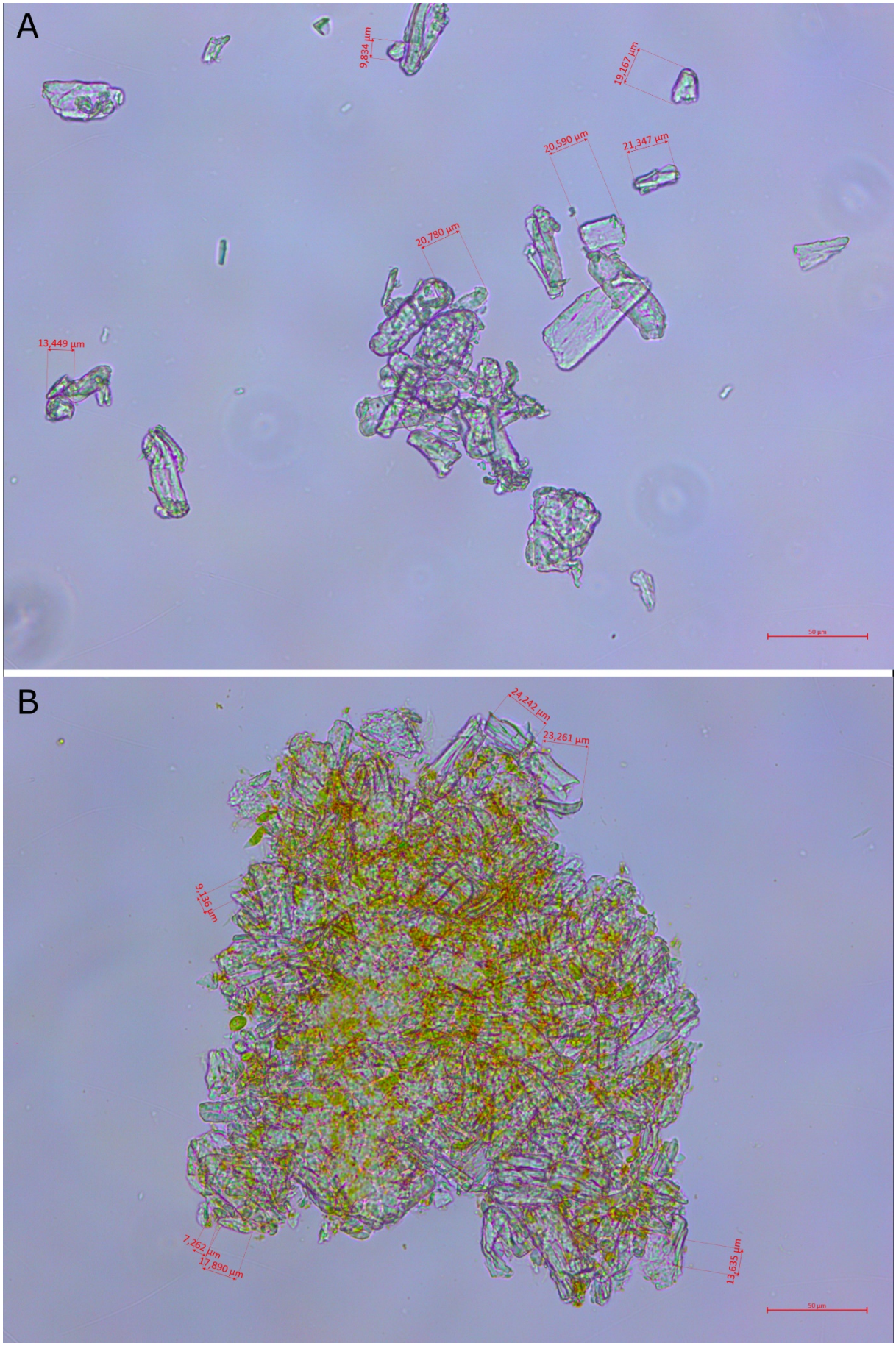

A
9,834 µm
19,167 µm
20,590 µm
21,347 µm
20,780 µm
13,449 µm
50 µm
B
24,242 µm
23,261 µm
9,136 µm
7,262 µm
17,890 µm
13,635 µm
50 µm

Figure SI 10: Microscopic images of cellulose particles (A) before (pristine) and (B) after gut passage through a *D. magna* individual. In (B), the fecal pellet is covered with digested algae remnants, but cellulose particles are still clearly visible in the same size range as the pristine ones. Images were captured with an optical microscope (Zeiss Axio Lab.A1, Carl Zeiss Microscopy GmbH, Jena, Germany), and size measurements were made using ZEN 2 core v2.5 / ZEN 2 starter v2.5 (Carl Zeiss Microscopy GmbH, Jena, Germany). Fecal pellets were collected immediately after release by the daphnid. Scale bars = 50 µ.

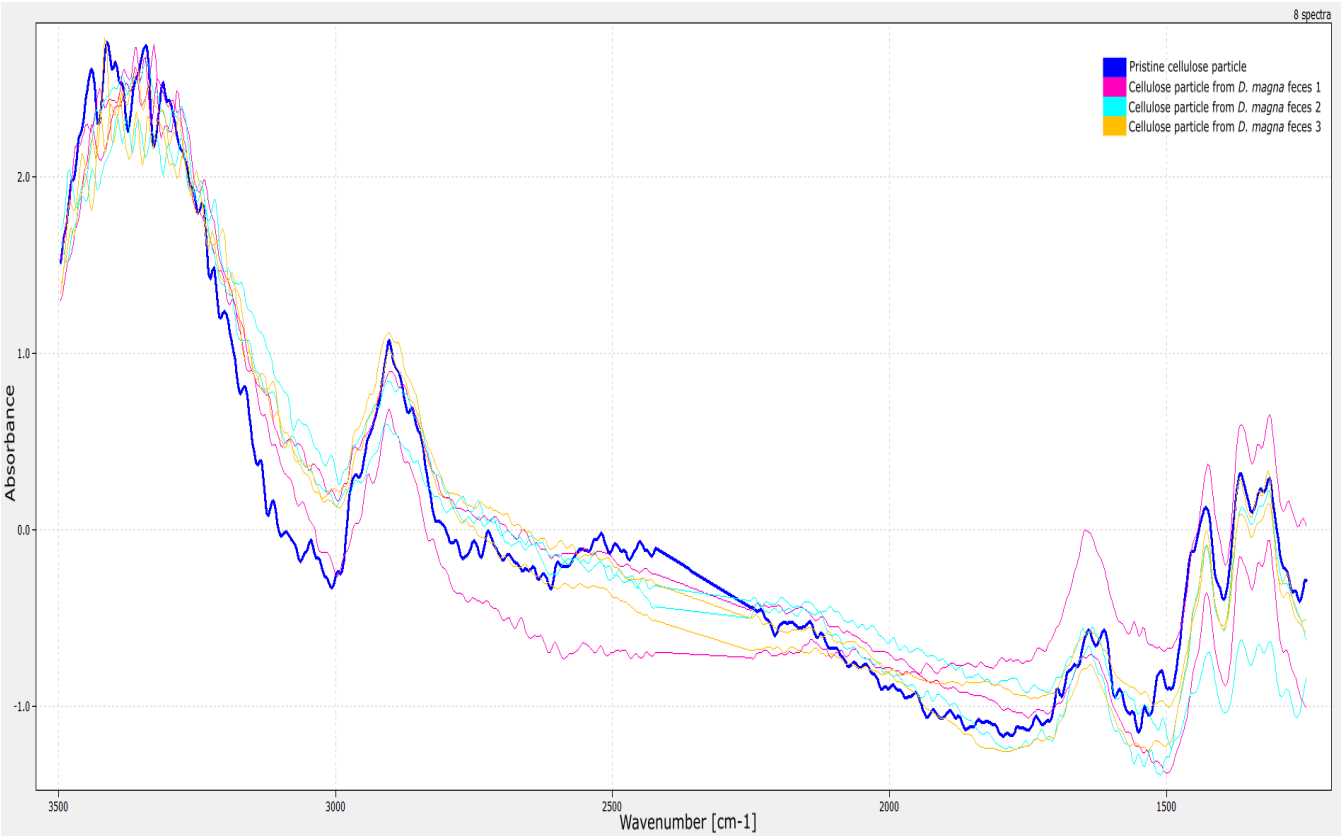


Figure SI 11: Spectra of pristine (blue) cellulose and cellulose particles after gut passage of *D. magna* (other colors). Fecal pellets were collected immediately after release by the individual daphnid and transferred onto an Anodisc 25 (0.2 µm pore size, 25 mm diameter; Product number: 6809-6022, Whatman, Germany), then dried at room temperature inside a petri dish overnight. Spectra were measured using a Lumos II FT-IR microscope (Bruker Corporation, USA). After a qualitative assessment, no missing or additional peaks were detected.

Analyses of clone-specific responses

The two clones may respond differently to the applied treatments. However, they also have an inherent difference; for example, clone BL2.2 is generally larger than the clone Aig, even under controlled conditions. To separate the inherent clone difference from the varying response to the treatment, we further centered the values of all individuals for each considered sublethal parameter (juvenile body length growth rate, body length, number of neonates) to the respective concentration 0. This approach removes the inherent clonal variation from the analyses. Consequently, significant treatment effects in the respective analyses of centered values indicate effects that occur despite inherent clonal differences. Similarly, significant effects or interactions involving the clone predictor suggest a distinct response pattern between the clones, regardless of their inherent differences.

Analyses of clone-specific responses - Results

The two *D. magna* clones showed intrinsic clonal variation in sublethal responses, with the clone BL2.2 being generally larger at the end of the experiment ($F_{(3,263)} = 5.86$, $p < 0.001$, Figure SI 12A and SI 13). For instance, the control mean length was 2850.9 ± 64.8 µm for BL2.2 and 2728.4 ± 108.7 µm for Aig. Generally, the BL2.2 clone also had a higher reproductive output compared to the Aig clone ($F_{(3,263)} = 172.5$, $p < 0.001$) with a reproduction at concentration 0 of 4.4 ± 1.8 neonates (BL2.2 clone) vs. 1.14 ± 1.4 neonates (Aig clone). Centering the responses of the two clones to their respective concentration 0 allowed the analysis and the comparison of the response pattern of the clones despite the intrinsic clonal differences (Figure SI 12, Table SI 5). Additionally, it allowed the identification of clone-specific differences in the response pattern.

Regarding reproductive output, the clone BL2.2 produced more neonates than the Aig clone, depending on the interaction of the polymer treatment ($F_{(3,263)} = 2.9$, $p = 0.032$) and particle concentration ($F_{(3,263)} = 9.1$, $p = 0.002$). Additionally, the response to the MP treatment was also dependent on the interaction with particle concentration ($F_{(3,263)} = 3.5$, $p = 0.015$).

The body length at the end of the exposure was reduced in both clones in the MP treatments ($F_{(3,263)} = 5.8$, $p < 0.001$) and the difference in body length increased with increased MP concentration ($F_{(3,263)} = 77.4$, $p < 0.001$), dependent on the plastic type, i.e., a significant interaction was detected ($F_{(3,263)} = 11.1$, $p < 0.001$) (Figure SI 12). However, there was no clonal difference in body length or the interactions with the other parameters (see Table 1). The juvenile body length growth rate was similarly affected by the treatment ($F_{(3,263)} = 3.7$, $p = 0.011$), the particle concentration ($F_{(3,263)} = 64.5$, $p < 0.001$), and by their interaction ($F_{(3,263)} = 4.1$, $p = 0.006$). The clonal difference, however, remained significant ($F_{(3,299)} = 41.5$, $p < 0.001$), but not its interactions with the other considered parameters (see Table SI 5).

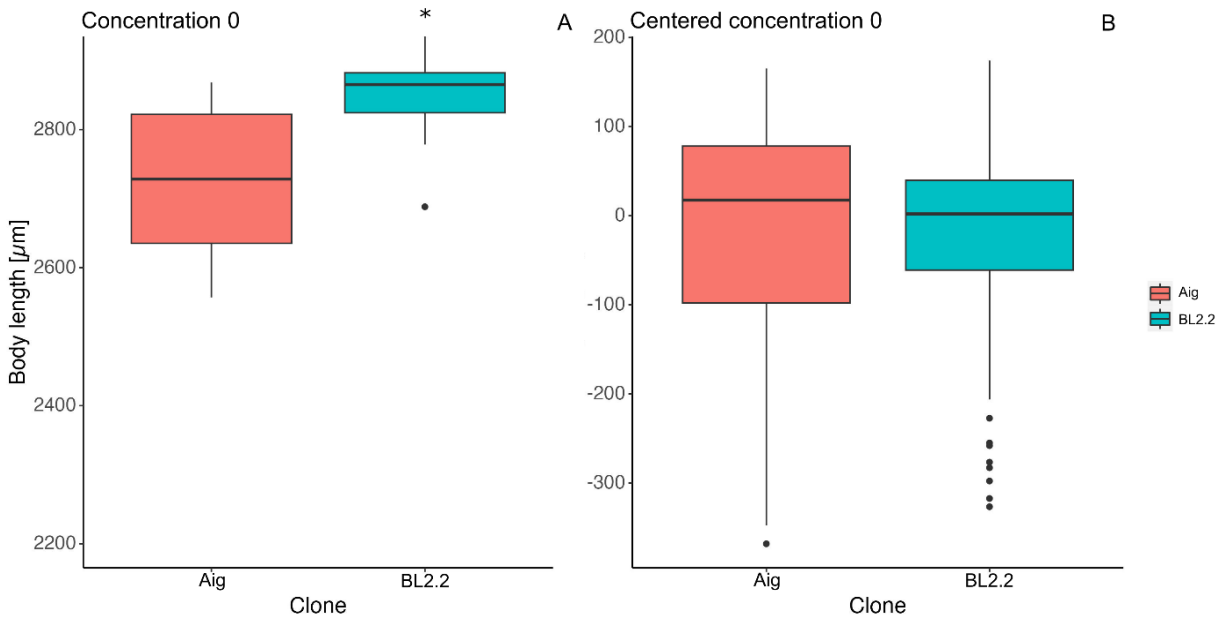


Figure SI 12: Body length of the 0 particles mL^{-1} concentration of both *D. magna* clones (A) before ($F_{(1,27)} = 13.80$, $p = 0.0009$) and (B) after being centered. Centering removes inherent clonal variation to focus on responses and clone-specific response patterns upon exposure to the treatment.

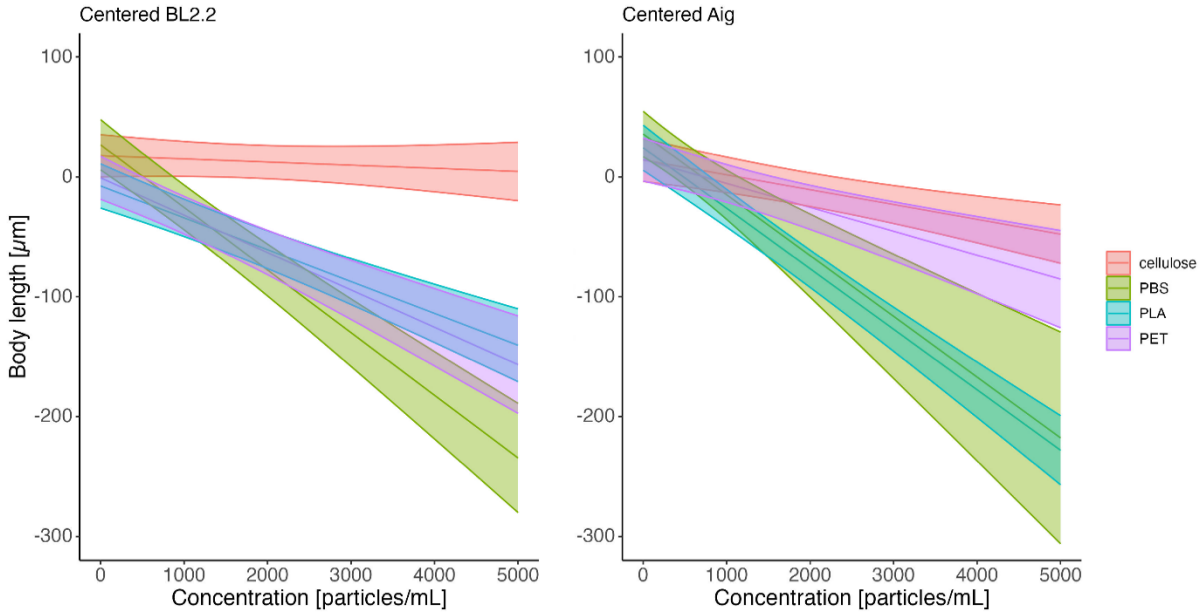


Figure SI 13: Body length of A) BL2.2 and B) Aig centered to the respective concentration 0 in order to overcome the effects of intraclonal variation.

Table SI 5: Parameters from the linear regression analysis of *D. magna*'s life history traits during the chronic exposure obtained using the values that were centered to the respective concentration 0. Bold values represent significance ($p < 0.05$).

Responses	***Fixed effects***	***DF***	***χ^2/F value***	***P value***
Num. of neonates	Treatment	3	1.4	0.226
	Concentration	1	0.05	0.810
	Clone	1	3.1	0.079
	Treatment:concentration	3	3.5	**0.015**
	Treatment:clone	3	2.9	**0.032**
	Concentration:clone	1	9.1	**0.002**
	Treatment:concentration:clone	3	0.6	0.589
Juvenile body length growth rate	Treatment	3	3.7	**0.011**
	Concentration	1	64.5	< **0.001**
	Clone	1	42.5	**<0.001**
	Treatment:concentration	3	4.1	**0.006**
	Treatment:clone	3	1.6	0.186
	Concentration:clone	1	0.9	0.322
	Treatment:concentration:clone	3	1.2	0.302
Body length 21 d	Treatment	3	5.8	**<0.001**
	Concentration	1	77.4	**<0.001**
	Clone	1	0.003	0.954
	Treatment:concentration	3	11.1	**<0.001**
	Treatment:clone	3	0.5	0.636
	Concentration:clone	1	2.6	0.102
	Treatment:concentration:clone	3	1.5	0.192

Article IV

Extracted chemicals from microplastics exert increased adverse effects on *Daphnia magna* compared to the pristine particles

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(*contributed equally to this work)

Extracted chemicals from microplastics exert increased adverse effects on *Daphnia magna* compared to the pristine particles

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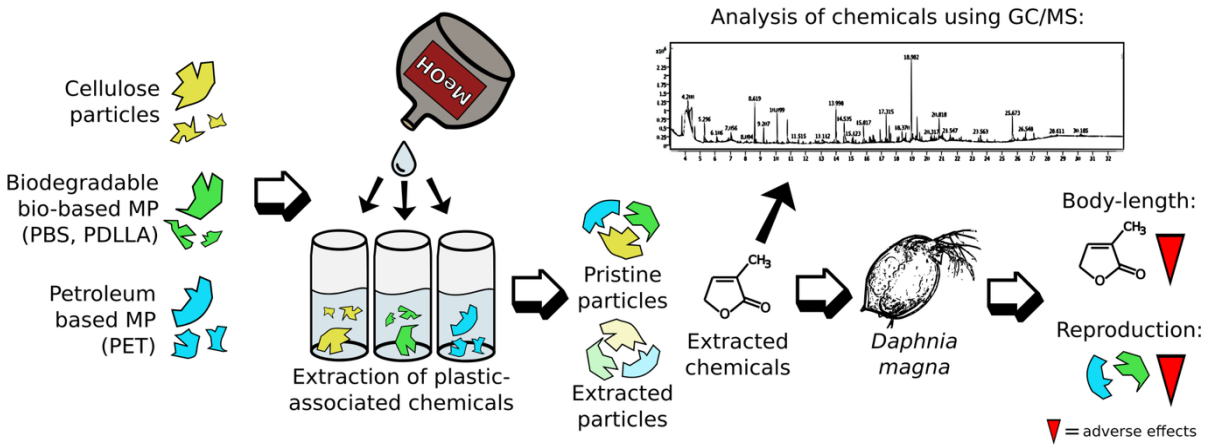
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Abstract
Microplastics (MP) are ubiquitous environmental contaminants with diverse physicochemical characteristics. Size, shape, and polymer type have already been found responsible for MP toxicity, but this also seems to differ among MP from the same plastic type. Plastic-associated chemicals, a broad class of compounds intentionally or unintentionally added to plastics during their production and manufacturing, may be co-responsible for these differences among studies. Therefore, to elucidate this aspect of MP toxicity, we exposed *Daphnia magna* individuals to pristine MP, extracted MP (eMP), and methanol-based extracts for 10 days. Our results indicate that, depending on the polymer and the endpoint considered, associated chemicals may be crucial in determining plastic toxicity. Body length in particular was significantly impaired by PET and PDLLA extracts, while reproduction is affected by all selected treatments. The experiment was conducted with reduced food availability to investigate plastic effects in an environmentally relevant scenario. The introduction of a control, fed *ad libitum,* suggests that a more realistic feeding regime might exacerbate plastic effects of the selected treatments.

Keywords: microplastics, extracts, *Daphnia magna,* food limitation, plastic-associated chemicals

Graphical Abstract



1. Introduction

Plastics are a diverse class of contaminants, characterized by complex chemical compositions. They are composed not only of one or more polymers but also of several associated chemicals. These chemicals can be intentionally added to enhance functionality, such as additives (e.g., fillers, flame retardants, pigments, or plasticizers) or non-intentionally added, including impurities, degradation products, or residual chemical compounds from the manufacturing process (Wagner et al. 2025). Most associated chemicals remain largely unknown, even though approximately 16,000 have been identified so far (Wagner et al. 2025, Monclús et al. 2025). Currently, producers are not required to disclose the list of chemicals used in the production phase of plastics (Beiras et al. 2021). However, some chemicals used in plastic manufacturing (e.g., HBCDs, PBDEs, polyphenols, or DEHP) are known to be toxic and are regulated under the Water Framework Directive (2000/60/EC) as priority substances. They have also been included in the Environmental Quality Standard (2008/105/EC; 2013/39/EU) for surface waters (Gunaalan, Fabbri, and Capolupo 2020). Associated chemicals are often not covalently bound to the plastic material (Zimmermann et al. 2019), increasing the likelihood that they leach from the material throughout its entire life cycle (Giametti and Finelli 2022). Given the ubiquity of plastic waste in all environmental compartments, and particularly of microplastics (MP, 1-1000 μm ISO 24187:2023), MP inevitably interact with the biota, causing adverse effects depending on shape, size, and polymer type (Schwarzer et al. 2022). However, adverse effects caused by associated chemicals from MP may be more severe, but only a few studies have considered them among the drivers of MP toxicity (e.g., Beiras et al. 2021; Giametti and Finelli 2022; Brehm et al. 2023). Plastic-associated chemicals can vary with the properties required for different plastic applications (Capolupo et al. 2023). Hence, materials made of the same polymers do not necessarily have the same chemical composition. This high diversity in chemical composition

could therefore be one factor responsible for the many inconsistent outcomes in MP ecotoxicology studies (e.g., De Felice et al. 2019, Trotter et al. 2021, Sørensen et al. 2023).

To shed light on the mechanisms behind MP toxicity, we compared the effects of pristine MP, extracted MP (eMP, i.e., particles depleted of extractable chemicals following solvent-based extraction), and MP extracts using *Daphnia magna* as a model organism. *D. magna* was selected for this experiment as it represents a keystone species for lentic ecosystems. Freshwater environments are reported to be among the most impacted by MP pollution (Global Plastic Outlook, OECD 2022). Given the widespread occurrence of MP in freshwater environments, it is important to assess the effects of both MP particles and their chemicals on freshwater organisms.

This study was carried out using irregular fragments (< 20 µm) of one petroleum-based (PB-) plastic: poly(ethylene terephthalate) (PET); and two biodegradable and bio-based (BB-): poly(butylene succinate) (PBS), and poly(D-lactic acid) (PDLLA, PLA). The MP effects were compared to those of one naturally occurring polymer in the same size range: cellulose. Both PB- and BB-plastics, as well as cellulose, underwent the same preparation steps to produce eMP and extracts. The inclusion of both PB- and BB-plastics was intended to determine whether the latter exhibit lower toxicity or effects similar to those of PET shown in earlier studies (e.g., An et al. 2025, Serra et al. 2025, Oscar et al. 2025, Mondellini et al. 2026). The inclusion of BB-MP in ecotoxicological research is currently of the utmost importance. As the market demand for sustainable "environmentally friendly" alternatives to conventional plastics continues to rise (Shruti et al. 2019), along with their production rates and environmental release, it is essential to assess the potential threat these polymers pose to the biota. Additionally, to achieve comparable physical properties to the conventional plastics they are intended replace, these so-called bioplastics require a similar, or even greater, amount of associated chemicals throughout all stages of production (Capolupo et al. 2023; Savva et al.

2023b). The plethora of chemicals present in both BB- and PB-plastics might contribute substantially to their overall hazard. Once these associated chemicals leach from the particles into the surrounding water, they enter the aqueous phase, potentially increasing their mobility, bioavailability, and subsequent bioaccumulation in biota compared to exposure through ingestion of MP containing the same compounds (Jang et al. 2021). Consequently, they may exert stronger biological effects (e.g., Schrank et al. 2019, Gunaalan et al. 2020, Capolupo et al. 2023). Given their properties and design, BB-plastics should degrade under specific sets of conditions (Tong et al. 2022). However, such conditions are often not met in natural environments where they disperse following intentional or improper disposal, resulting in persistence times comparable to those of PB-MP. Experimental evidence indicates that, over comparable timeframes, BB-plastics generate greater quantities of smaller MP than PB-items (Tong et al. 2022). These smaller MP are characterized by a higher surface-to-volume ratio, which can promote faster leaching of associated chemicals (Frazzetto et al. 2022, Allan et al. 2022).

While these characteristics may increase the potential hazard posed by BB-MPs, the magnitude of their biological effects is likely to depend on the environmental context in which exposure occurs. In this regard, most ecotoxicological studies on *D. magna*, conducted according to OECD guidelines (i.e., OECD No. 211), provide food *ad libitum* (2 mg C L^{-1}). However, such conditions do not accurately represent natural environments, where food availability is typically limited and subject to seasonal fluctuations (Giebelhausen and Lampert, 2001). Therefore, to better reflect environmentally relevant conditions, this study was conducted under reduced food availability (0.5 mg C L^{-1}), selecting a concentration that still supports survival and reproduction in *Daphnia* (Lampert 1978; Schür et al. 2020). Previous studies have shown that MP effects are more severe under food-restricted regimens compared to standard toxicity test conditions (Silva et al. 2022a, Lyu et al. 2022, Mondellini

et al. 2026). Consequently, standard conditions might underestimate the hazard posed by MP and their associated chemicals to the biota in a natural setting by mitigating their potential effects (Silva et al 2022a, Ghosh et al. 2025). Therefore, this study seeks to investigate the drivers of MP toxicity by comparing the effects of MP, eMP, and extracts and by incorporating reduced food availability as an environmentally relevant stressor, with the objective of generating results that more accurately reflect conditions under a realistic feeding regime.

Accordingly, the aim of this study is threefold: (i) to compare the effects of MP, eMP, and their extracts in order to identify the main component driving MP toxicity; (ii) to investigate the potential differences between the effects induced by PB- and BB-MP and those of their associated chemicals; and (iii) to analyze the effects of MP under reduced food availability as an additional environmentally relevant stressor.

2. Materials and Methods

2.1 *Daphnia magna* cultivation

The individuals of *D. magna* used in this experiment came from a well-established in-house husbandry. The chosen clone was BL2.2, which originated from a pond (Oud Meren) in Leuven (Belgium) and has been kept in husbandry since 1997 (Imhof et al. 2017).

For the experiment, animals were randomly selected from the third brood of age-synchronized mothers that were held in 1.5 L glass beakers (Weck GmbH u. Co. KG, Wehr-Öflingen, Germany).

All animals were maintained in M4 artificial medium (Elendt and Bias 1990) with the addition of 4.48 µg/L of selenium dioxide (SeO_2) and kept at 20±0.5 °C with a photoperiod of 14h light : 9h dark with 30 minutes of dusk and dawn. The *D. magna* culture was fed *ad libitum* with the green algae *Acutodesmus obliquus.* The mothers were separated from their offspring 24 h before the start of the experiment to prevent further hatching and to ensure the use of similarly aged daphnids for the experimental procedure.

2.2 Particle suspension and extraction of plastic-associated chemicals

In this study, we employed three different plastic types (fragments < 20 µm) and fragments from cellulose (microcrystalline powder 20 µm, CAS: 9004-34-6, Sigma Aldrich Chemie GmbH, Steinheim, Germany) as a particle control. As plastic treatments, fragments of two BB- and one PB- plastics were employed: poly (butylene succinate) (PBS), poly (D- lactic acid) (PDLLA, PLA), and poly (ethylene terephthalate) (PET). Plastic fragments were produced in-house by milling (Ultra-Centrifugal Mill ZM 200, Retsch, Haan, Germany) and sieving (<20 µm, ALPINE Air Jet sieve e200 LS, FRITSCH, Idar-Oberstein, Germany). The particle size distribution was determined via a combination of LD (laser diffraction) and DIA

(dynamic image analysis) (FLOWSYNC, Microtrac MRB, Montgomeryville and York, USA).

The extracts were prepared via solvent-based extraction (Zimmermann et al. 2020) by incubating the respective amount of MP (determined from their densities in order to achieve an exposure concentration of 500 MP mL^{-1}) in 50 mL of methanol (LiChrosolv Methanol for liquid chromatography, CAS-No: 67-56-1, Supelco, Merk KGaA, Darmstadt, Germany), replicated three times. After 45 minutes of incubation in an ultrasonic bath, the glass vials containing the incubated particles were left to settle for 15 minutes. 100 mL of the supernatant was then removed, filtered through a 0.2 µm syringe filter (Filtropur S, PES membrane, Sarstedt AG & Co. KG, Nümbrecht, Germany), and divided into two new 50 mL glass vials. The methanol was then removed with a gentle flow of N_2 for approximately 6 hours by evaporation (Reacti-Therm III #TS-18824, Thermo Scientific, Waltham, Massachusetts, USA) until only 1 mL of each particle extract remained. The final methanol evaporation was performed in 1.5 mL glass vials (Caliper Turbo Vap LV evaporator, Marschall Scientific, Hampton, USA) and was stopped before complete evaporation. We then added 1 mL DMSO (CAS: 67-68-5, Merk KGaA, Darmstadt, Germany) and completed the evaporation of the methanol. Methanol and DMSO have different boiling points (64.7°C and 189°C respectively), meaning that methanol is highly volatile and is therefore removed from the solution at room temperature without any DMSO evaporation. These extracts contained all chemicals extractable in methanol, representing a worst-case scenario relative to what might be leached in a freshwater system, as this type of extraction is reported to release most of the plastic chemicals (Zimmermann et al. 2020). The particles used to create the extracts (i.e., the extracted particles that should not contain other methanol-extractable compounds) were left to dry in the glass vials used for the methanol incubation and then retrieved to be used as extracted MP (eMP) for the exposure experiments. A procedural blank was included.

Before the start of the experiment, particle suspensions were prepared by adding 100 mg of each particle to 100 mL of previously autoclaved M4 medium. Prior to use, stock suspensions were shaken for 48 hours at 100 rpm. A haemocytometer (Neubauer Improved, Brand GmbH & Co. KG., Wertheim, Germany) was used to determine particle concentration in the stock suspensions.

2.3 Particle characterisation

2.3.1 Gas Chromatography/Mass Spectrometry for Extracts Characterization

To avoid masking the signals with DMSO, GC–MS analysis was performed on methanol extracts obtained as described above, with minor modifications, scaling down the methanol volumes to one-fifth of those previously used. Briefly, the particles were incubated in glass vials containing 10 mL of methanol (LiChrosolv Methanol for liquid chromatography, CAS No. 67-56-1, Supelco, Merck KGaA, Darmstadt, Germany), in triplicate. After 45 minutes of incubation in an ultrasonic bath, the glass vials were allowed to settle for 15 minutes. Subsequently, 6.6 mL of the supernatant from each vial was collected and combined in a glass beaker to obtain a final volume of 20 mL. The 20 mL extract was filtered through a 0.2 µm syringe filter (Filtropur S, PES membrane, Sarstedt AG & Co. KG, Nümbrecht, Germany) and then divided into two new 10 mL glass vials. Methanol was removed by gentle evaporation under a nitrogen (N_2) stream (Thermo SCIENTIFIC REACTI-VAP I #TS-18825 Evaporation Unit, Thermo Fisher Scientific Inc., Darmstadt, Germany) for approximately 6 hours, until a final volume of 250 µL was reached.

Half a microlitre of each Methanol extract was injected by an AOC20i+s autosampler (Shimadzu, Kyoto, Japan) splitless into the GC-MS (GC2030 gas chromatograph connected to a QP2020NX mass spectrometer; both Shimadzu). The GC oven was fitted with a low-polar phase; cross-linked diphenyldimethylpolysiloxane capillary column (Rtx-5MS; length = 30 m, inner diameter = 0.25 mm, film thickness = 0.25 µm; Bad Homburg v. d. Höhe,

Germany) and the oven temperature was increased from 70 °C to 280 °C at a rate of 5 °C/min, then held for 7 minutes. Helium was used as the carrier gas at a linear velocity of 50 cm/s. Mass spectrometry was measured in TIC mode in a mass range between 35 and 550 m/z. Blank measurements have been performed before and after each measurement to verify the absence of carryover. A reference mixture of C7-C33 n-alkanes (Sigma-Aldrich, St. Louis, USA) was used for calculating the Kovats index of the unknown substances.

2.3.2 Nuclear magnetic resonance

Both MP and eMP, but not the extracts, were analysed with 1H (300 MHz) Nuclear magnetic resonance (NMR). Spectra were recorded on an Ultrashield-300 spectrometer (Bruker) in CDCl3. The residual peak of the undeuterated component in CDCl3 was used for calibration.

2.3.3 Scanning electron microscopy analyses

The morphology and surface characteristics of both pristine and extracted particles were analysed at a scanning electron microscope (SEM, JEOL JSM-IT500 Scanning Electron Microscope, JEOL Ltd., Japan). Particles were fixed to aluminum stubs (∅12mm, Plano GmbH, Ernst-Befort-Straße 12, Wetzlar, Germany) on conductive carbon pads (G3347, Plano GmbH) and were coated with 2 nm C and 2 nm Pt (EM ACE600, Leica Microsystems GmbH, Wetzlar, Germany).

2.4 10-day *Daphnia magna* exposure

The exposure to 500 particles mL^{-1}, or the equivalent of extracts, was carried out for 10 days. The animals were exposed in 900 mL glass beakers (Weck GmbH u. Co. KG, Wehr-Öflingen, Germany) filled with 500 mL of modified M4 medium, each containing 10 daphnids. Every treatment was replicated 5 times. The exposed animals were fed every second day with 0.5 mg C L^{-1} of the green algae *Acutodesmus obliquus.* In addition to the MP, eMP, and extracts, we included a blank control, a solvent control (DMSO, 0.002%), and a food-level control, in which the organisms were fed *ad libitum* (2 mg C L^{-1}). After 6 days of exposure, we acquired

pictures of five animals/replicate using a dissecting microscope equipped with a digital camera (M50 Leica; camera: DP26, Olympus, Hamburg, Germany; Light: KL 300 LED, Leica) to analyse morphological parameters (cellSens Dimension, v1.11, Olympus). The number of offspring produced at the first clutch was also recorded to assess reproduction alterations.

2.5 Biochemical analyses

Reactive oxygen species (ROS) levels were analysed as described in Mondellini et al. (2022). In short, at the end of the 10-day exposure, organisms of each treatment were pooled and homogenized in 1 mL of potassium phosphate buffer (100 mM, pH = 7.4, with 100 mM KCl, 1 mM EDTA, 1 mM Dithiothreitol (DTT, CAS: 3483-12-3, Sigma Aldrich, Missouri, USA) and protease inhibitors 1/100 v/v (cOmplete™ ULTRA Tablets, Mini, *EASYpack* Protease Inhibitor Cocktail, 05892970001**,** Merck KGaA, Darmstadt, Germany), an aliquot of 250 µL of the homogenate was separated before centrifugation for lipid peroxidation (LPO) analyses. The remainder of the homogenate was centrifuged at 15000g for 30 min at 4°C, and the supernatant was used for biochemical analyses, replicated twice. Protein quantification was performed prior to analysis using the Bradford (1976) method, and the resulting protein concentration was used for data normalisation. ROS levels were quantified by assessing the fluorescence change ($\lambda_{ex} = 595$ nm, $\lambda_{em} = 530$ nm) of 2'7' diclhorodihydrofluorescine diacetate (2'7'DCFH-DA) (Sigma-Aldrich, St. Louis, Missouri, USA) determined by the presence of pro-oxidative molecules (Deng et al. 2009). The concentration of ROS was normalized to the protein content and expressed as arbitrary units AU DCF mg proteins^{-1}. LPO was assessed according to Ohkawa et al. (1979) by quantifying thiobarbituric acid reactive substances (TBARS). All the biochemical analyses were conducted on a microplate reader (Thermoscientific Varioskan LUX 3020-82516) at λ = 595 nm for protein

quantification, λ_{ex} = 485 nm and λ_{em} =530 nm for ROS quantification, and λ = 535 nm for LPO analyses.

2.6 Statistical analyses

All statistical analyses were conducted using R (version 4.2.2, R Core Team 2022). First, we assessed whether the particle control (cellulose), the solvent (DMSO), or the food level affected the experimental animals. Therefore, we applied linear regression models with a Gaussian distribution (Poisson distribution for offspring numbers) with the respective controls as treatment levels and the beaker as a random intercept to account for the nested design. Then, we employed pairwise comparisons after Tukey HSD with Holm adjustment for multiple comparisons, using the emmeans package (Lenth et al. 2020). We did not find significant differences between the controls, except for the higher food concentration and the number of neonates in the DMSO-treated animals. Thus, we decided to proceed with the statistical analyses using the particle control (cellulose) as the most adequate control group.

To differentiate between the effects of plastic particles and plastic additives and compare their effects across different plastic types, we applied Gaussian-distributed linear regression models (Poisson distribution for offspring numbers). We used the particle type (i.e., cellulose, PBS, PDLLA, PET), particle occurrence (i.e., yes/no), and additive occurrence (i.e., yes/no) as fixed terms with a three-way interaction and the beaker as a random intercept to account for the nested design. For instance, PBS particles containing their additives have been declared as plastic type = PBS; particle occurrence = yes; additive occurrence = yes. In contrast, the extract of PBS particles (without the particles) was declared as plastic type = PBS; particle occurrence = no; additive occurrence = yes; and so on. Therefore, the ANOVA results were directly and easily interpretable, indicating whether particles and/or extracts affected the responses of the experimental animals. To compare results across plastic types, we used a Tukey HSD post hoc test with Holm-adjustment for multiple comparisons (Lenth et al. 2020).

All models have been validated by checking the residuals for normal distribution and homogeneity of variance. For the body length of the animals, we observed a violation of the homogeneity of the variance. Therefore, we applied a variance structure (varIdent) for the extract term using the package nlme (v. 3.1-160; Pinheiro et al. 2022). Plots were created with the package ggplot2 (Wickham et al. 2016). The significance level was set to 0.05.

3. Results

3.1 Particle characterisation

SEM imaging of the particles before and after the extraction process did not reveal any visible changes in shape, size, or surface roughness in all particles except for PDLLA eMP. In case of PDLLA the eMP appeared to be smoother than the original MP (Figure 1, 2, 3 & 4, Figure SI 1). The selected particles were also analysed using NMR. The NMR spectra of the polymers did not show any alteration after undergoing the solvent-based extraction protocol (Figure SI 2). GC-MS analyses revealed 19 peaks: 4 for PET, 10 for PBS, 4 for PDLLA, and 1 for cellulose. Of these peaks, only 4 were uniquely identified via their literature Kovats indices (Kováts 1958). For details, see Table 1.

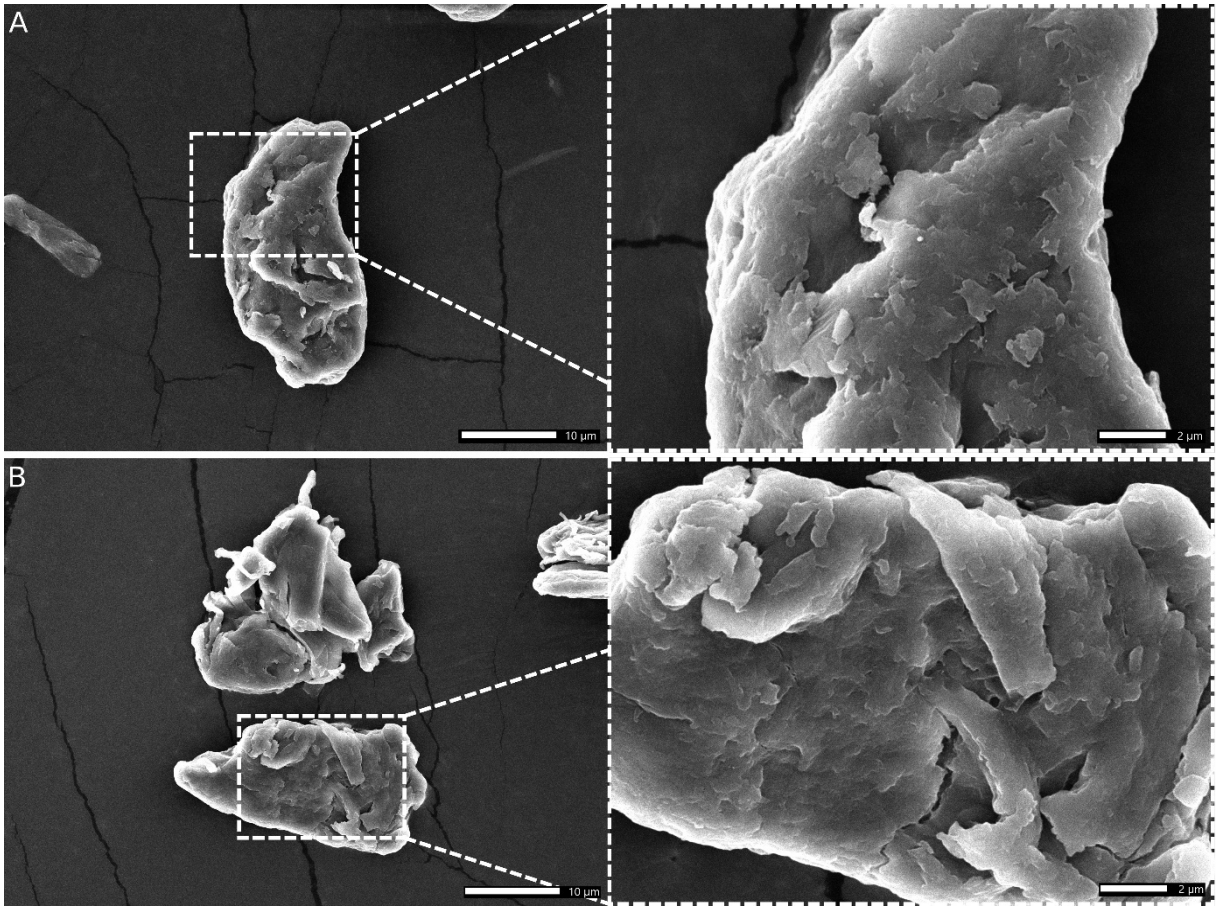


Figure 1: SEM images of cellulose particles. (A) pristine cellulose. (B) eCellulose. Left: Overview, scale bar = 10 µm; Right: Detail, scale bar = 2 µm.

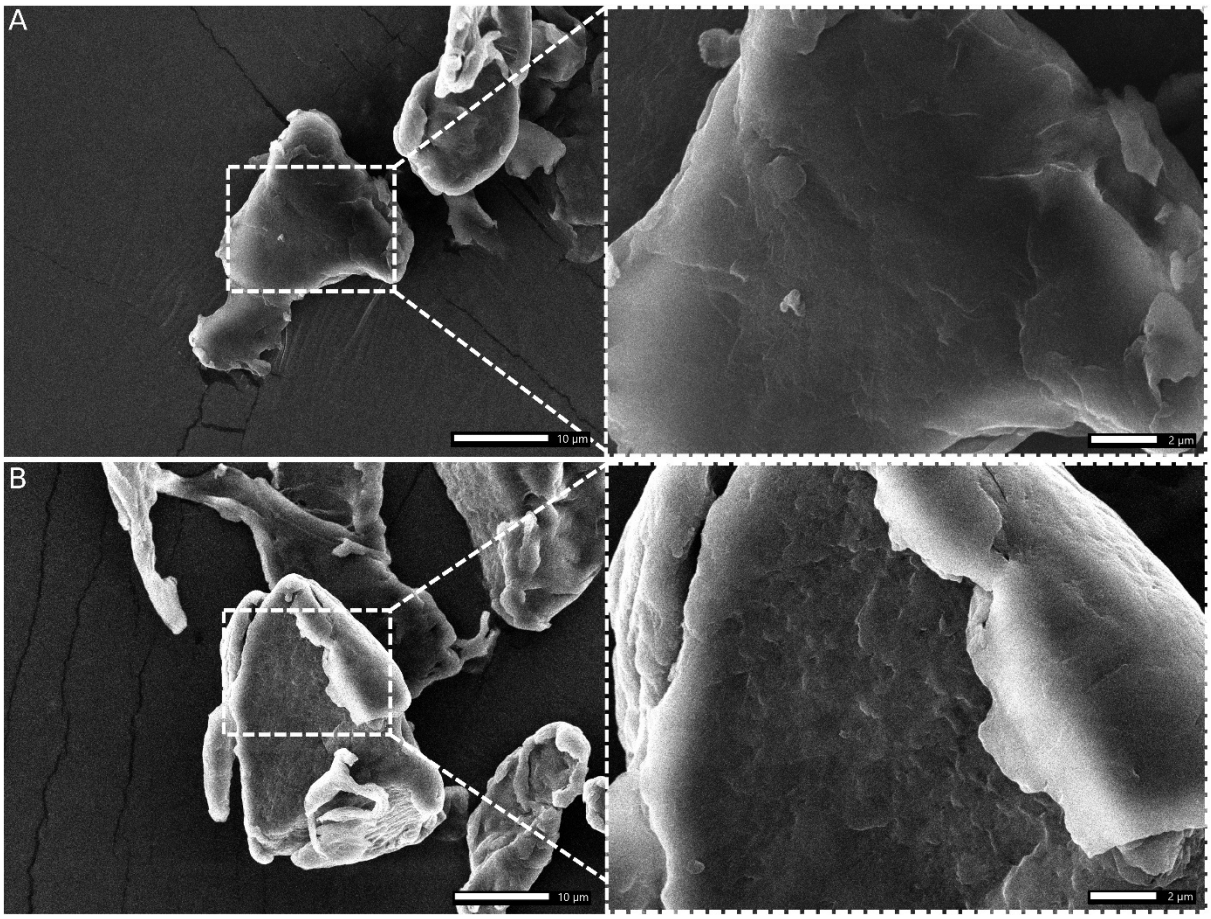


Figure 2: SEM images of PET particles. (A) pristine PET. (B) ePET. Left: Overview, scale bar = 10 µm; Right: Detail, scale bar = 2 µm.

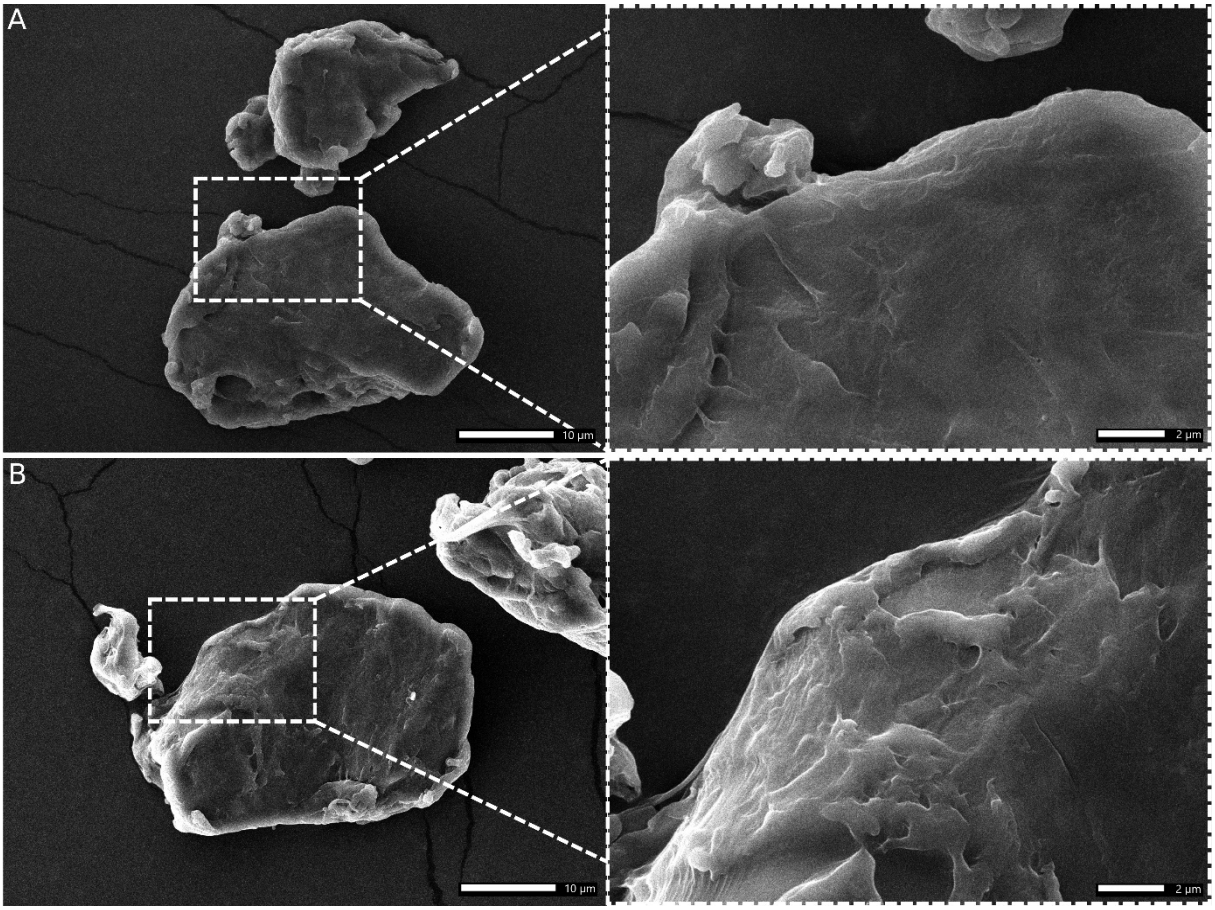


Figure 3: SEM images of PBS particles. (A) pristine PBS. (B) ePBS. Left: Overview, scale bar = 10 µm; Right: Detail, scale bar = 2 µm.

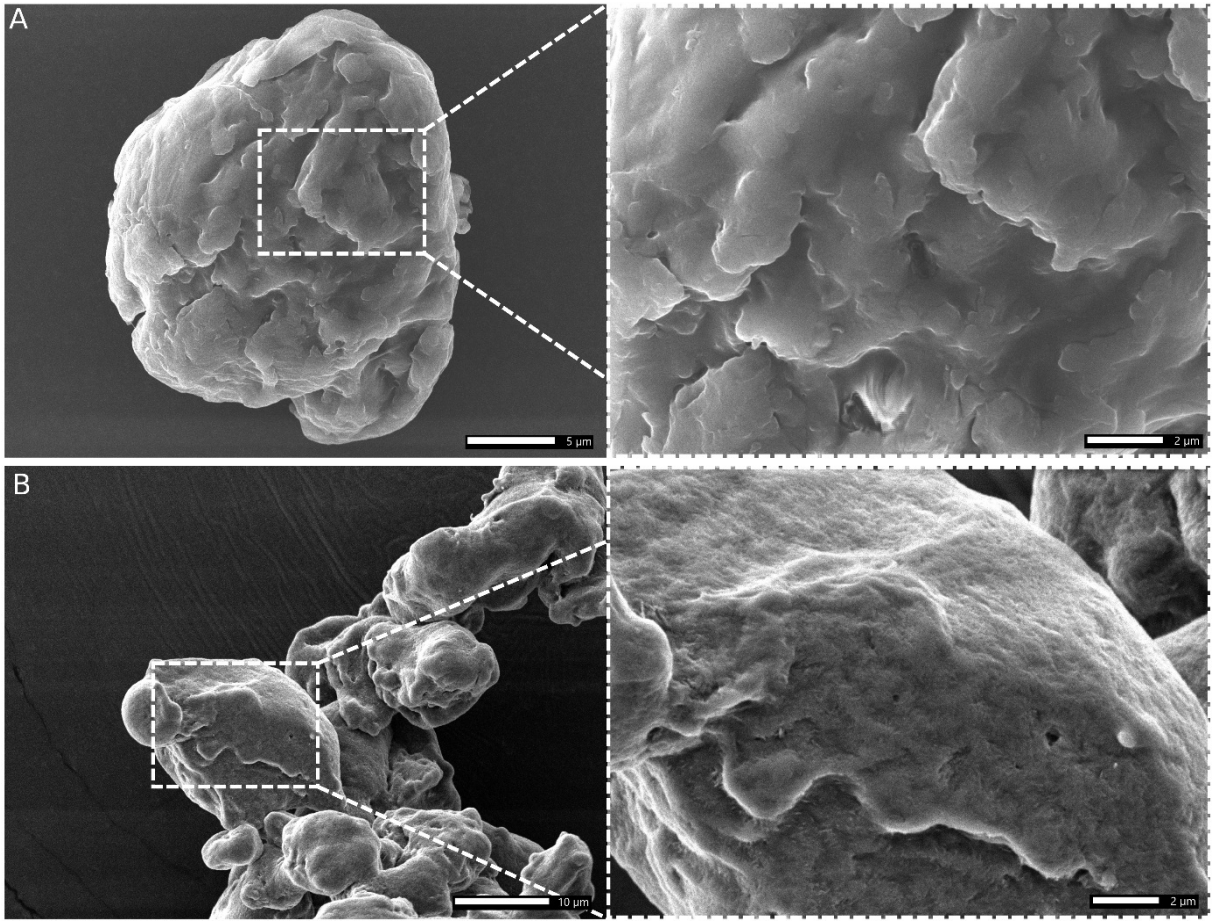


Figure 4: SEM images of PDLLA particles. (A) pristine PDLLA, scale bar = 5 µm. (B) ePDLLA, scale bar = 10 µm. Left: Overview; Right: Detail, scale bar = 2 µm.

Table 1: Results of the GC/MS analyses conducted on the MP extracts. AC: associated chemical, RT: retention time, m/z: mass to charge ratio of base peak, Calculated Kovats: calculated Kovats index of measured peak (Kováts 1958). S/N: signal-to-noise ratio.

Polymer	***AC***	***CAS***	***RT***	***S/N***	***m/z***	***Calculated Kovats***	***Literature Kovats***
PET	Thiodiglycol	111-48-8	9.979	167.72	61.00	1184,73	1190.1
	Peak 1	NA	14.757	109.63	163.00	1507,34	1550.4
	Docosane	629-97-0	21.897	15.28	57.00	2198,55	2200
	Peak 2	NA	26.109	3.95	55.00	2731,44	NA

PBS	Peak 3	NA	4.728	505.45	42.00	947,17	NA
	Butanedioic acid, dimethyl ester	123-25-1	6.986	341.49	115.00	1042,44	1035.5
	Thiodiglycol	111-48-8	9.970	98.88	61.00	1184,37	1190.1
	Peak 4	NA	12.649	129.10	101.00	1322,50	NA
	Peak 5	NA	15.510	204.13	115.00	1570,83	1705
	Peak 6	NA	21.190	238.26	73.00	2120	NA
	Peak 7	NA	21.749	132.37	115.00	2124,35	NA
	Peak 8	NA	22.276	9.74	43.00	2243,05	NA
	Peak 9	NA	24.976	397.33	55.00	2578,70	NA
	Peak 10	NA	26.105	139.63	115.00	2730,92	NA
PDLLA	2(5H)-Furanone, 3-methyl-	22122-36-7	5.700	10.50	41.00	988,18	982
	Peak 11	NA	9.005	740.25	45.00	1134,11	NA
	Peak 12	NA	9.161	378.11	56.00	1142,23	NA
	Peak 13	NA	14.403	96.91	45.00	1480,53	NA
Cellulose	Peak 14	NA	25.443	9.73	43.00	2641,50	NA

3.2 Control comparison

The comparison of the body length at day 6 of the separate controls (Figure 5) showed that animals from the food level control were significantly larger than animals from the other control treatments (cellulose-food control $t = -4.687$, $p < 0.0001$; blank-food control $t = -$

5.381, p < 0.0001; DMSO-food control t = -7.008, p < 0.0001). The other control treatments did not differ significantly from one another.

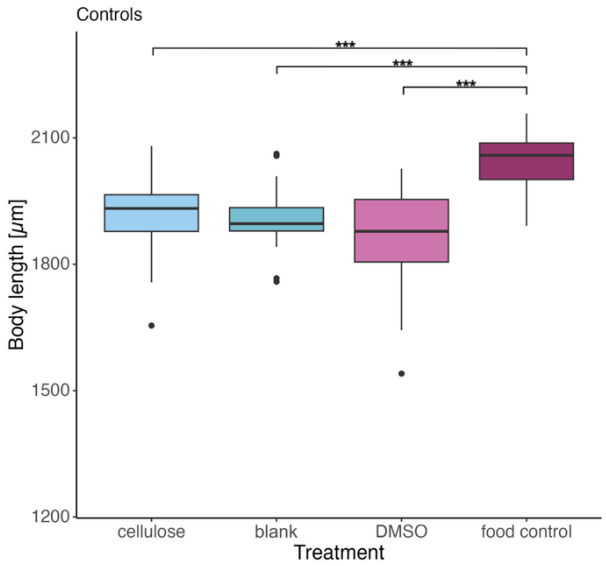


Figure 5: D. magna body length control treatments after 6 days of exposure. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$). N= 25 animals per treatment.*

The comparison of the reproductive success in the first clutch (Figure 6) showed that animals from the food control had a higher reproductive output compared to the ones from the other control treatments (cellulose-food control t = -6.689, p < 0.0001; blank-food control t = -5.482, p < 0.0001; DMSO-food control t = -4.614, p < 0.0001). Animals from the DMSO control also had higher reproductive output than those from the cellulose treatment (p = 0.01). The remaining control treatments did not differ significantly from one another.

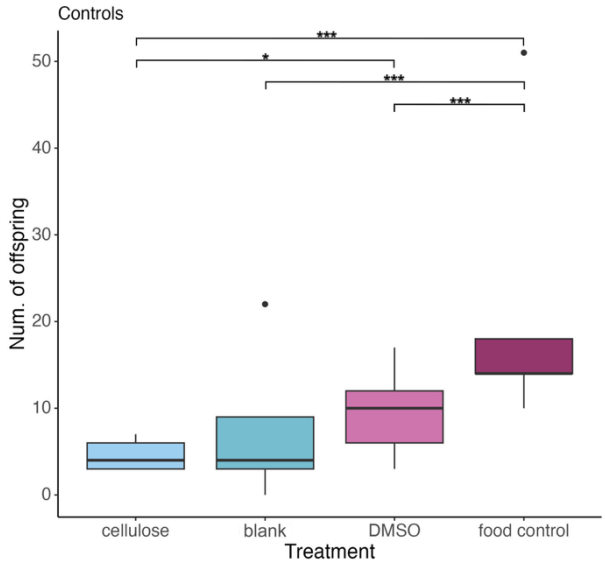


Figure 6: Number of produced offspring in each treatment beaker (N=10 per replicate) at the first clutch in the chosen control treatments. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$).*

Biochemical analyses of the control treatments showed no significant difference in ROS levels among treatments, despite the food control appearing to have lower ROS levels overall (Figure 7). When considering the LPO, animals of the food control treatment had significantly higher levels of LPO, compared to animals from the other control treatments (cellulose-food control $t = -6.639$, $p < 0.0001$; blank-food control $t = -6.658$, $p < 0.0001$; DMSO-food control $t = -6.745$, $p < 0.0001$; Figure 7).

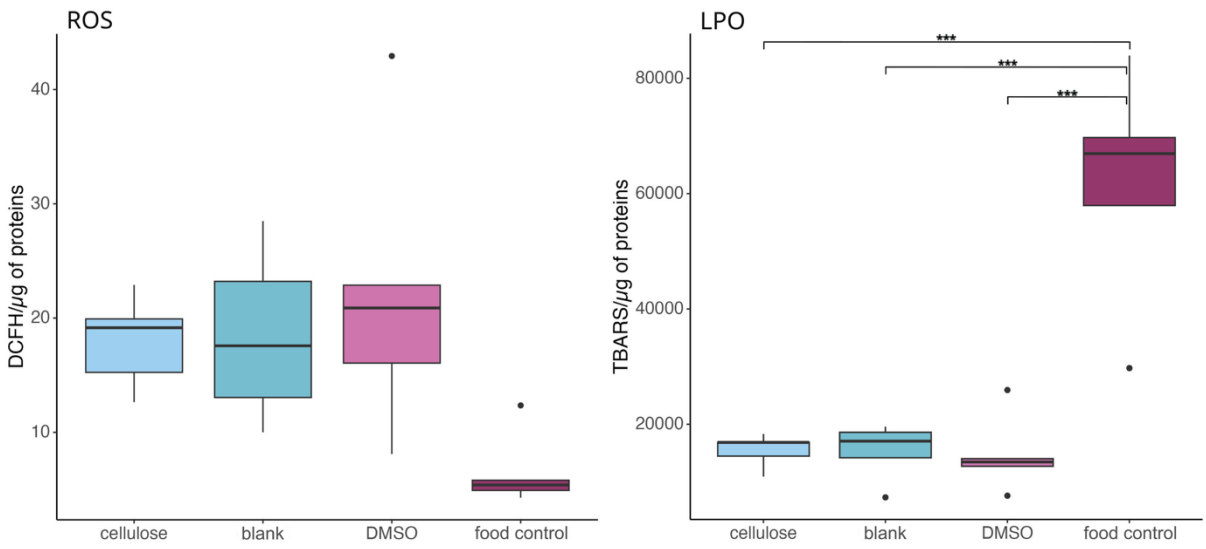


Figure 7: Levels of ROS and LPO in the chosen control treatments. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$). N= 10 animals per replicate.*

3.3 Body length at day 6

Investigations on the body length showed significant effects determined particle type, type of extract, and the interactions of these parameters with themselves and the treatment (all $p < 0.0001$; further details in the SI Table 1, 2 & 3).

3.3.1 Within treatment

The comparison of the different polymers within the same treatment type (Figure 8) highlighted significant differences only when considering the extracts. In particular, compared to cellulose, animals from the PDLLA treatment ($t = 12.37$, $p < 0.0001$) and the PET treatment ($t = 5.62$, $p < 0.0001$) were significantly smaller. Also, compared to animals in the PBS treatment, both groups were significantly smaller (PBS-PDLLA $t = 10.49$, $p < 0.0001$; PBS-PET $t = 3.73$, $p = 0.0004$). Comparing animals from the PDLLA treatment to the ones from the PET treatment, the first ones were significantly smaller ($t = -6.75$, $p < 0.0001$).

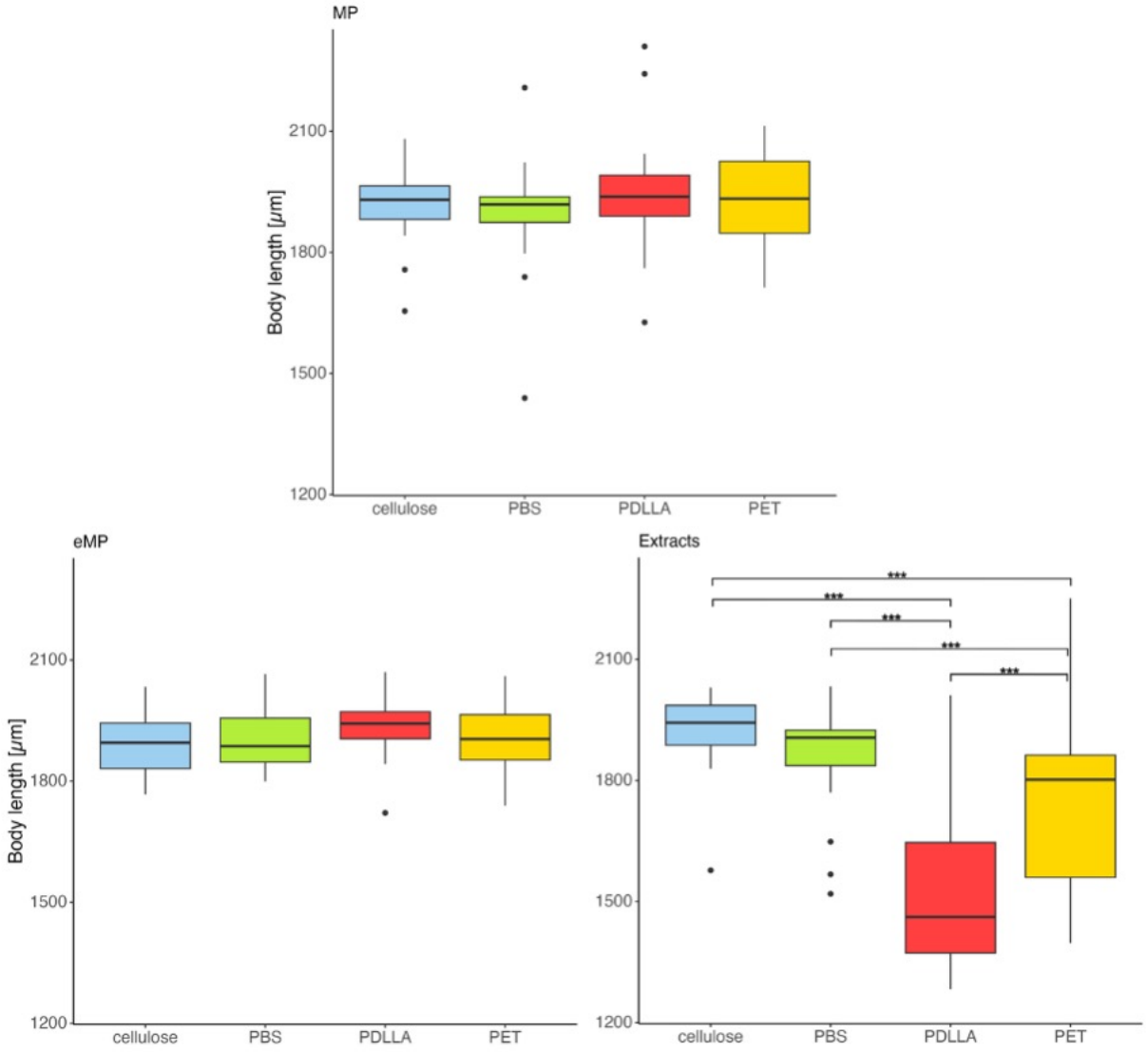


Figure 8: Body length in µm recorded at day 6 comparing MP, eMP, and extracts. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$). N= 25 per treatment.*

3.3.2 Within polymer

When considering the pairwise comparisons of the different treatments within the same polymer type (Figure 9) we observed significant differences only in the PDLLA and PET treatments, where animals from the extract treatments were significantly smaller than the ones from the blank control (PDLLA $t = 11.81$, $p < 0.0001$, $D = 3.3$; PET $t = 5.06$, $p < 0.0001$) and the respective eMP treatments (PDLLA $t = 12.54$, $p < 0.0001$; PET $t = 5.00$, $p < 0.0001$) and MP (PDLLA $t = -12.92$, $p < 0.0001$; PET $t = -5.91$, $p < 0.0001$).

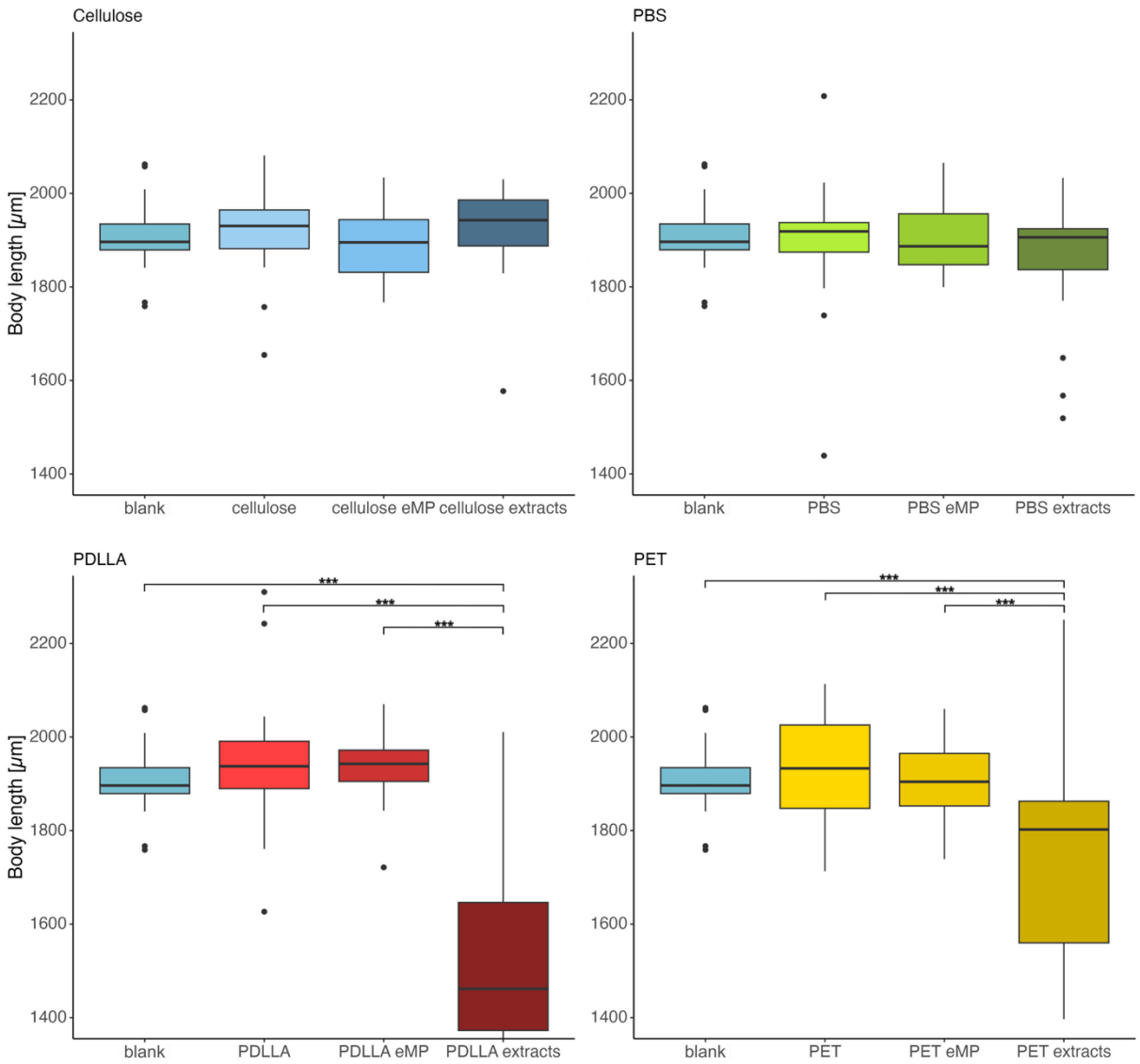


Figure 9: Body length in µm recorded at day 6, divided by the different polymers, specifically cellulose, PDLLA, PET, and PBS. The horizontal lines represent the respective treatment

median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$). N = 25 per treatment.*

3.4 Reproductive success at the first clutch

Investigation of the reproductive success of the first clutch highlighted significant negative effects of the treatment, particle type, and the interactions of treatment and particle type, treatment and extracts, particle type and extracts, and treatment, particle type, and extracts (Table SI 1, 2, & 3).

3.4.1 Within treatment

The reproductive outcome of the first clutch of the different treatment types (Figure 10) was significantly different within the MP and extract treatments, but not within the eMP treatments. In particular, animals treated with PBS-MP had significantly fewer offspring than those in the cellulose treatment ($p = 0.009$). Moreover, animals in the PDLLA treatment appeared to have higher reproductive output than in the PBS ($p = 0.0002$) and PET ($p = 0.01$) treatments. Within the extract treatments, all animals from the MP treatments had significantly fewer neonates than those from the cellulose treatment (PBS $p = 0.01$, PDLLA $p = 0.0001$, PET $p = 0.002$).

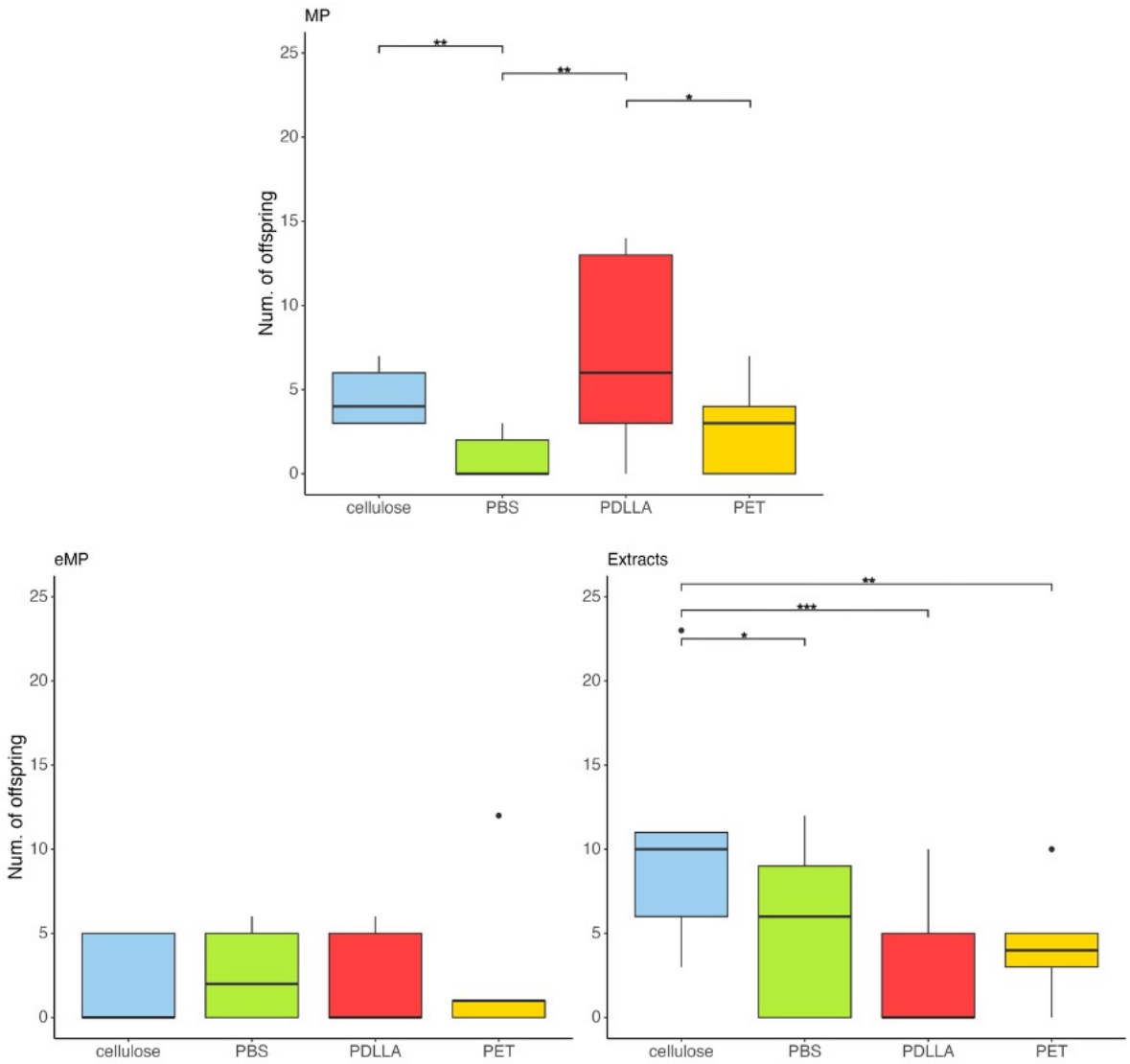


Figure 10: Number of produced offspring in each treatment beaker (N=10 per replicate) at the first clutch comparing MP, eMP, and extracts. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$).*

3.4.2 Within polymer

When considering the different treatments within the same polymer type (Figure 11), we observed several significant differences. Among cellulose treatments, animals in the cellulose eMP treatment had significantly fewer neonates than the blank (p = 0.0009) and the extract

treatments ($p = 0.0001$). Contrary to cellulose eMP, animals from the cellulose extract treatment had significantly more neonates than animals from the cellulose particle treatment ($p = 0.003$). Among the PBS treatments, animals in the PBS MP ($p = 0.0001$) and PBS eMP treatments ($p = 0.003$) had significantly fewer neonates than the blank animals. PBS MP also had fewer neonates than the extract treatment ($p = 0.002$). Among PDLLA treatments, animals from the blank had significantly more neonates than the PDLLA eMP ($p = 0.002$) and the extract PDLLA treatment ($p = 0.009$). Animals from the PDLLA MP treatment had a significantly higher reproductive output than the ones from the extract ($p = 0.01$) and PDLLA eMP treatments ($p = 0.003$). Among PET treatments, the PET eMP animals had significantly fewer neonates than the blank ($p = 0.008$) and the extract PET treatment ($p = 0.008$). The remaining pairwise comparisons revealed no further significant differences.

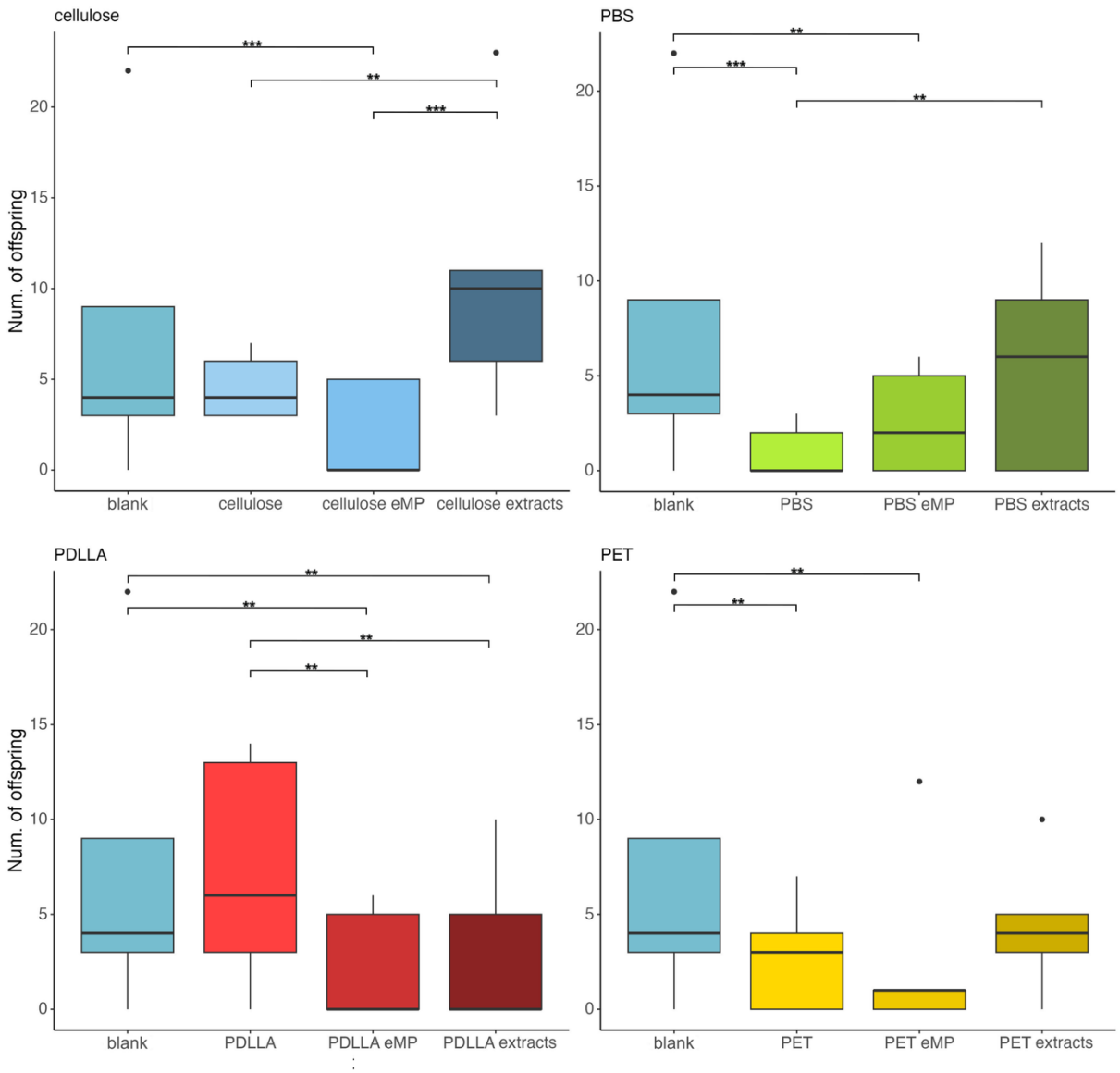


Figure 11: Number of produced offspring in each treatment beaker (N=10 per replicate) at the first clutch divided by the different polymers, specifically cellulose, PDLLA, PET, and PBS. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (= $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$).*

3.5 Biochemical analyses

The analyses of ROS levels highlighted a significant effect of the treatment ($\chi^2_{3,79} = 8.47$, p = 0.037). However, no significant differences were observed in the pairwise comparisons

among the treatments and polymers. When considering the levels of LPO, our analyses reported a significant effect of the treatment ($\chi^2_{3,79} = 10.306$, $p = 0.016$) and of the particle type ($\chi^2_{3,79} = 4.075$, $p = 0.043$). Here, pairwise comparisons between the treatments and polymers showed no significant differences (Figure SI 3-6, Table SI 1-3).

4. Discussion

This study aimed to elucidate the drivers of MP toxicity in *D. magna* by comparing MP, eMP, and their extracts. The experimental work was conducted in a setup with environmentally realistic low food availability. Our results indicate that the chemicals in the extracts exert a generally stronger negative effect than the pristine and extracted MP.

Qualitative SEM particle characterization analyses show that eMP did not undergo visible surface alterations before and after the extraction process, except for PDLLA. Micrographs reveal that the particles before and after extraction maintain a similar size, shape, and surface roughness, except for PDLLA, whose eMP appears to have a smoother surface compared to the original MP. Therefore, any variation in the biological response of the organisms exposed to either MP or eMP may probably not be attributed to the surface morphology of the said particles. In the case of PDLLA, however, this may be different. MP surface characteristics, like their surface roughness, could influence their potential toxicity (Burrows et al. 2020). Rough and sharp particle edges, for instance, can be associated with damage to cellular membranes, leading to cytolysis and death by necrosis (Choi et al. 2020). Rougher particle surfaces have also been associated with enhanced physical interaction and inflammatory responses, whereas smoother particles may be taken up by organisms more frequently than rougher ones (Khan and Jia 2023; Kim et al. 2021). Consequently, differences between the effects of PDLLA MP and PDLLA eMP (Figure 4) could also be attributed to changes in surface properties of the particles after the extraction procedure. Furthermore, surface

roughness could be associated with the particle's capacity to absorb various chemicals and biological factors, which might affect MP toxicity and organisms' interactions, particularly in the environment where plastic particles coexist with a wide range of substances (Hu et al. 2024; Burrows et al. 2020).

NMR analyses were conducted to investigate potential differences in the basic chemical structure and confirmed that the extraction process did not alter the polymers' chemical profile. Despite the absence of substantial surface or chemical modifications, the experiment revealed significant negative effects on both body length and offspring production. These differences may be associated with chemicals linked to the polymers.

GC/MS analyses of the employed extracts showed several plastic-associated chemicals. We were able to confirm the identity of four of them when the calculated Kovats indices matched those reported in the literature. Exposure to mixtures of plastic chemicals, such as those found in our extracts, could significantly affect organisms, since these chemicals may interact in antagonistic, additive, or synergistic ways, even if they are not individually identified as potentially harmful. This is due to the complex interactions among the chemicals present in the mixtures (Silva et al. 2022b), which make it difficult to predict outcomes solely from their chemical composition. These complex interactions should be better assessed in future investigations. In total, 19 chemicals were detected inside the employed extracts. PBS contained the most chemicals, with 10, followed by PET and PDLLA (4 each), and cellulose, which contained only 1. It is essential to note that the materials used in this study were derived from pre-production pellets, which do not contain the same amount of chemicals that are eventually incorporated into the final plastic products, which tend to have higher amounts of intentionally and unintentionally added compounds (UNEP, 2023). We chose to use pre-production pellets rather than fragments obtained from ground plastic materials to better compare and interpret the results in relation to a previous study conducted on *D. magna* using

the same materials (Mondellini et al. 2026), for which increased negative effects were observed for all MP, particularly from those derived from BB- plastics (i.e., PDLLA and PBS). Nevertheless, several chemicals are already employed in the production of these pellets, particularly for BB-plastics, where mixtures of chemicals are often used to achieve properties comparable to those of PB-plastics (Savva et al. 2023a). Accordingly, even though PDLLA had the same amounts of associated chemicals as PET, the BB-MP PBS had the highest number of associated chemicals in our study. The employed extraction protocol should ensure that the majority of extractable chemicals are obtained from the original material using an organic solvent (i.e., methanol; Zimmermann et al. 2020). Therefore, the obtained peaks should be representative of the chemicals incorporated into these pre-production pellets. One of these chemicals was Thiodiglycol, which was detected in both PET and PBS. This compound is commonly used in plastic manufacturing, particularly to manipulate the microstructures of polyesters (Fuoco and Finne-Wistrand 2020), such as PET and PBS. It is considered to exhibit low toxicity, with a reported EC_{50} on *D. magna* above 500 mg/L (OECD 2004). In PET, we also identified docosane. This compound is an n-alkane commonly detected in plastic debris. Like other alkanes, docosane is used either as an external lubricant, to help polymers slide over one another, or as a solvent and starting compound for diverse additives and polymers, particularly during olefin polymerization (Espinosa, Esteban, and Cuesta 2016). This compound is generally regarded as of low acute hazard (ECHA, 2018). The GC/MS analyses of PBS extracts also highlighted the presence of dimethyl ester of butanedioic acid. This compound is used as a monomer in the synthesis of polyesters (Miller, Nguyen, and Short 2021; Nikolic and Djonlagic 2001) and is generally considered to be of low toxicity. According to ECHA (2021), no effect was observed in a 48-hour acute toxicity test conducted in accordance with OECD Guideline 202 (2004). Nevertheless, it is classified as an eye irritant and potentially harmful to the upper respiratory tract in humans (NCBI).

In PDLLA, four peaks were identified, but the identity of only one of them, 3-methyl-2(5H)-furanone, could be confirmed. While this compound is commonly employed in the flavor and fragrance industry (Effenberger et al. 2019), there is no evidence that it is used in the process of plastic manufacturing. Data on its potential effects are very limited, and no data are available for aquatic organisms, but it is reported to be a skin, eye, and respiratory tract irritant in humans (Sigma Aldrich, 2026).

One peak was also found in the cellulose extracts. However, we were unable to identify this compound with certainty. Nevertheless, this treatment had no adverse effects on the exposed daphnids.

Although all identified substances are typically described as non-toxic or of low toxicity, no data are available on their potential hazard in the context of MP and environmental samples, where they appear as mixtures with uncertain combined toxicity. Plastics represent a complex class of synthetic pollutants, and many of the toxic effects reported in the scientific literature may be due to plastic-associated chemicals rather than plastic particles per se (Gao, Huang, and Han 2025). Although few studies have investigated plastic chemical extracts, several reports have documented effects of MP leachates (e.g., Qiu et al. 2023; Jang et al. 2024; Schiavo et al. 2020). For instance, Qiu et al. (2023) investigated the endocrine-disrupting effects of leachates from 15 plastic products, both virgin and aged using a xenon lamp, on larvae of *Oryzias melastigma,* showing that leachates, particularly from aged plastics, pose endocrine disruption risks in marine environments. Another study from Laranjeiro et al. (2024) investigated the acute toxicity of PLA, PHBv, and PP leachates on marine plankton. The results of this study showed that leachates from the bio-based polymer PHBv contained higher concentrations of chemicals and exhibited the highest toxicity, compared with those from PP and PLA, which exhibited minimal toxicity. Another study conducted on *D. magna* (Schrank et al. 2019) compared the effects of leachates obtained from both rigid and flexible

PVC. Schrank and co-authors demonstrated that flexible PVC caused significant negative effects, whereas its rigid counterpart had minimal effects, indicating that not only the polymer but also the chemicals within pose a risk to aquatic life. Our results on growth and reproduction after exposure to plastic extracts align with those from the studies, demonstrating that plastic-associated chemicals play a major role in determining the particles' effects. Nevertheless, the effect of the particles alone cannot be excluded, especially when considering the observed impairment of reproduction caused by all treatments in our study. Notably, while body length was exclusively affected by the extract treatment across all plastic types, the number of offspring was negatively affected by the extracts (particularly from PET and PDLLA), the particles, and the extracted particles. Extracted particles from cellulose also adversely affected offspring numbers, whereas cellulose particles and extracts did not. While an effect of MP on *Daphnia* reproduction is unsurprising and widely reported in the scientific literature (e.g., Brehm et al. 2023; Schwarzer et al. 2022; Funke, Webb, and Wolinska 2024), the negative effect of eMP was partly unexpected. Nevertheless, high concentrations of suspended particles, not necessarily MP, combined with reduced food availability, can impair feeding efficiency, alter energy allocation, and ultimately decrease population growth rate in *D. magna* (Zhang et al. 2013). Accordingly, particle presence alone might account for the observed effects on reproduction, acting in conjunction with the effects of the associated chemicals when considering the untreated MP.

Overall, the results presented in this study are consistent with those reported for the same materials in a previous investigation (Mondellini et al. 2026), in which significant negative effects of all MP (both PB- and BB-) were observed across all investigated endpoints. Specifically, although all plastic particles significantly impaired the assessed parameters, PBS-MP exerted the strongest negative effects on survival and reproduction, which was also observed for reproduction in the present study.

Contrary to our hypothesis, the investigated biochemical parameters, ROS and LPO levels, were not affected by any of the plastic treatments. MP are known to cause oxidative stress in several organisms (e.g., Liu et al. 2019; Silva et al. 2021; Roy et al. 2023; Oduro et al. 2025). The stable ROS levels, together with the lack of lipid-related oxidative damage found in our study, might be connected to the concentration tested, which could be too low to elicit an alteration of the daphnids' oxidative status (e.g., De Felice et al. 2022; Esterhuizen et al. 2024). Previous investigations that report an alteration of this endpoint often use higher MP concentrations, compared to the one used in this study (e.g., Zhu et al. 2022; Jeyavani et al. 2022). It is important to note that the concentration tested (500 MP mL^{-1}) is already elevated compared to those currently found in the environment (Gupta et al. 2023), representing a possible worst-case scenario. Another possible explanation for the lack of significant ROS and LPO responses is the reallocation of energy toward detoxification and antioxidant defense systems. Such compensatory mechanisms may prevent measurable oxidative damage while diverting energetic resources away from growth and reproduction (De Coen and Janssen 2003; Barata et al. 2005), which aligns with the observed results regarding body length and number of offspring in our study. Further studies investigating the levels of the main antioxidants and detoxifying enzymes should be carried out to shed light on this aspect.

To investigate the impact of reduced food availability, a food control was included in our experiment. In line with our initial hypothesis, the control fed *ad libitum* grew larger and had a higher number of offspring compared to the other control treatments (i.e., blank, DMSO, and cellulose). Regarding ROS and LPO levels, we observed no significant differences in ROS, although pro-oxidant levels appeared lower in the food control than in the others. However, we recorded a significant increase in lipid damage (LPO), which could be attributed to higher lipid content in animals in the food control treatment, as they had more resources available to store as lipid droplets. The limited food supply probably depleted the fatty acid

reservoirs in the organisms subjected to such a feeding regime (Yang et al. 2021), thereby lowering their pool of peroxidable substrate. These differences among the control groups suggest that the limited amount of food provided in this study might have exacerbated the negative effects of our particle treatments (Mondellini et al. 2026).

5. Conclusion

This study compared the effects of MP, eMP, and extracts from PET (PB-MP), PBS, and PDLLA (BB-MP), and cellulose on *D. magna*. The experiment was conducted under food-limited conditions to enhance environmental relevance. The study had multiple aims: (i) to investigate whether the associated chemicals (extracts), the MP, or just the particle presence (eMP) are responsible for MP toxicity, (ii) to evaluate these drivers of MP toxicity under environmentally relevant conditions, and (iii) to assess whether BB-MP are less harmful than PB-MP under these scenarios. Our findings demonstrate that BB-MP cannot be considered a safer alternative to PB-MP, as they exert comparable negative effects on daphnids. Furthermore, our results underscore the importance of incorporating environmentally realistic scenarios in future assessments like a reduced food availability, which may exacerbate plastic-related effects that could otherwise remain overlooked under standardized testing protocols. Finally, the data highlight the critical role of associated chemicals in plastic toxicity, indicating that, for certain endpoints, the observed toxic effects can be attributed primarily to these chemical components rather than to the particles themselves. Taken together, these findings call for a reassessment of current hazard evaluation frameworks, emphasizing the need to account for chemical co-exposure and environmental realism when determining the ecological safety of MP derived from both conventional and biodegradable bio-based plastics.

Authors contribution statement

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Seema Agarwal: Project administration, Funding acquisition, Resources, Writing - Review & Editing

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Declaration of Competing Interest

M.W. is an unremunerated member of the Scientific Advisory Board of the Food Packaging Forum Foundation and received travel support for attending annual board meetings. The other

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

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Supplementary material pertaining to Article IV

Extracted chemicals from microplastics exert increased adverse effects on *Daphnia magna* compared to the pristine particles

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(*contributed equally to this work)

Supplementary information

Extracted chemicals from microplastics exert increased adverse effects on *Daphnia magna* compared to the pristine particles

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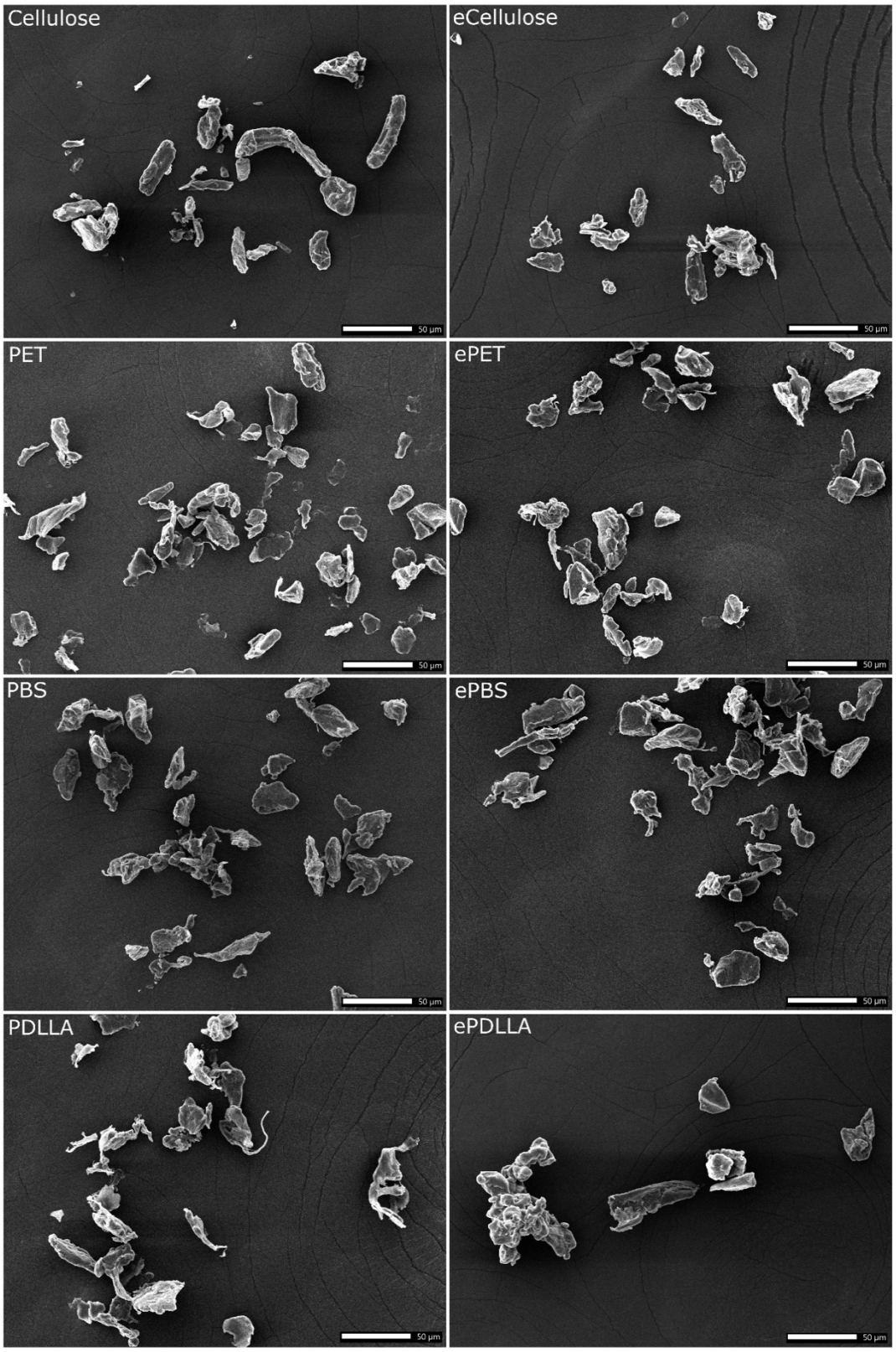


Figure SI 1: *Overview SEM micrographs of cellulose and MP particles. Left column: pristine particles; right column: particles that went through the extraction process. Scale bars = 50 µm.*

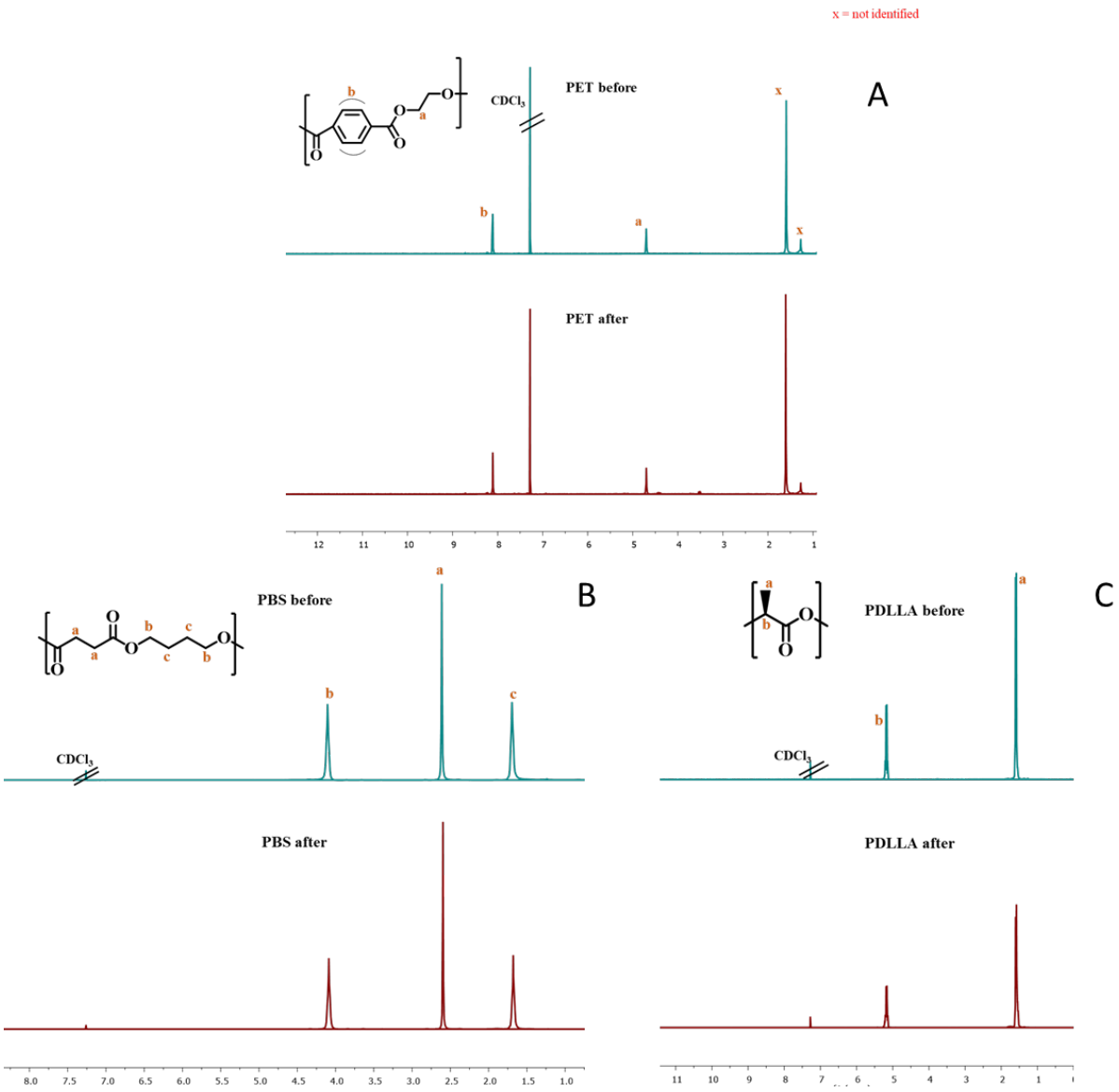


Figure SI 2: *NMR spectra in CDCl* $_3$ *before (blue spectra) and after (red spectra) the methanol extraction process of A) PET, B) PBS and C) PDLLA. Not identified peaked are indicated with x.*

Table SI 1: parameters from the linear regression analyses of *D. magna*'s life history parameters obtained during the 10-day exposure. Bold values represent significance ($p < 0.05$).

Response	***Fixed effects***	***DF***	χ^2 ***/F***	***p-value***

			value	
Body length (day 6)	Treatment	3	4.07	0.253
	Particle type	1	13.63	**0.0002**
	Extracts	1	30.6	**<0.0001**
	Treatment:particle type	3	22.0	**<0.0001**
	Treatment:extracts	3	45.5	**<0.0001**
	Particle type:extracts	1	48.2	**<0.0001**
	Treatment:particle type:extracts	3	44.7	**<0.0001**
Num. of offspring	Treatment	3	9.8	**0.019**
	Particle type	1	52.9	**<0.0001**
	Extracts	1	0.06	0.801
	Treatment:particle type	3	16.0	**0.001**
	Treatment:extracts	3	13.9	**0.002**
	Particle type:extracts	1	12.0	**0.0005**
	Treatment:particle type:extracts	3	16.2	**0.0001**
ROS	Treatment	3	8.4	**0.037**
	Particle type	1	2.0	0.147
	Extracts	1	1.0	0.315
	Treatment:particle type	3	2.7	0.428
	Treatment:extracts	3	0.4	0.928
	Particle type:extracts	1	1.2	0.260
	Treatment:particle type:extracts	3	1.5	0.669

LPO	Treatment	3	10.3	**0.016**
	Particle type	1	4.1	**0.043**
	Extracts	1	0.5	0.471
	Treatment:particle type	3	2.5	0.472
	Treatment:extracts	3	1.4	0.694
	Particle type:extracts	1	0.4	0.551
	Treatment:particle type:extracts	3	0.8	0.853

Table SI 2: pairwise comparison between the separate polymers divided by treatment of the *D. magna'*s life history parameters obtained during the 10-day exposure. Bold values represent significance ($p < 0.05$) and a detectable effect ($D > 0.3$).

Contrasts	***Cohen'D***	***t/z value***	***p value***
		Body length (day 6)	
		MP	
Cellulose-PBS	0.211	0.747	1.000
Cellulose-PDLLA	-0.165	-0.586	1.000
Cellulose-PET	-0.094	-0.336	1.000
PBS-PDLLA	**-0.376**	-1.333	1.000
PBS-PET	**-0.306**	-1.083	1.000
PDLLA-PET	0.070	0.250	1.000
		eMP	
Cellulose-PBS	-0.072	-0.256	1.000
Cellulose-PDLLA	**-0.300**	-1.061	1.000
Cellulose-PET	-0.078	-0.276	1.000
PBS-PDLLA	-0.227	-0.805	1.000
PBS-PET	-0.005	-0.020	1.000
PDLLA-PET	0.222	0.785	1.000
		Extracts	
Cellulose-PBS	**0.533**	1.885	0.060

Cellulose-PDLLA	**3.500**	12.377	**<0.0001**
Cellulose-PET	**1.590**	5.624	**<0.0001**
PBS-PDLLA	**2.967**	10.492	**<0.0001**
PBS-PET	**1.057**	3.739	**0.0004**
PDLLA-PET	**-1.910**	-6.753	**<0.0001**
Num. of offspring			
MP			
Cellulose-PBS	**0.618**	3.093	**0.009**
Cellulose-PDLLA	-0.181	-1.678	0.186
Cellulose-PET	0.201	1.465	0.186
PBS-PDLLA	**-0.799**	-4.136	**0.0002**
PBS-PET	**-0.417**	-1.976	0.144
PDLLA-PET	**0.382**	2.999	**0.010**
eMP			
Cellulose-PBS	-0.106	-0.624	1.000
Cellulose-PDLLA	-0.038	-0.218	1.000
Cellulose-PET	-0.136	-0.813	1.000
PBS-PDLLA	-0.067	0.408	1.000
PBS-PET	-0.030	-0.192	1.000
PDLLA-PET	-0.097	-0.599	1.000
Extracts			
Cellulose-PBS	**0.533**	2.853	**0.017**

Cellulose-PDLLA	**3.500**	4.316	**0.0001**
Cellulose-PET	**1.590**	3.467	**0.003**
PBS-PDLLA	**2.967**	1.825	0.203
PBS-PET	**1.057**	0.713	0.505
PDLLA-PET	**-1.910**	-1.144	0.505
	ROS		
	MP		
Cellulose-PBS	**-0.744**	-1.105	1.000
Cellulose-PDLLA	**-1.407**	-2.325	0.141
Cellulose-PET	-0.169	-0.269	1.000
PBS-PDLLA	**-0.726**	-1.079	1.000
PBS-PET	**0.575**	0.854	1.000
PDLLA-PET	**1.301**	2.057	0.220
	eMP		
Cellulose-PBS	**-0.808**	-0.256	0.826
Cellulose-PDLLA	**-1.250**	-1.061	0.316
Cellulose-PET	**-0.355**	-0.276	1.000
PBS-PDLLA	**-0.443**	-0.805	1.000
PBS-PET	**0.453**	-0.020	1.000
PDLLA-PET	**0.896**	0.785	0.809
	Extracts		
Cellulose-PBS	**-0.998**	-1.577	0.720

Cellulose-PDLLA	**-0.804**	-1.273	0.833
Cellulose-PET	-0.027	-0.043	1.000
PBS-PDLLA	0.193	0.305	1.000
PBS-PET	**0.971**	1.535	0.720
PDLLA-PET	**0.777**	1.230	0.833
		LPO	
		MP	
Cellulose-PBS	-0.744	-1.105	1.000
Cellulose-PDLLA	-1.470	-2.325	0.141
Cellulose-PET	-0.169	-0.268	1.000
PBS-PDLLA	**-0.726**	-1.079	1.000
PBS-PET	**0.574**	0.854	1.000
PDLLA-PET	**1.301**	2.057	0.220
		eMP	
Cellulose-PBS	**-0.808**	-1.577	0.826
Cellulose-PDLLA	**-1.250**	-1.977	0.316
Cellulose-PET	**-0.355**	-0.561	1.000
PBS-PDLLA	**-0.443**	-0.700	1.000
PBS-PET	**0.453**	0.716	1.000
PDLLA-PET	**0.896**	1.416	0.809
		Extracts	
Cellulose-PBS	**-0.998**	1.885	0.720

Cellulose-PDLLA	**-0.805**	12.377	0.833
Cellulose-PET	-0.027	5.624	1.000
PBS-PDLLA	0.193	10.429	1.000
PBS-PET	**0.971**	3.739	0.720
PDLLA-PET	**0.778**	-5.753	0.833

Table SI 3: pairwise comparison between the treatments divided by polymer of the *D. magna*'s life history parameters obtained during the 10-day exposure. Bold values represent significance ($p < 0.05$) and a detectable effect ($D > 0.3$).

contrasts	*Cohen'D*	*t/z value*	*p-value*
		Body length (day 6)	
		Cellulose	
Blank-MP	-0.146	-0.516	1.000
Blank-eMP	0.095	0.337	1.000
Blank-Extracts	-0.154	-0.559	1.000
eMP-Extracts	-0.253	-0.896	1.000
eMP-MP	-0.241	-0.853	1.000
Extracts-MP	0.012	0.042	1.000
		PBS	
Blank-MP	0.065	0.231	1.000
Blank-eMP	0.023	0.081	1.000
Blank-Extracts	**0.375**	1.326	1.000
eMP-Extracts	**0.352**	1.245	1.000
eMP-MP	0.042	0.150	1.000
Extracts-MP	**-0.310**	-1.095	1.000
		PDLLA	
Blank-MP	**-0.312**	-1.102	0.813
Blank-eMP	-0.205	-0.724	0.939
Blank-Extracts	**3.342**	11.818	**<0.0001**

eMP-Extracts	**3.548**	12.542	**<0.0001**
eMP-MP	-0.107	-0.378	0.938
Extracts-MP	**-3.310**	-12.920	**<0.0001**
	PET		
Blank-MP	-0.241	-0.852	1.000
Blank-eMP	0.017	0.061	1.000
Blank-Extracts	**1.433**	5.065	**<0.0001**
eMP-Extracts	**1.416**	5.004	**<0.0001**
eMP-MP	-0.256	-0.913	1.000
Extracts-MP	**-1.673**	-5.917	**<0.0001**
	Num. of offspring		
	Cellulose		
Blank-MP	0.203	1.901	0.115
Blank-eMP	**0.540**	3.756	**0.0009**
Blank-Extracts	-0.135	-1.565	0.117
eMP-Extracst	**-0.675**	-4.837	**<0.0001**
eMP-MP	**-0.337**	-2.199	0.083
Extracts-MP	**0.338**	3.343	**0.003**
	PBS		
Blank-MP	**0.821**	4.263	**0.001**
Blank-eMP	**0.434**	3.338	**0.003**
Blank-Extracts	0.138	1.358	0.175
eMP-Extracts	-0.256	-2.165	0.091
eMP-MP	**0.387**	1.816	0.139
Extracst-MP	**0.682**	3.464	**0.003**

	PDLLA		
Blank-MP	0.022	0.232	0.869
Blank-eMP	**0.502**	3.621	**0.002**
Blank-Extracts	**0.376**	3.048	**0.009**
eMP-Extracts	-0.126	-0.781	0.869
eMP-MP	**-0.480**	-3.441	**0.003**
Extracts-MP	**-0.354**	-2.849	**0.013**
	PET		
Blank-MP	**0.404**	3.194	**0.008**
Blank-eMP	**0.404**	2.040	**0.008**
Blank-Extracts	0.221	3.194	0.165
eMP-Extracts	-0.183	-1.322	0.558
eMP-MP	0.000	0.000	1.000
Extracts-MP	0.183	1.322	0.558
	ROS		
	Cellulose		
Blank-MP	0.071	0.112	1.000
Blank-eMP	**-0.776**	-1.227	1.000
Blank-Extracts	-0.233	-0.368	1.000
eMP-Extracts	**0.543**	0.859	1.000
eMP-MP	**0.847**	1.340	1.000
Extracts-MP	**0.304**	0.481	1.000
	PBS		
Blank-MP	**0.867**	1.288	1.000
Blank-eMP	**0.589**	0.932	1.000

Blank-Extracts	**0.510**	0.807	1.000
eMP-Extracts	-0.078	-0.125	1.000
eMP-MP	0.277	0.412	1.000
Extracts-MP	**0.356**	0.529	1.000
		PDLLA	
Blank-MP	0.288	0.457	1.000
Blank-eMP	-0.219	-0.347	1.000
Blank-Extracts	**-0.503**	-0.796	1.000
eMP-Extracts	-0.284	-0.449	1.000
eMP-MP	**0.508**	0.803	1.000
Extracts-MP	**0.792**	1.253	1.000
		PET	
Blank-MP	**0.987**	1.560	0.744
Blank-eMP	**0.722**	1.142	1.000
Blank-Extracts	0.142	0.225	1.000
eMP-Extracts	**-0.580**	-0.917	1.000
eMP-MP	0.265	0.418	1.000
Extracts-MP	**0.845**	1.336	0.934
		LPO	
		Cellulose	
Blank-MP	-0.018	-0.029	1.000
Blank-eMP	0.282	0.446	1.000
Blank-Extracts	**0.424**	0.670	1.000
eMP-Extracts	0.142	0.224	1.000

eMP-MP	**-0.300**	-0.475	1.000
Extracts-MP	**-0.442**	-0.699	1.000
		PBS	
Blank-MP	**-0.762**	-1.132	1.000
Blank-eMP	**-0.526**	-0.831	1.000
Blank-Extracts	**-0.574**	-0.907	1.000
eMP-Extracts	-0.048	-0.076	1.000
eMP-MP	-0.237	-0.352	1.000
Extracts-MP	-0.189	-0.280	1.000
		PDLLA	
Blank-MP	-0.188	-0.297	1.000
Blank-eMP	-0.073	-0.115	1.000
Blank-Extracts	**0.397**	0.628	1.000
eMP-Extracts	**0.469**	0.724	1.000
eMP-MP	-0.115	-0.182	1.000
Extracts-MP	**-0.585**	-0.924	1.000
		PET	
Blank-MP	**-1.488**	-2.354	0.132
Blank-eMP	**-0.381**	-1.531	0.524
Blank-Extracts	**-0.381**	-0.602	1.000
eMP-Extracts	**0.587**	0.929	1.000

eMP-MP	**-0.520**	-0.823	1.000
Extracts-MP	**-1.108**	-1.751	0.424

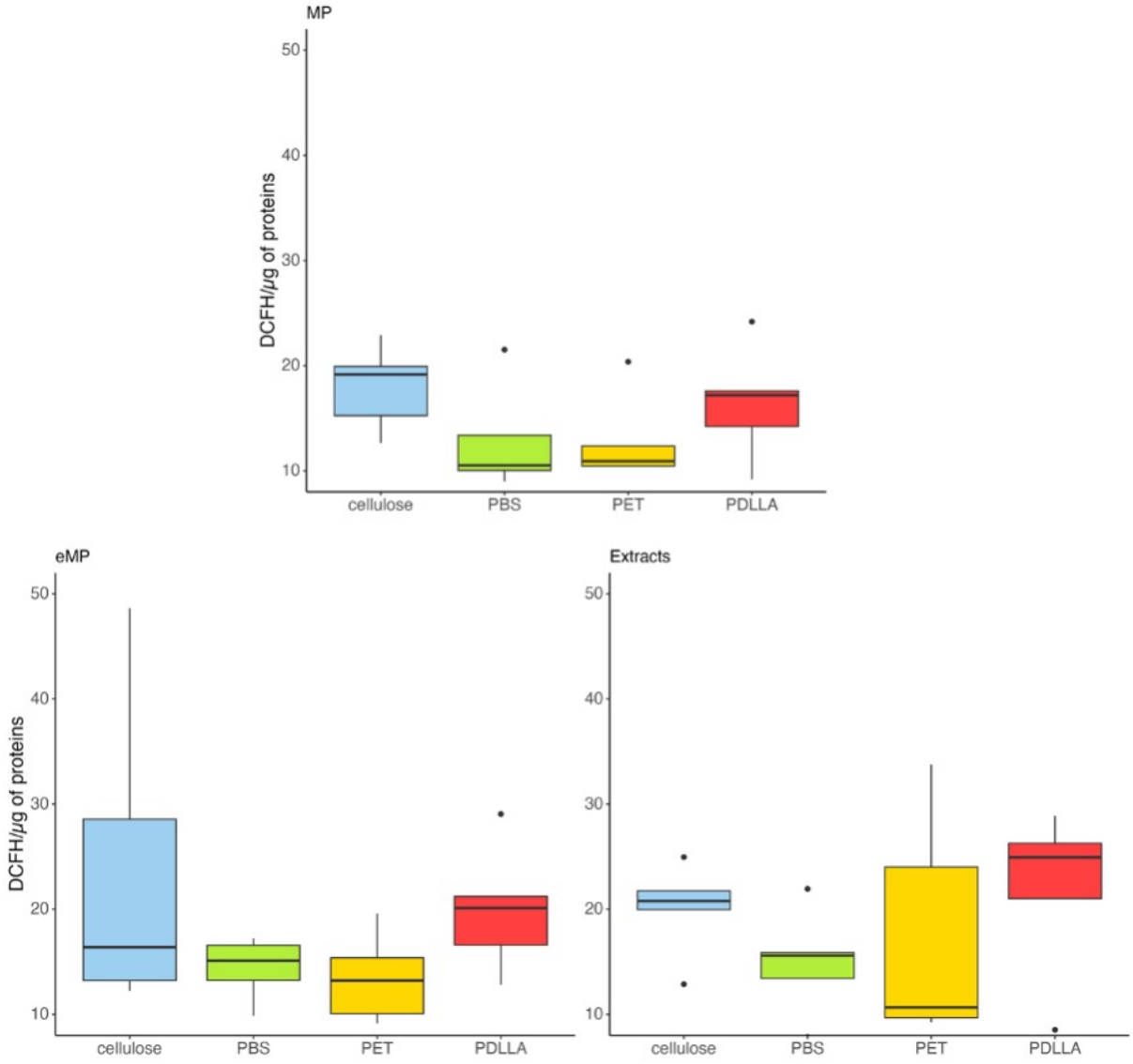


Figure SI 3*: Levels of ROS measured at the end of the exposure expressed in DCFH/µg of proteins comparing MP, eMP, and extracts. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (* = p ≤ 0.05; ** = p ≤ 0.01; *** = p ≤ 0.001). N= 10 per replicate.*

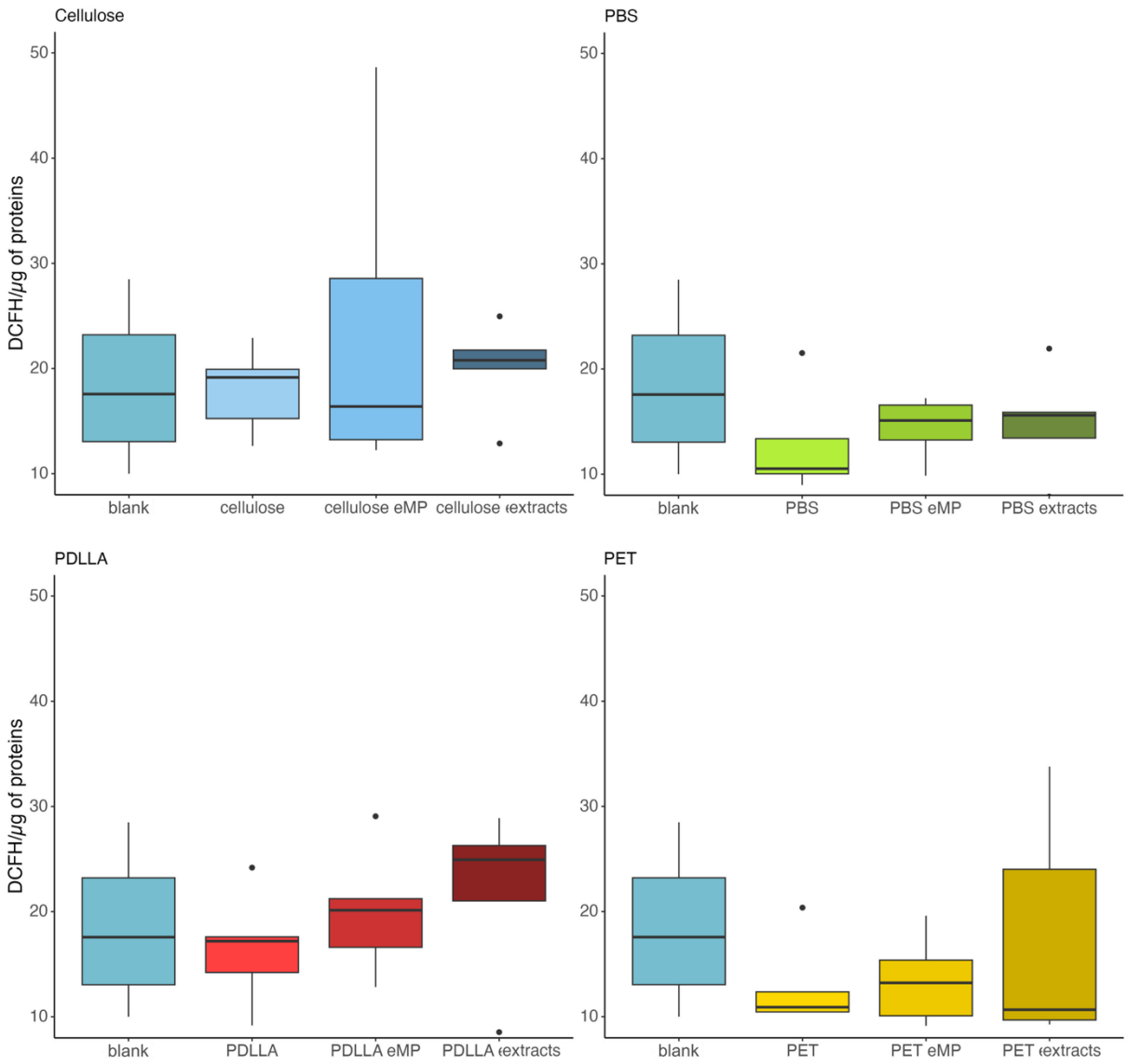


Figure SI 4: *Levels of ROS measured at the end of the exposure expressed in DCFH/µg of proteins, divided by the different polymers, specifically cellulose, PDLLA, PET, and PBS. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$). N = 10 per replicate.*

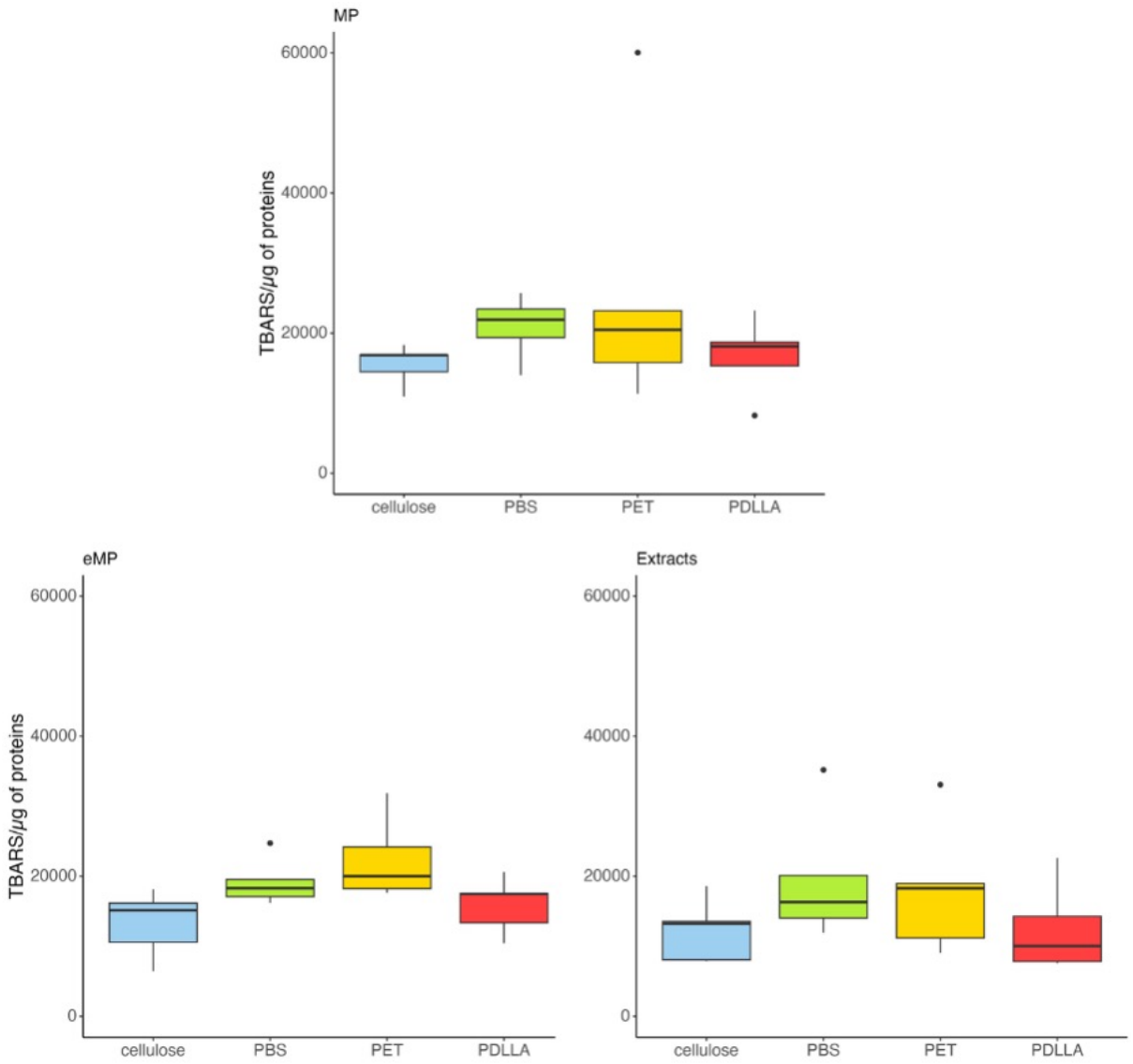


Figure SI 5: *Levels of LPO measured at the end of the exposure expressed in TBARS/µg of proteins comparing MP, eMP, and extracts. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$). N= 10 per replicate.*

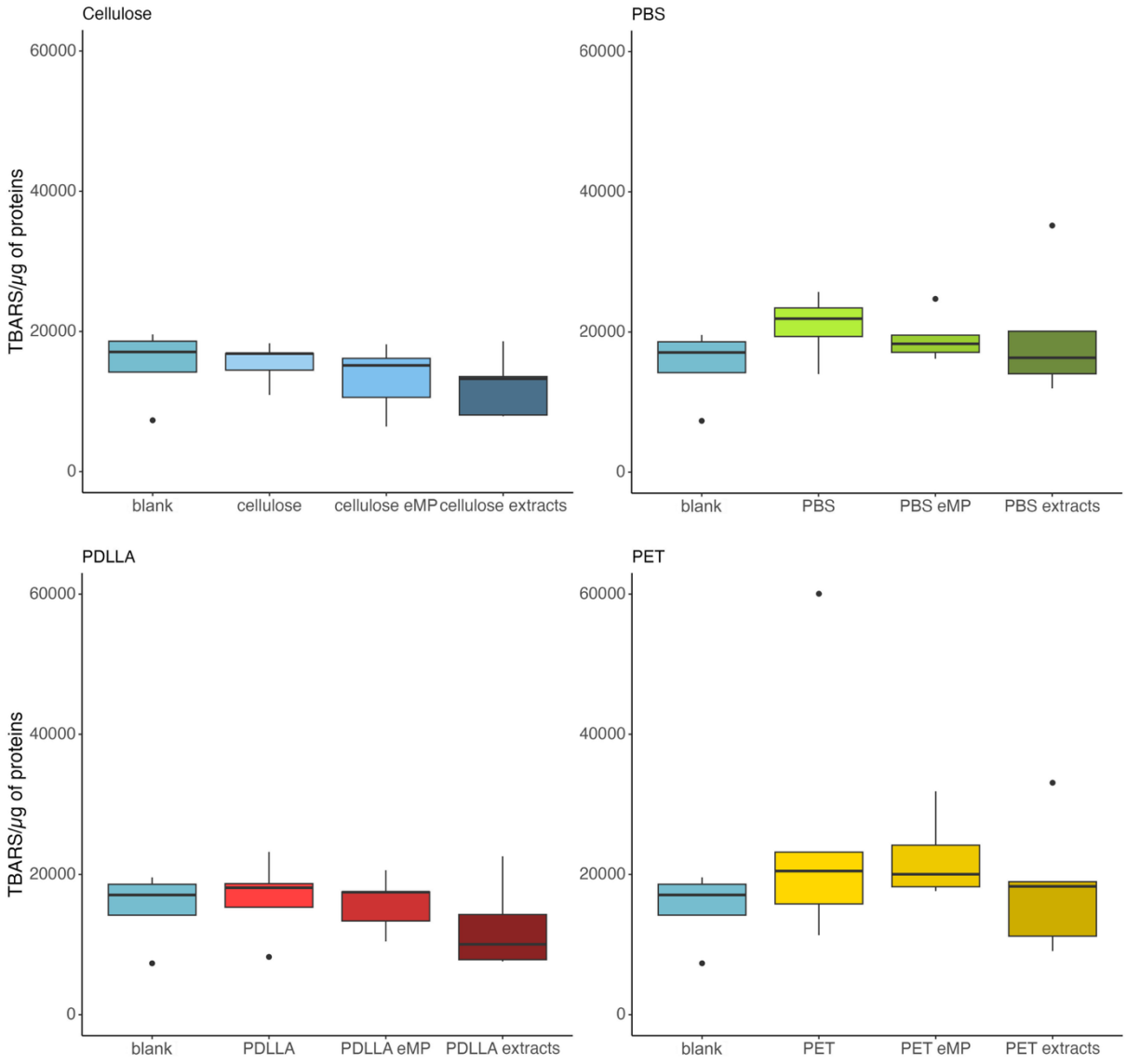


***Figure SI 6**: Levels of LPO measured at the end of the exposure expressed in TBARS/µg of proteins, divided by the different polymers, specifically cellulose, PDLLA, PET, and PBS. The horizontal lines represent the respective treatment median, while the boxes represent the intermediate quartiles of the data. Asterisks indicate significance (* = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$). N = 10 per replicate.*

Article V

Beyond microplastics: Water soluble synthetic polymers exert sublethal adverse effects in the freshwater cladoceran *Daphnia magna*

Authors: Simona Mondellini, Matthias Schott, Martin G.J. Löder, Seema Agarwaal, Andreas Greiner, Christian Laforsch

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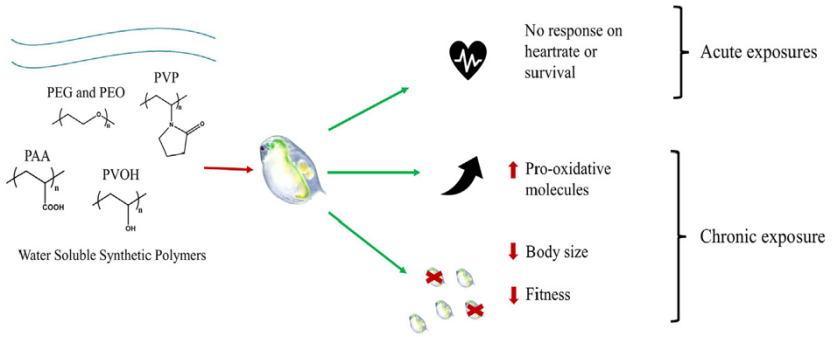
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HIGHLIGHTS

- Including water soluble synthetic polymers to hazard assessment is urgently needed.
- Toxicity of water soluble synthetic polymers is assessed in *D. magna*.
- 21-d exposure to water soluble synthetic polymers impacted *D. magna*'s life history.
- Water soluble synthetic polymers did not exert acute effects on *D. magna*.

GRAPHICAL ABSTRACT



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ABSTRACT

Plastic pollution is considered one of the causes of global change. However, water soluble synthetic polymers (WSSPs) have been neglected so far, although they are used in several industrial, dietary, domestic and biomedical products. Moreover, they are applied in wastewater treatment plants (WWTPs) as flocculants and coagulant agents. Hence, their presence in the aquatic environment as well as their uptake by aquatic organisms is probable, whereas no data are available regarding their potential adverse effects. Here we show in the freshwater key species *D. magna* exposed to five different WSSPs life history changes along with an altered level of reactive oxygen species, although acute mortality was not observed. Since daphnids act as keystone species in lake ecosystems by controlling phytoplankton biomass, even sublethal effects such as WSSPs induced changes in life history may result in cascading effects, from lower to higher trophic levels, which in turn could affect the whole food web.

1. Introduction

Water soluble synthetic polymers (WSSPs) account for ca. 6 % of the global polymer market (Wang et al., 2021) and find application in a variety of different fields and industries like food, cosmetics, pharmaceuticals, paint, textile, paper, construction, adhesive, coatings and water treatment (Kadajji and Betageri, 2011). Unlike the majority of the chemicals that are employed in Europe, WSSPs are not registered under REACH

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(Regulation, Evaluation, Authorization and Restriction of Chemicals) (Huppertsberg et al., 2020). This is because, according to the Regulation (EC) No 1907/2006 (Regulation EC No 1907/2006 of the European Parliament and of the Council of 18 December 2006 Concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), Establishing a European Chemical Agency, Amending Directive 1999/4 2006), polymers should be exempted from registration and evaluation until they pose any risk to human health or the environment. However, according to Huppertsberg et al. (2020), an estimation of the annual production volume of WSSPs in Europe can be made if the educts of polymer synthesis that are REACH registered are taken into account, leading to a production volume in the range of millions of tons (e.g., poly(ethylene glycol (PEG) > 10^6 t/a, poly(acrylic acid) (PAA) 10^5–10^6 t/a).

Although WSSPs are massively used, information regarding their concentrations and prevalence in the natural environment is still scarce, so their fate and impact remains mostly unclear.

Nevertheless, some studies confirm their presence in the freshwater compartment, for example poly(vinyl pyrrolidone) (PVP) which is present in a variety of medicines, food products and supplements (Julinová et al., 2018) was detected in a concentration range comprised between 0.9 and 7.1 mg/L in the effluents of wastewater treatment plants (WWTPs) in Düren (Germany) (Antić et al., 2011). Also recently, Wang et al. (2021) revealed the presence of PEG (widely used in pharmaceuticals and personal care products, for blood and organs storage and in the automotive industry (Jang et al., 2015; Kadajji and Betageri, 2011; Wang et al., 2021)) in fresh falling snow with a concentration of 2.19 μg/L in the city of Montreal (Canada), confirming air-born dispersion. Once they enter our water bodies, they are free to interact with the biota on different levels of the biological hierarchy. Moreover, their ability to bind heavy metals or organic pollutants gives these toxins the potential to increase their mobility and thus increase their bioavailability (Julinová et al., 2018).

Despite the fact that these polymers cannot be seen washing up on shores like microplastics (MP) do, they occur in our rivers, lakes and other water depositories. (Gross and Kalra, 2002). Once they reach the environment they can possibly exert - unnoticed - a plethora of different adverse effects (possibly affecting different levels of the biological hierarchy, from molecular to individual, up to population or community), rendering them potentially hazardous for the biota and the entire ecosystem (Gross and Kalra, 2002). Given the paucity of reports regarding possible repercussions of WSSPs, we investigated the effects of five synthetic commonly used water soluble polymers on the freshwater model organism *Daphnia magna*. In detail we focused our research on the effects of poly(vinyl alcohol) (PVOH), PVP, PAA, PEG and poly(ethylene oxide) (PEO), (Fig. SI 1). All polymers chosen were of low molecular weights (M_w (weight average molecular weight) < 10,000). To additionally study the effect of molecular weight on *Daphnia magna*, the polymers with ethylene oxide repeat units (PEO and PEG) were used in low and high molecular weights (M_w 1000 and 900,000).

We conducted acute toxicity tests on *D. magna* and a 21-day reproduction test to assess any life-history alteration on the exposed cladocerans. At the end of the chronic test we also measured the levels of reactive oxygen species (ROS). Moreover, we performed a heartrate acute assay on 48-hour old daphnids, as it was proven to be a reliable and quick test for rapid screening on possible adverse effects of toxic compounds (Park et al., 2019). With this work, we aim to investigate and compare for the first time the effects of five widely employed WSSPs on different endpoints in *D. magna* since it is not known if these frequently used compounds evoke adverse effects on aquatic organisms.

2. Material and methods

2.1. Daphnia magna cultivation

A single clone of *D. magna* BL 2.2 was chosen for the experiments from the large and well-established laboratory collection. This *Daphnia* clone originated in Belgium and is maintained in husbandry since 1997 (Imhof et al., 2017).

The organisms used originated from aged-synchronized mothers held in 1,5 L glass beakers (Weck GmbH u. Co. KG, Wehr-Öflingen, Germany).

All the animals were maintained in M4 artificial medium (Elendt and Bias, 1990) with the addition of 4.48 μg/L of selenium dioxide (SeO_2) and kept at 20 ± 0.5 °C with a photoperiod of 14 h light: 9 h dark with 30 min of dusk and dawn. The *Daphnia* culture was fed ad libitum with the green algae *Acutodesmus obliquus* to achieve 2 mgC/L. The mothers were separated from the offspring (<24 h) so that no more hatching occurs and to ensure the use of similar-aged daphnids for the experimental procedure.

All the specimens that were used during these experiments were born from the same mothers and randomly selected for the treatments.

2.2. Chemicals and reagents

The WSSPs that were used during this work (PVOH (75 % hydrolyzed, M_w ~ 2000, Acros Organics), PVP (Kollidon 17PF, M_w ~ 7000–10,000, BASF), PAA (Mw ~ 1800, Sigma Aldrich), PEG (M_w ~ 1000, Sigma Aldrich) and PEO (M_w ~ 900,000, Sigma Aldrich)) are shown in Fig. SI 1. The stock solutions were prepared by dissolving 0.5 g of each WSSP in 99.5 g of MilliQ water, in order to obtain five stock solutions at 0.5 w% containing one polymer each. The so prepared stock solutions where diluted in ultra-pure water (0.055μS) (PURELAB Flex, ELGA LabWater).

For the protein quantification Bradford protein assay solution ROTI-Quant (5× concentrated, Carl Roth, Karlsruhe, Germany) was used (Bradford, 1976; Song et al., 2021). 2′-7′ Diclorofluorescin–Diacetate (2,7-DCFH-DA) (Sigma-Aldrich, St. Louis, USA) was used for the determination of the reactive oxygen species content (Deng et al., 2009).

2.3. Acute tests

2.3.1. Acute exposure experiment

A 48-hour acute exposure experiment was performed following the OECD 202 guidelines (Test No. 202: Daphnia Sp. Acute Immobilisation Test, 2004). Four concentrations (1 mg/L, 5 mg/L, 10 mg/L and 50 mg/L) of the selected WSSPs were used for this test. The procedure was performed on four replicates with 5 neonates each per treatment, exposed in beakers containing 100 mL of the modified M4 medium. During this test mortality and motility within 48-hour were considered as the only endpoints.

2.3.2. Heartrate assay

A heartrate acute assay was conducted on 48-hour old daphnids. Ten animals per concentrations plus ten for control were randomly selected for the exposure to each polymer (forty animals for each WSSP treatment, two hundred animals in total). For the first 48-hour the animals were maintained in the local husbandry and fed with 1 mgC/L of *A. obliquus*. After this period, they were mounted on sterile syringe needles (Ø 0.8 × 22 mm 21G × 7/8", B. Braun Melsungen AG, D-34209 Melsungen, Malaysia) using a drop of Vaseline (MOLYDUVAL, Ratingen, Germany) as adhesive and inserted in 2.5 mL cuvettes (disposable cuvettes, PS 12.5 × 12.5 × 45 mm, BRAND GMBH, Wertheim, Germany) filled with the modified M4 medium and algae as a food source (see more details in the extended methods in the SI). After a 45-minute acclimatization time the animals were recorded with a high-speed camera (i-Speed 3 Olympus Shinjuku, Japan at 60 fps to avoid aliasing), focusing on their hearts, for at least 300 frames (5 s) of which only 300frames were used for further analysis. The organisms were then moved to new cuvettes containing three different concentrations of each of the WSSPs (1 mg/L, 5 mg/L and 10 mg/L) and a blank that consisted of culture medium with the addition of 0.2 % ultra-pure water. This addition corresponded to the same volume of polymer solution that was added in the highest concentration. Ultra-pure water was chosen as control because it was used as solvent for the preparation of the WSSPs' stock solutions. After another 45 min of acclimatization in the treatments the animals were recorded again as described above.

2.4. Chronic toxicity test and ROS quantification

A 21-day chronic exposure experiment was carried out according to OECD 211 (Test No. 211: Daphnia magna Reproduction Test, 2008) using three different concentrations (1 mg/L, 5 mg/L and 10 mg/L) of the selected WSSPs. Due to handling the experiment was set up on two consecutive days. On the first day the exposure started with PVOH, PEO and PEG, on the consecutive day started the procedures with PAA and PVP. For each exposure group an individual control was set up, resulting in two control groups.

Each treatment and the two controls had fifteen replicates, each containing one individual of *D. magna* (with an age < 24-hour). The experiments were performed in 160 mL beakers (Weck GmbH u. Co. KG, Wehr-Öflingen, Germany) containing 50 mL of the modified M4 medium. In order to guarantee constant exposure conditions, the exposure medium was renewed every second day. *Daphnia* were fed with 0.5 mgC/L of *Acutodesmus obliquus* every second day. As life history parameters we recorded different morphological parameters at primiparity and at the end of the exposure, the number of reproductive events, the amount of daphnids hatched daily (neonates) and the levels of ROS.

The measurements of the specimens at primiparity and at the end of the chronic exposure was carried out using a dissecting microscope equipped with a digital camera (Leica M50, Wetzlar, Germany; camera: OLYMPUS DP26, Hamburg, Germany; Light: Leica KL 300 LED, Wetzlar, Germany). The following morphological parameters were considered: body length (measured from the anterior edge of the compounded eye to the base of the tail-spine) and body width (defined as the maximal length between the dorsal and ventral edge of the carapace at the region of the heart) (Fig. SI 2). These morphological parameters were recorded using the software cellSens Dimension (v1.11, OLYMPUS, Hamburg, Germany). For the measurements at primiparity the animals were caught and then released immediately after the picture for the measurements was taken. At the end of the exposure the specimens were photographed and then sacrificed in liquid nitrogen.

At the end of the chronic exposure the animals of each treatment were pooled and homogenized with a pestle in potassium phosphate buffer (100 mM, pH = 7.4, with 100 mM KCl, 1 mM EDTA and protease inhibitors 1/100 v/v) directly in a 1.5 mL Eppendorf tube (Eppendorf Group, Hamburg, Germany). The homogenate was then centrifugated at 15000*g* for 30 min at 4 °C. The supernatant was used to perform biochemical analyses with two technical replicates. Before quantifying the ROS levels, we assessed the sample's protein content according to the Bradford's method (Bradford, 1976). To be able to evaluate the amount of protein we had to use all the exposed animals, hence for this procedure we relied on the use of technical replicates. The protein quantification was performed using a microplate reader (Synergy HT, BioTek Instruments, Friedrichshall, Germany) (λ_{ab} = 595 nm). The data obtained via this assay were then used for normalization. Then we quantified the amount of ROS using the fluorimetric method described by Deng and coauthors (Deng et al., 2009). This method relies on the fluorescence change (λ_{ex} = 585 nm, λ_{em} = 530 nm)) determined in the 2'7' DCFH-DA (Sigma-Aldrich, St. Louis, Missouri, USA) by the presence of pro-oxidative molecules. ROS concentration was normalized to the protein content and expressed in arbitrary units AU DCF/mg proteins.

2.5. Statistical analyses

The statistical analyses were performed using R (R version 4.0.2, © 2020 The R Foundation for Statistical Computing). To test for normal distribution and homogeneity of the residuals we used the Shapiro Wilk and Levene Test together with a visual inspection using Q-Qplot (quantile-quantile plot) for estimating normal distribution. Since the conditions of normality and homogeneity were met, we used a linear regression model (ANOVA) followed by Tukey HSD post-hoc test with Holm's correction method for multiple comparison by using the package *emmeans* (Lenth et al., 2020).

3. Results

3.1. Acute toxicity tests

3.1.1. 48-hour acute toxicity test

After the 48-hour acute toxicity test neither immobilization nor mortality were registered in the test organisms for the control and any of the four concentrations tested (1 mg/L, 5 mg/L, 10 mg/L and 50 mg/L) of all used WSSPs.

3.1.2. Heartrate acute assay

The heartrate acute assay did not reveal any significant alteration on the heart beat rate of the exposed daphnids for any of the used treatments as shown in Fig. 1. The only treatment where there could be a trend is the one with PVP, for which we could see a decrease in the heartrate with an increase of the concentration of exposure which however is not significant (Table SI 1 and Fig. SI 3 for additional statistical information).

3.2. 21-day chronic toxicity test

3.2.1. Effects on reproduction

At the end of the chronic exposure experiment significant alterations in the number of neonates were registered (Fig. 2). In particular we observed a decrease in the number of offspring in the animals exposed to PVOH 5 mg/L ($t = -4.451$, $p = 0.0001$) and 10 mg/L ($t = -4.585$, p = 0.0001), PEO 10 mg/L ($t = -2.827$, $p = 0.027$), PEG 5 mg/L ($t = -3.580$, $p = 0.002$) and PEG 10 mg/L ($t = -5.103$, $p < 0.0001$) when compared to the corresponding control group 1 (Table SI 2).

Alterations were also detected in the total number of reproductive cycles (Fig. 3). In detail we observed a reduction in the number of reproductive events in the organisms exposed to PVOH 10 mg/L ($t = -3.120$, $p = 0.018$) and to PEG 10 mg/L ($t = -3.629$, $p = 0.003$) when compared to the corresponding control group, and in the animals exposed to PAA 10 mg/L ($t = -2.862$, $p = 0.026$) and to PVP 5 mg/L ($t = -3.103$, $p = 0.015$) when compared to the corresponding control group 2 (Table SI 3).

No significant alteration was recorded in the number of days to primiparity.

During the exposure we registered one dead individual in the 15 individuals in "control group 1", in PEO 1 mg/L, in PEO 5 mg/L and in PAA 5 mg/L.

3.2.2. Effects of WSSPs on morphological parameters

Regarding morphological parameters no alterations were detected at primiparity (defined as the age at first reproduction) neither in terms of body length nor body width (Table SI 4 and 5).

Alterations were however recorded at the end of the 21-day exposure.

In detail a significant reduction in body-length (Fig. 4) was registered for the cladocerans exposed to PVOH 10 mg/L ($t = -3.495$, $p = 0.005$) compared to the corresponding control group, while an increase in length was observed in the organisms exposed to PVP 10 mg/L ($t = 3.284$, $p = 0.008$)) compared to the corresponding control group (Table SI 6). A reduction in body-width was observed just for PVOH 5 mg/L ($t = -3.073$, $p = 0.023$) compared to the corresponding control group (Fig. 5) (Table SI 7).

3.2.3. ROS production

After the 21-day chronic exposure the surviving *Daphnia* of each treatment were pooled together and homogenized to perform an evaluation on the levels of ROS (Fig. 6). The levels of pro-oxidative molecules appear to increase along with the concentration of exposure, for every treatment except for the one with PVOH, where it seems the lowest concentration determined a major increase of ROS when compared to the two higher ones.

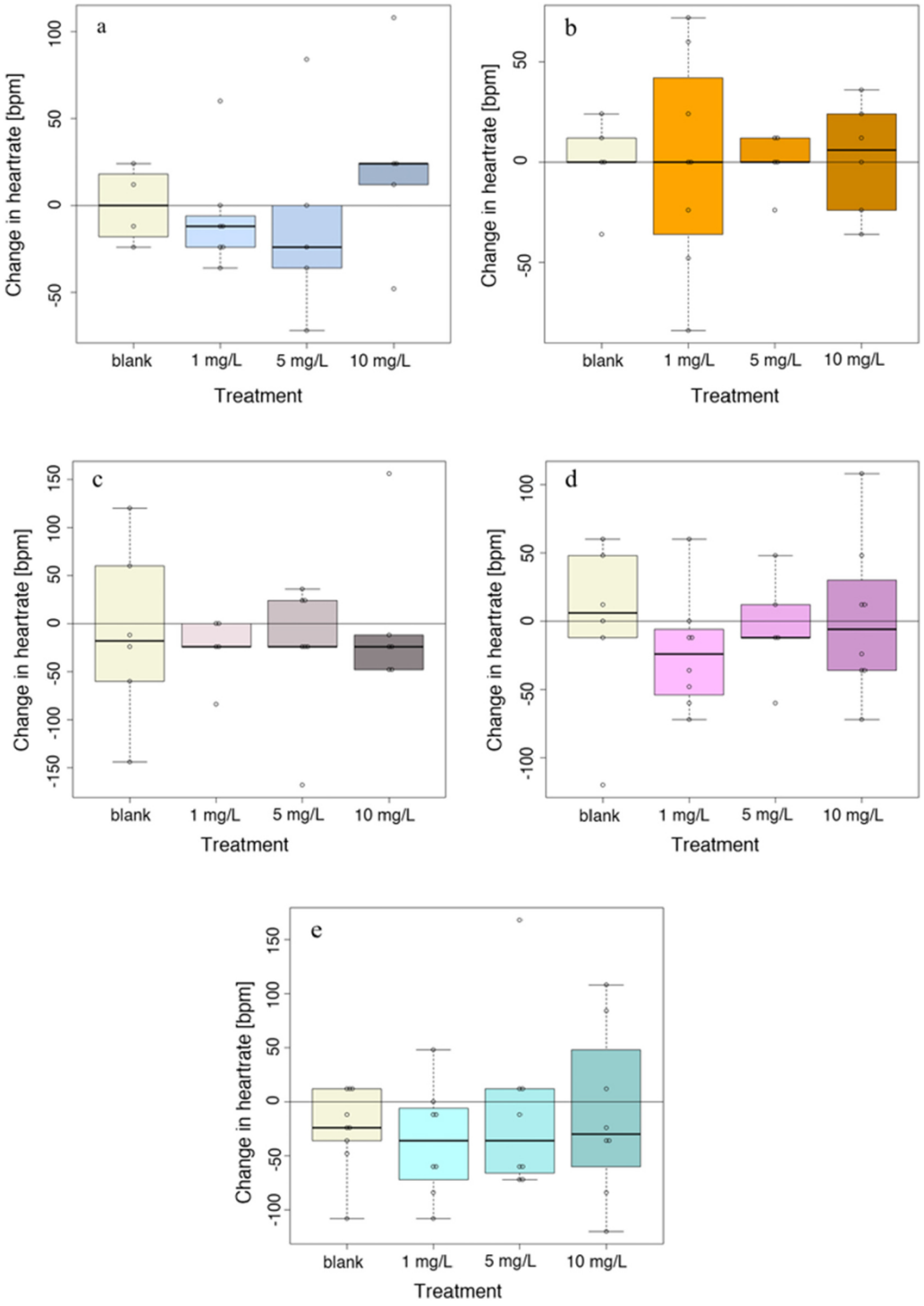


Fig. 1. Change in the heartrate (bpm) as a result of the heartrate acute assay. The boxplots represent the delta in the heartrate before and after the exposure. (a) Treatment with PVOH. (b) Treatment with PEO. (c) Treatment with PEG. (d) Treatment with PAA. (e) Treatment with PVP. Labels on the x-axis indicate treatment, points indicate delta data of one animal. Additional statistical information can be found in Table SI 1 and Fig. SI 3.

4. Discussion

Nowadays plastics in the environment are one of the most discussed topics, both by researchers and the public. However, the discussion so far has been focused mainly on the potential threat represented by particulate plastics, neglecting the hidden risk that WSSPs might pose.

Given their extensive use they also occur in the environment (Antić et al., 2011; Wang et al., 2021). In contrast to particulate microplastics, WSSPs do homogeneously dissolve in water (Kvale et al., 2020). Thus, aquatic organisms are expected to be uninformedly exposed to these polymers and not predominantly at the surface or the sediments of the water body as observed for microplastics.

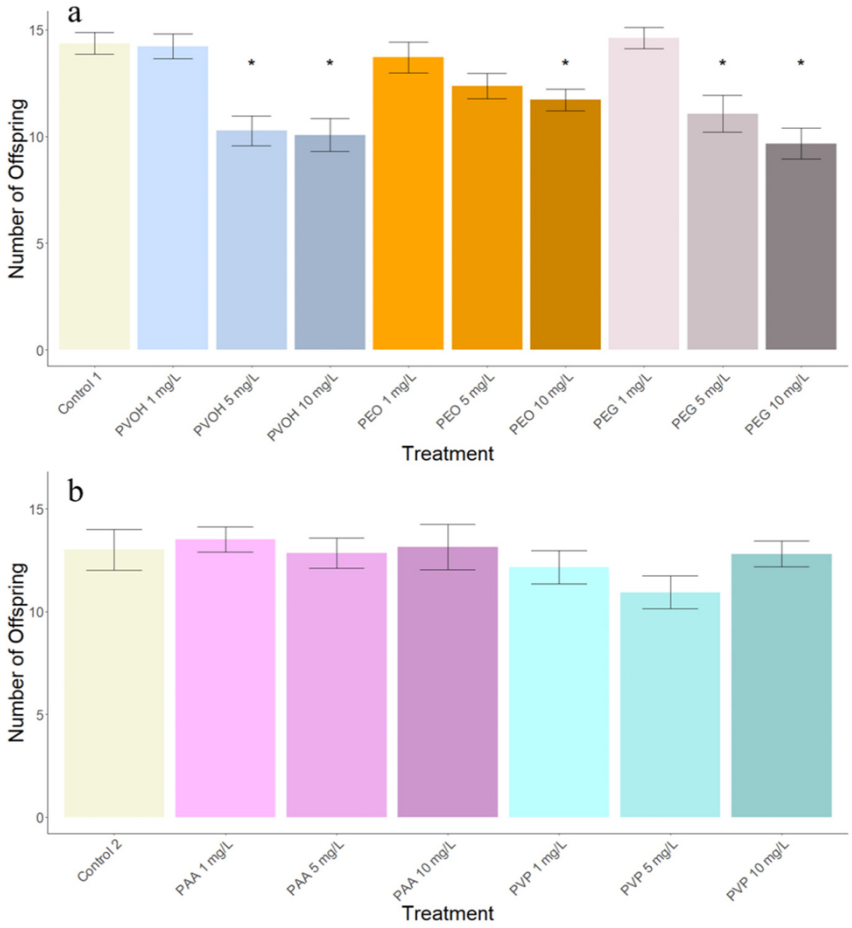


Fig. 2. Number of offspring during the chronic exposure. (a) number of offspring of the animals treated with PVOH, PEO and PEG compared to Control 1. Asterisks represents significance ($p < 0.05$) compared to the control. Error bar represents standard error. (b) number of offspring of the animals treated with PAA and PVP compared to Control 2. Asterisks represents significance ($p < 0.05$) compared to the control. Error bar represents standard error. Additional statistical information can be found in Table SI 2.

Acute toxicity testing with model organisms like *D. magna* is a standardized procedure for the assessment of the ecological risk for non-target organisms resulting from different chemicals. The scientific literature has plenty of examples of these kind of standardized test being used to analyze the effects of a plethora of water-soluble compounds, like pharmaceuticals, pesticides etc. (Daniel et al., 2019; Horton et al., 2018; Haap et al., 2008; Pretti et al., 2009; Okamoto et al., 2015; Cappelli et al., 2020; Han et al., 2006). Hence, we employed these tests first also for WSSPs. However, the investigated WSSPs do not exert any acute effect up to 50 mg/L on *D. magna*. Since the highest concentration of a WSSP (PVP) measured at the outflow of a sewage treatment plant was reported as 7.1 mg/l (Antić et al., 2011) it is unlikely that the tested WSSPs show any acute toxicity in nature. However, even if not acute toxic, any chemical compound can interfere with the physiology of an organism and therefore might affect its fitness. Therefore, the heartrate assay was included in our investigations since the cardiac rhythm could represent a reliable measure to assess the physiological effects of the exposed (Fekete-Kertész et al., 2018; Xu et al., 2017; Duong et al., 2021). In our research, however, the results we obtained do not show any significant alteration to the heartrate of *Daphnia* for the concentrations of WSSPs under investigation.

Despite the fact that this test suggests that WSSPs did not induce any acute physiological alterations we did observe a chronic impairment in the life history of the exposed organisms for most of the employed polymers, both in terms of number of living offspring and reproductive events. In particular, a lower amount of produced neonates was observed for the animals that were exposed to PVOH (5 mg/L and 10 mg/L), PEO (10 mg/L) and PEG (5 mg/L and 10 mg/L). A reduced amount of reproductive cycles was recorded in the treatments with PVOH (10 mg/L), PEG (10 mg/L), PAA (10 mg/L) and PVP (5 mg/L). Further, a reduction in body size was recorded for the animals exposed to PVOH (5 mg/L and 10 mg/L), while the organisms exposed to PVP (10 mg/L) were observed to grow bigger compared to the control.

Differences between PEO and PEG, that might be due to the different molecular weight of these polymers composed of ethylene oxide repeated units (M_w respectively1000 and 900,000), can be observed just in terms of reproductive outcome. In fact, while just the cladocerans exposed to the highest concentration of PEO show a significant decrease in the number of produced offspring, for the animals treated with PEG we registered a decrease in the amount of neonates at both concentrations 5 mg/L and 10 mg/L, together with a reduction in the number of reproductive events at 10 mg/L. These results suggest that the toxicity increases with the increase of the molecular weight. No significant differences were however recorded on the investigated morphological parameters.

The reproductive success and *Daphnia* body measurements are important parameters to consider, as they are frequently assessed as endpoints in ecotoxicological studies and as their impairment might ultimately translate on multiple levels of the biological hierarchy. Numerous studies have in fact showed the toxicity of different compounds assessing these variables. For example Sancho et al. (2018) demonstrated that the exposure to the pyrethroid etofenprox impaired both the reproductive outcome and

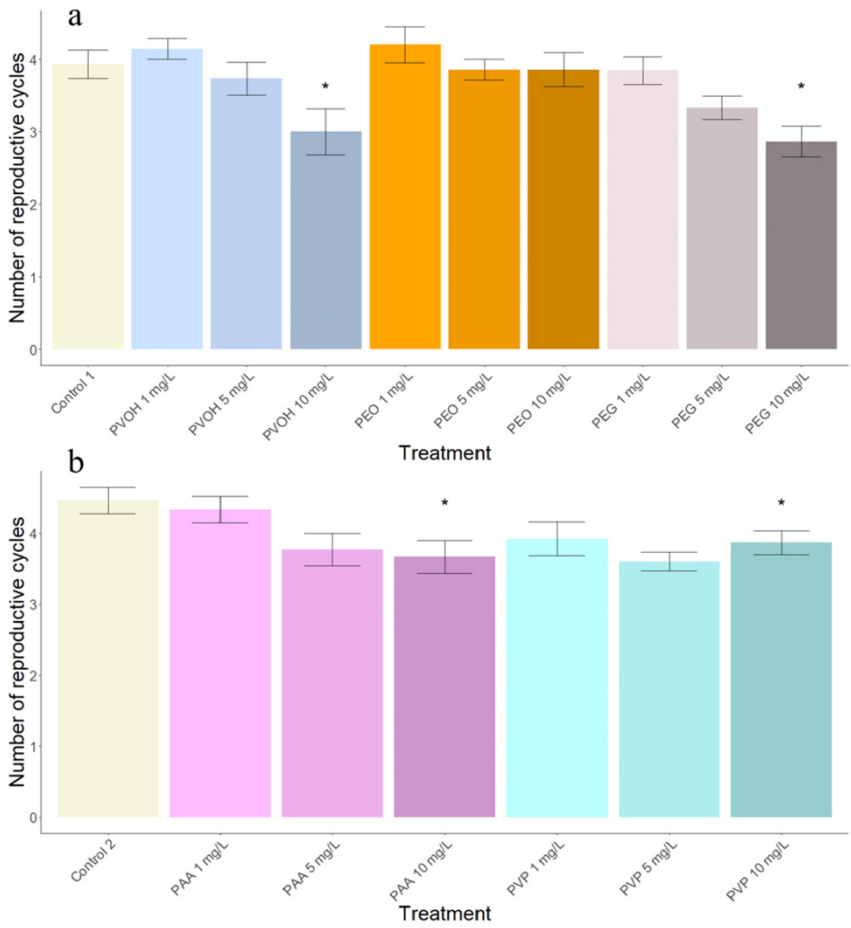


Fig. 3. Number of reproductive cycles during the 21-day chronic toxicity test. (a) number of the reproductive cycles of the animals treated with PVOH, PEO and PEG compared to Control. Asterisks represents significance ($p < 0.05$) compared to the control. Error bar represents standard error. (b) number of reproductive cycles of the animals treated with PAA and PVP compared to Control 2. Asterisks represents significance ($p < 0.05$) compared to the control. Error bar represents standard error. Additional statistical information can be found in Table SI 3.

the growth in *D. magna* at environmentally relevant concentrations. The same was recorded by Rocha et al. (2014) on *Daphnia* exposed to anticholinesterasic drugs. Further, also microplastics exposure showed to have detrimental effects over growth and reproduction (Zimmermann et al., 2020; Jaikumar et al., 2019; Bosker et al., 2019; Schwarzer et al., 2022). Detrimental effects on the reproductive success might manifest at first on the population level, with a decline of *Daphnia* biomass. This could then translate over the biological community and the ecosystem as an essential link of the freshwater food web would be diminished (Bosker et al., 2019).

Alterations in morphological parameters might also have relevant environmental repercussions, both in case of a size reduction and increase. A decrease in body size like we observed in the organisms exposed to PVOH might in fact make the daphnids more susceptible to gape-limited predators like *Mesocyclops* (Chang and Hanazato, 2003; Krylov, 1992) contributing so to the reduction of their biomass(Schwarzer et al., 2022). By contrast, an increased in size, as was recorded for the animals exposed to PVP, might render them more vulnerable to visual predators e.g. the small sunfish *Lepomis macrochirus* or *Notonecta undulata,* for which a smaller body size was observed as an inducible defense in different exposed *Daphnia* species (Dodson, 1989). However, if size alterations as a consequence of PVOH or PVP exposition would overweigh an inducible defense in nature must be investigated in future.

A potential reason for the effects we observed on life history may be oxygen depletion since water soluble polymers may cover the gill surface or interact with oxygen transfer. A study conducted by Goodrich et al. (1991) on *Oncorhynchus mykiss* (rainbow trout), proved that cationic WSSPs are able to bind via ionic interactions onto the fishes' gills, resulting in a toxic effect on the organisms due to an impaired oxygen transfer through damaged gill surfaces. However, since the compounds employed in this work are not charged and *Daphnia*'s gills are physically separated on the thoracopoda it is unlikely that they collapse and stick together as it was previously observed on *O. mykiss* and hence exert negative effect on the cladocerans. Further, the fact that the heartrate of *D. magna* was not increased during exposure renders an impaired oxygen transfer unlikely.

A further potential reason why we observed an impairment in the reproductive success and a reduction in size when it comes to the PVOH treatment might be the onset of an altered oxidative status, as suggested by the increased amount of ROS within the organisms. Several studies (e.g. Trestrail et al., 2020) have shown how exposure to different contaminants can enhance the intracellular ROS production, leading to the activation of different energy expensive physiological defenses (De Felice et al., 2020; Xia et al., 2020; Parolini et al., 2018). Under normal conditions the organisms use most of their energy reserves for growth, reproduction and for their basal metabolism (Arzate-Cárdenas and Martínez-Jerónimo, 2012). However, when they have to face the adverse effects induced by the presence of one or more toxic compounds, such as WSSPs, the energy required for the basal metabolism is much higher. Ultimately this results in an energy depletion and a

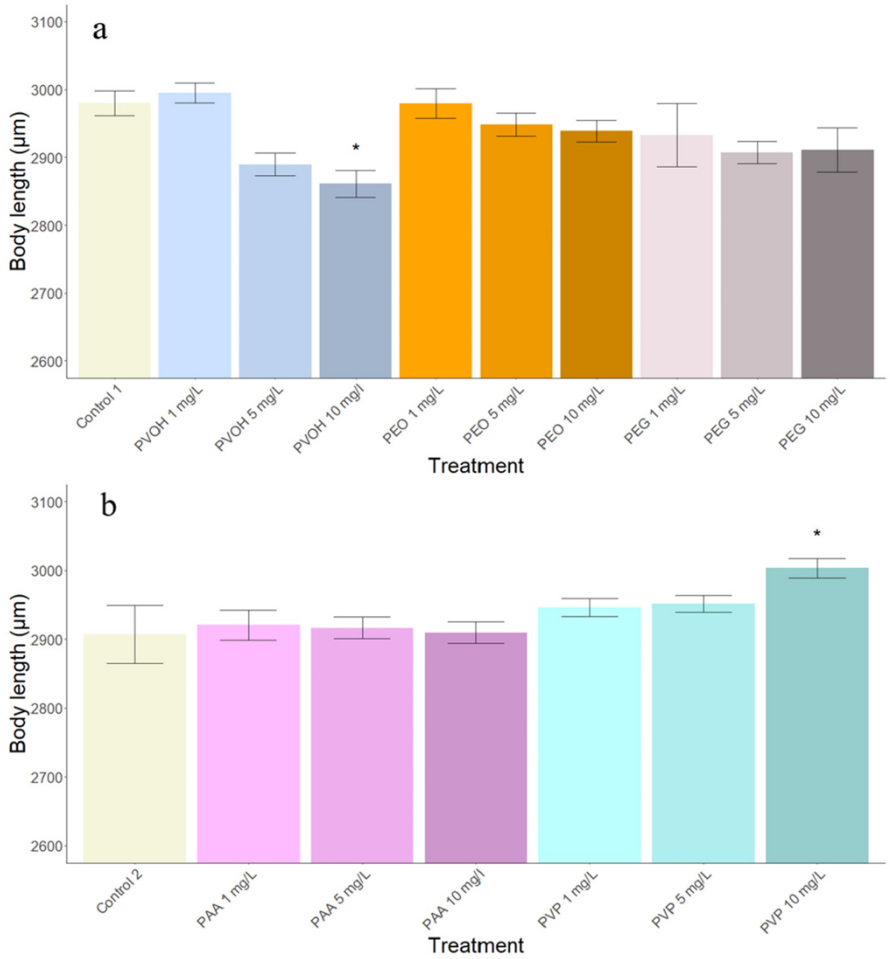


Fig. 4. Body length of the exposed animals at day 21. The measures are expressed in µm. (a) Body length of the animals treated with PVOH, PEO and PEG compared to Control 1. Asterisks represents significance ($p < 0.05$) compared to the control. Error bar represents standard error. (b) Body length of the animals treated with PAA and PVP compared to Control 2. Asterisks represents significance ($p < 0.05$) compared to the control. Error bar represents standard error. Additional statistical information can be found in Table SI 6.

different allocation of the resources to detoxification and antioxidant enzymes (Pieters et al., 2006; Knops et al., 2001; Nogueira et al., 2004). The preferential allocation of resources in favor of the biochemical defenses of the organisms might so explain the impairment of the reproductive success we observed in our study. This phenomenon was previously described for different substances, by e.g. Parolini et al. (2018) after the exposure of *D. magna* to benzoilegonine or by Liu et al. (2022)after the exposure of the same model organism to poly (vinyl chloride) (PVC) microplastics.

However, an altered oxidative status does not explain the findings concerning *Daphnia* exposed to PVP, for which we observed an increase in body length at the higher concentration over the 21-day exposure, together with the highest concentration of ROS among the tested animals. The increased growth rate of the organisms exposed to PVP might be due to a phenomenon called hormesis (Calabrese and Baldwin, 1998; Gems and Partridge, 2008; Mattson, 2008). This term refers to the biphasic response that can be observed after the exposure to some chemicals: lower doses might show a beneficial effect in terms of growth rate, fitness and stress resistance, while higher concentrations of the same compound could exert a toxic effect.

Nevertheless, a difference in resource allocation in favor of the antioxidant defenses is consistent with a lower reproductive outcome. Even though there was no induced mortality or any alteration in days to primiparity among all exposed daphnids, the reduction in the number of offspring and reproductive events could still have an impact over population and ecosystem dynamics. *D. magna* is relevant for ecotoxicological studies, as it represents a key species in lentic pelagic ecosystems. They are primary consumers for phytoplankton and an essential food source for secondary consumers (Miner et al., 2012). They therefore represent a crucial link for carbon and energy transfer between primary producers and higher trophic levels (Diel et al., 2020). A decrease in the number of daphnids might impair the top-down control of the phytoplankton, possibly leading to algal blooms and eutrophication (Ekvall et al., 2014). Moreover, a reduction in daphnids biomass could alter the bottom-up control of higher trophic levels, disrupting so the entire ecosystem function (Bosker et al., 2019).

5. Conclusion

Our study showed that, even though no acute effect was detected in terms of mortality, immobilization or alterations on the heartrate, the chronic exposure to five commercially available and extensively used WSSPs exerts sublethal effects on *D. magna*. We did record impairments on life history traits and morphological parameters of the exposed organisms. This may be concerning as the WSSPs mediated impairment of *Daphnia*'s fitness would lead to negative effects on the population level, which,

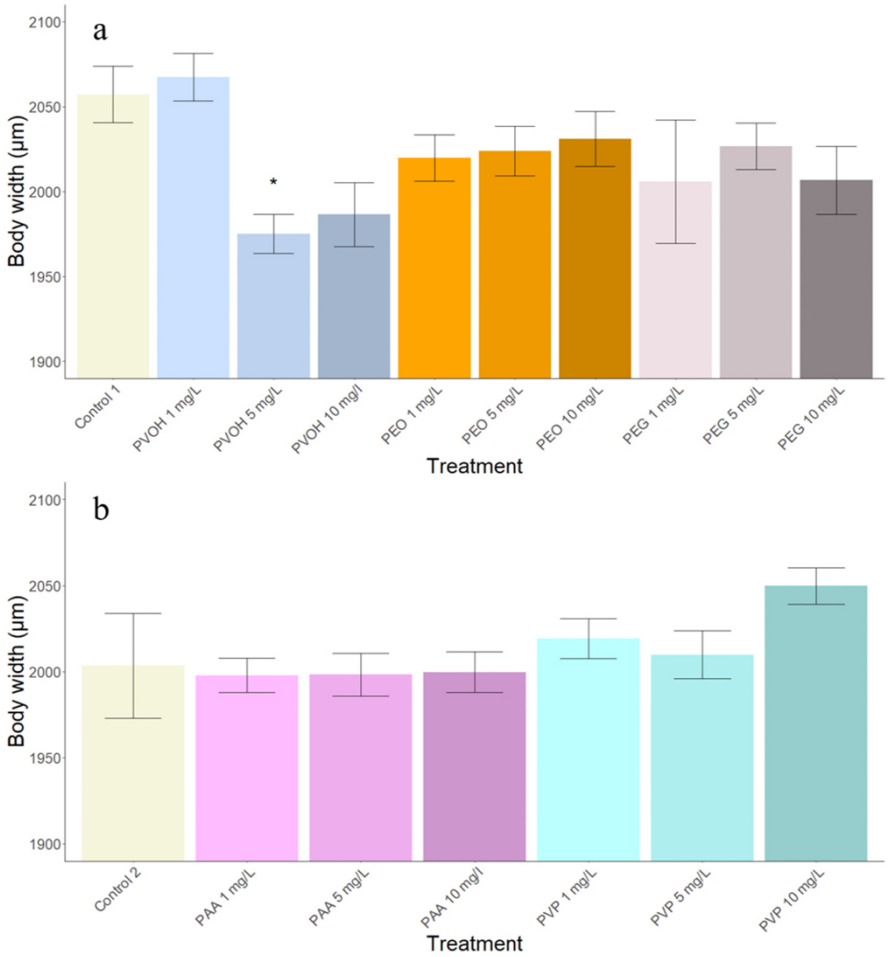


Fig. 5. Body width of the exposed animals at day 21. The measures are expressed in µm. (a) Body width of the animals treated with PVOH, PEO and PEG compared to Control 1. Asterisks represents significances ($p < 0.05$) compared to the control. Error bar represents standard error. (b) Body width of the animals treated with PAA and PVP compared to Control 2. Asterisks represents significance ($p < 0.05$) compared to the control. Error bar represents standard error. Additional statistical information can be found in Table SI 7.

over the long run, might potentially cascade over higher trophic levels, hence impairing the entire community and ecosystem. Nevertheless, a huge gap of knowledge still exists what impact the WSSPs could have and what are their mode of action, since our results suggest that the molecular weight of WSSPs may alter their toxicity. Hence, it would be highly desirable to have further investigations on the potential threat represented by these and other WSSPs, using not only *D. magna,* but also including other organisms.

CRediT authorship contribution statement

Simona Mondellini: Conceptualization, Methodology, Investigation, Writing - Original Draft, Writing - Review & Editing, Formal analysis, Visualization, Project administration, Validation, **Matthias Schott:** Conceptualization, Methodology, Investigation, Writing - Review & Editing, Formal analysis, Validation, **Martin G. J. Löder:** Conceptualization, Resources, Writing - Review & Editing, Validation, Project administration, Supervision, Funding acquisition, **Seema Agarwal:** Project administration, Funding acquisition, Resources, Development of Reference Material, **Andreas Greiner:** Project administration, Funding acquisition, Resources, Development of Reference Material, **Christian Laforsch:** Conceptualization, Resources, Writing - Review & Editing, Validation, Project administration, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Christian Laforsch reports financial support was provided by Horizon 2020. Christian Laforsch reports financial support was provided by Deutsche Forschungsgemeinschaft (DFG, German Research Foundation).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.157608.

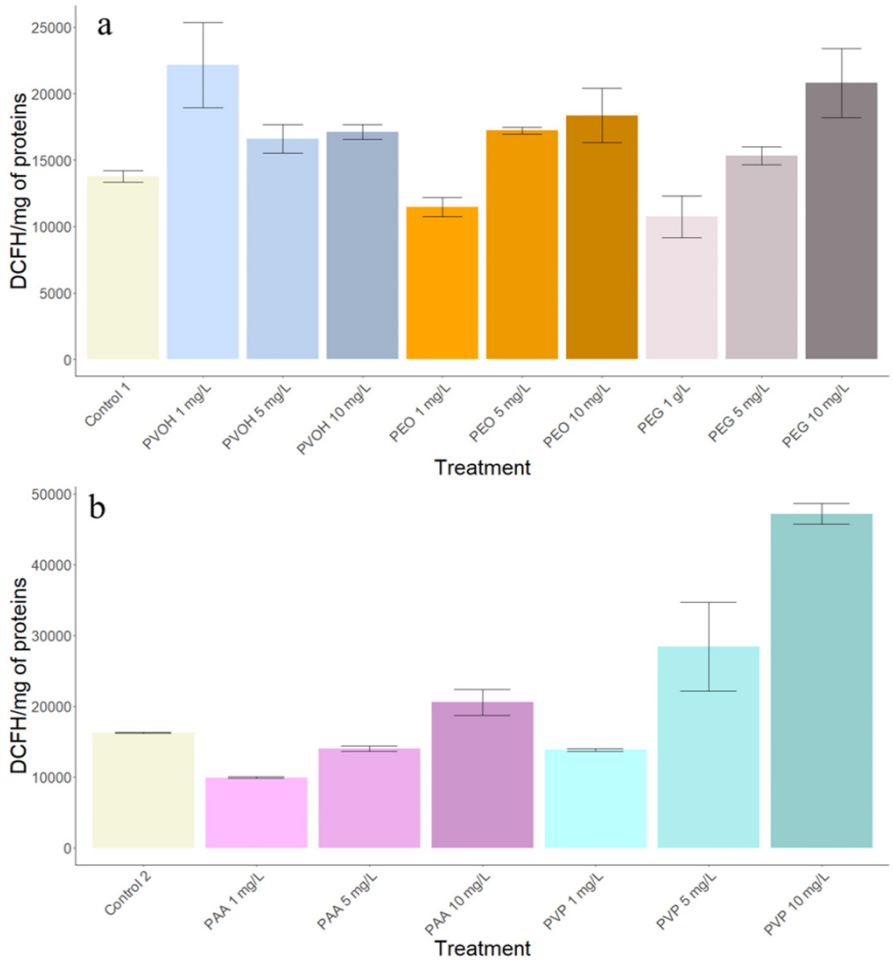


Fig. 6. Levels of ROS expressed as arbitrary fluorescence units and normalized to de protein content of each sample. (a) Levels of ROS of the animals treated with PVOH, PEO and PEG compared to Control 1. Error bar represents standard error. (b) Levels of ROS of the animals treated with PAA and PVP compared to Control 2. Error bar represents standard error.

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Supplementary material pertaining to Article V

Beyond microplastics: Water soluble synthetic polymers exert sublethal adverse effects in the freshwater cladoceran *Daphnia magna*

Authors: Simona Mondellini, Matthias Schott, Martin G.J. Löder, Seema Agarwaal, Andreas Greiner, Christian Laforsch

Supplemental information

Figure SI 1: Repeat unit structures of A) PEG and PEO, B) PVP, C) PVOH, and D) PAA.

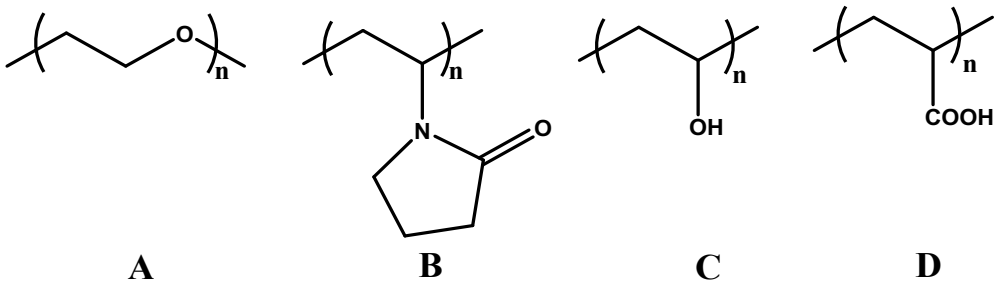


Figure SI 2: Picture of a D. magna specimen measured with cellSens Dimension. The red lines represent the measures of the body parameters.

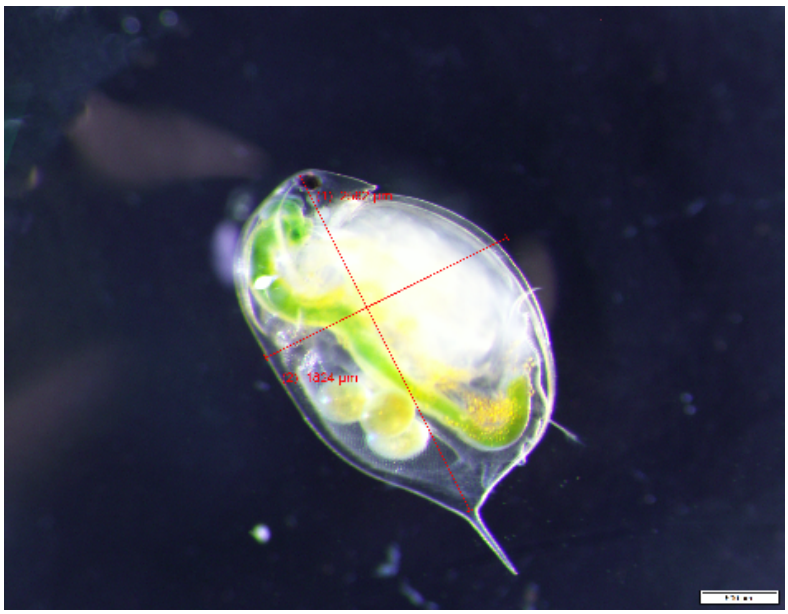
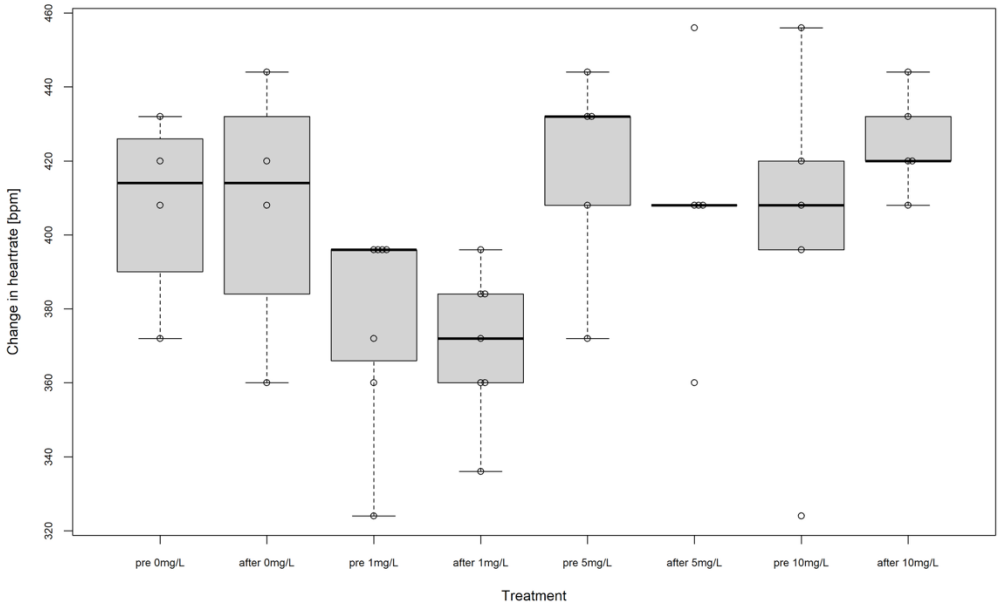
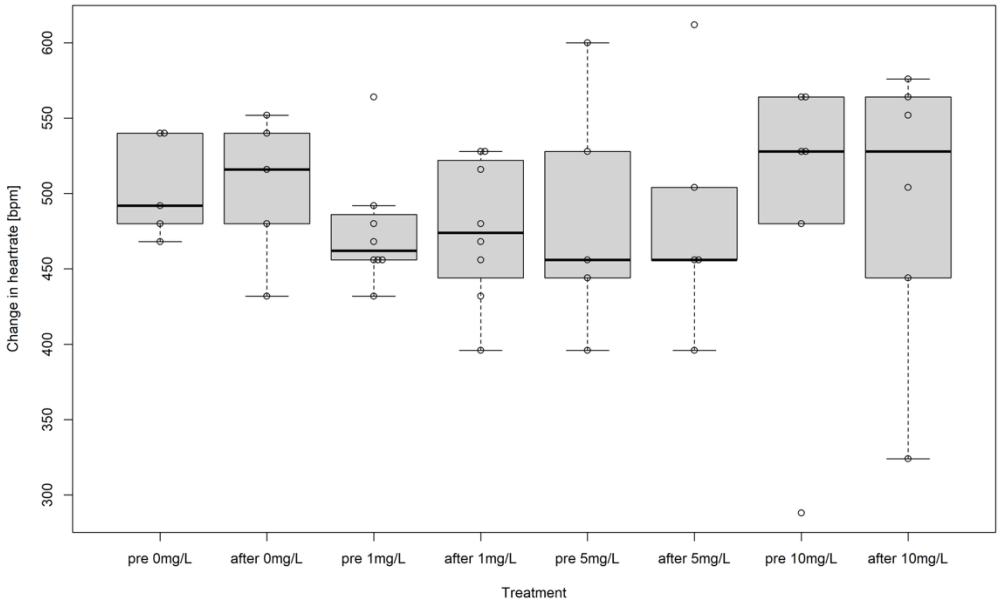


Figure SI 3: change heartrate (bpm) as a result of the heartrate acute assay. The boxplots represents the heartrate pre and after exposure (x-axis) to each polymer. Labels on the x-axis indicate treatment, points indicate data of one animal.

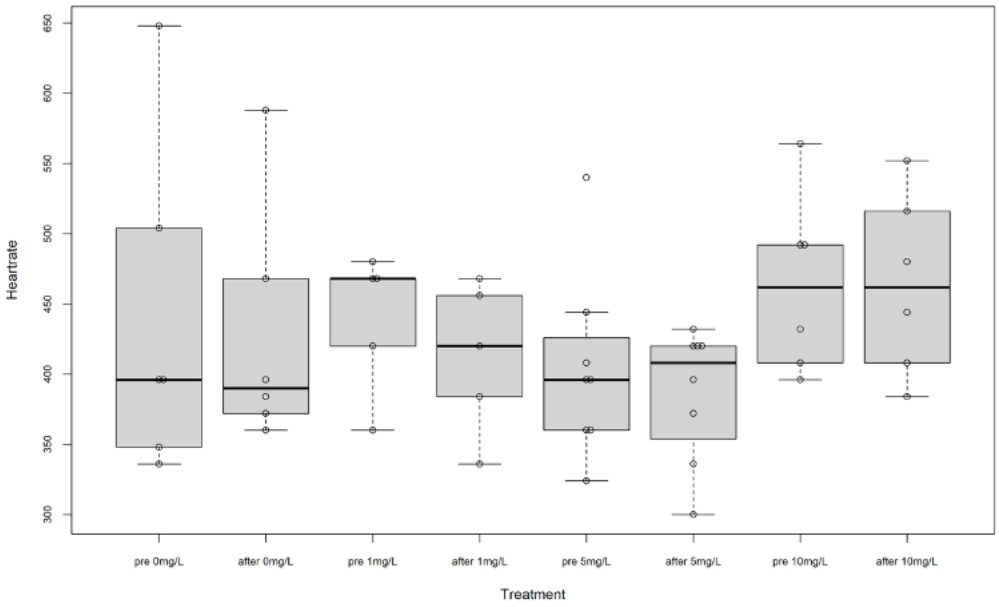
(A) Treatment with PVOH.



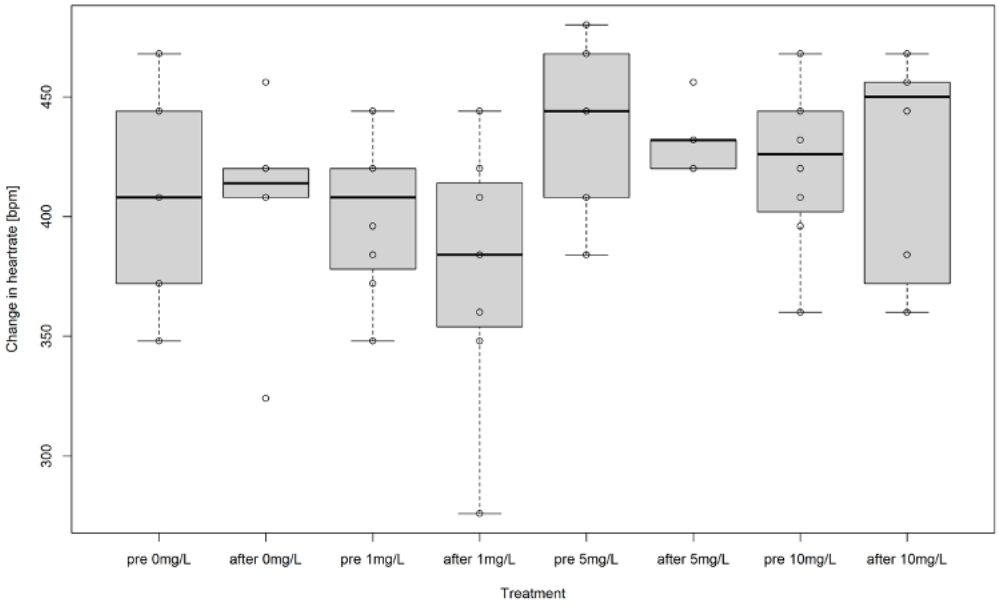
(B) Treatement with PEO



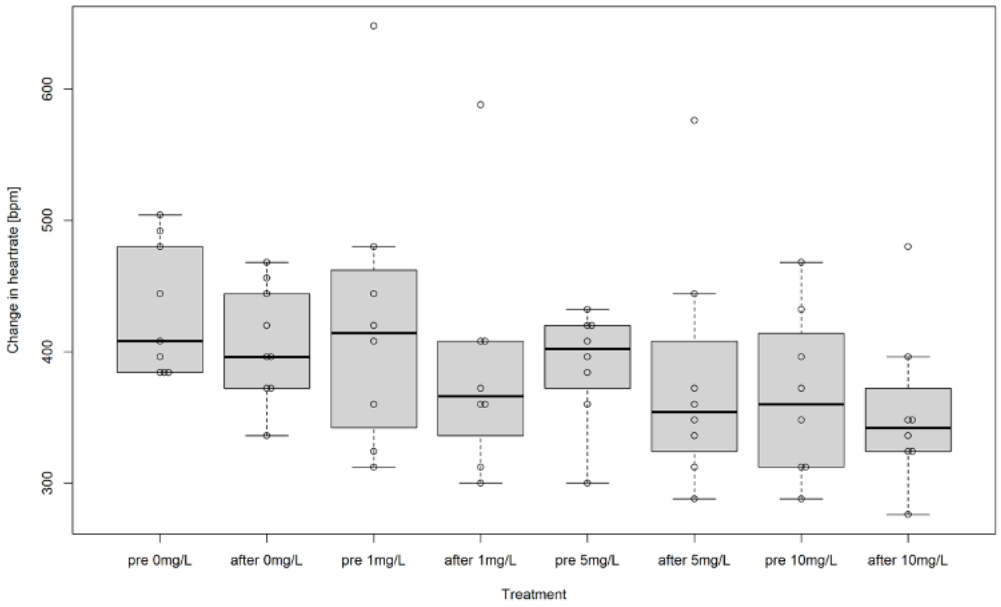
(C) Treatment with PEG



(D) Treatment with PAA



(E) Treatment with PVP



Details of the heartrate assay

Every cuvette used for the test was inserted in a 3D printed (Prusa i-3 MK-8, YOTINO, Guangdong, CN) non-transparent cuvette holder (made with Ø 1.75 mm white PLA filament, Form Futura, Nijimegen, Netherlands), with a dent specially designed to hold a red led light as light source in a 45 ° angle towards the animal and a window to record the heartbeat.

The acclimatization time was determined by pre-experiments and ensured that the test organisms recovered from the stress caused by the transfer to the cuvette (data not shown).

The videos were then cut to 300 frames and heartbeat movements were amplified by means of eulerian video magnification [1]. The heart beats were automatically counted from full extension of the heart as one beat with a self-coded Python3 program. If the standard deviation of the beat to beat duration exceeded 0.5 bpm, this was an indication of an unstable algorithm output. These videos were additionally manually counted in slow motion (1/3 of the original speed) to verify the heartrate.

Table SI 1: summarised statistical analyses of the heartrate acute assay. Each treatment is compared to its own control group (BH-method used for p-value adjustment).

Treatment	***df***	***t-value***	***Pr(> |t|)***
PVOH 1 mg/L	17	-0.247	0.808
PVOH 5 mg/L	17	-0.322	0.808
PVOH 10 mg/L	17	0.806	0.808
PEO 1 mg/L	20	0.000	1.000
PEO 5 mg/L	20	0.000	1.000
PEO 10 mg/L	20	0.092	1.000
PEG 1 mg/L	20	-0.371	0.815
PEG 5 mg/L	20	-0.303	0.815
PEG 10 mg/L	20	0.238	0.815
PAA 1 mg/L	23	-0.733	0.930
PAA 5 mg/L	23	-0.089	0.930
PAA 10 mg/L	23	0.125	0.930
PVP 1 mg/L	29	-0.388	0.701
PVP 5 mg/L	29	0.436	0.701
PVP 10 mg/L	29	0.388	0.701

Table SI 2: summarized statistical information of the number of offspring recorded during the 21-day chronic toxicity test (Holm method used for p-value adjustment). Each polymer group is compared to its control (C).

Treatment	***df***	***t-ratio***	***p-value***
PVOH 1 mg/L – C1	128	-0.153	1.0000
PVOH 5 mg/L – C1	128	-4.415	0.0001
PVOH 10 mg/L – C1	128	-4.585	0.0001
PEO 1 mg/L – C1	128	-0.642	1.0000
PEO 5 mg/L – C1	128	-2.140	0.1372
PEO 10 mg/L – C1	128	-2.827	0.0237
PEG 1 mg/L – C1	128	0.271	1.0000
PEG 5 mg/L – C1	128	-3.580	0.0029
PEG 10 mg/L – C1	128	-5.103	<.0001
PAA 1 mg/L – C2	89	0.401	1.0000
PAA 5 mg/L – C2	89	-0.126	1.0000
PAA 10 mg/L – C2	89	0.113	1.0000
PVP 1 mg/L – C2	89	-0.692	1.0000
PVP 5 mg/L – C2	89	-1.749	0.5025
PVP 10 mg/L – C2	89	-0.169	1.0000

Table SI 3: summarized statistical information of the number of reproductive cycles during the 21-day chronic toxicity test (Holm method used for p-value adjustment).

Treatment	***df***	***t-ratio***	***p-value***
PVOH 1 mg/L – C1	128	0.720	1.0000
PVOH 5 mg/L – C1	128	-0.667	1.0000
PVOH 10 mg/L – C1	128	-3.120	0.0179
PEO 1 mg/L – C1	128	0.833	1.0000
PEO 5 mg/L – C1	128	-0.240	1.0000
PEO 10 mg/L – C1	128	-0.240	1.0000
PEG 1 mg/L – C1	128	-0.272	1.0000
PEG 5 mg/L – C1	128	-2.034	0.3080
PEG 10 mg/L – C1	128	-3.629	0.0037
PAA 1 mg/L – C2	89	-0.437	0.6632
PAA 5 mg/L – C2	89	-2.409	0.0723
PAA 10 mg/L – C2	89	-2.862	0.0262
PVP 1 mg/L – C2	89	-1.873	0.1286
PVP 5 mg/L – C2	89	-3.103	0.0154
PVP 10 mg/L – C2	89	-2.142	0.1047

Table SI 4: summarized statistical information of body length at primiparity registered during the 21-day chronic toxicity test (Holm method used for p-value adjustment). Each polymer group is compared to its control (C).

Treatment	***df***	***t-ratio***	***p-value***
PVOH 1 mg/L – C1	126	0.319	1.0000
PVOH 5 mg/L – C1	126	-0.336	1.0000
PVOH 10 mg/L – C1	126	0.982	1.0000
PEO 1 mg/L – C1	126	-1.921	0.5128
PEO 5 mg/L – C1	126	0.963	1.0000
PEO 10 mg/L – C1	126	-0.176	1.0000
PEG 1 mg/L – C1	126	1.147	1.0000
PEG 5 mg/L – C1	126	-0.711	1.0000
PEG 10 mg/L – C1	126	1.311	1.0000
PAA 1 mg/L – C2	89	-1.075	1.0000
PAA 5 mg/L – C2	89	-0.823	1.0000
PAA 10 mg/L – C2	89	-1.997	0.2932
PVP 1 mg/L – C2	89	1.120	1.0000
PVP 5 mg/L – C2	89	0.796	1.0000
PVP 10 mg/L – C2	89	0.636	1.0000

Table SI 5: summarized statistical information of body width at primiparity registered during the 21-day chronic toxicity test (Holm method used for p-value adjustment). Each polymer group is compared to its control (C).

Treatment	***df***	***t-ratio***	***p-value***
PVOH 1 mg/L – C1	126	-0.286	1.0000
PVOH 5 mg/L – C1	126	-0.641	1.0000
PVOH 10 mg/L – C1	126	0.951	1.0000
PEO 1 mg/L – C1	126	-1.061	1.0000
PEO 5 mg/L – C1	126	0.194	1.0000
PEO 10 mg/L – C1	126	-0.649	1.0000
PEG 1 mg/L – C1	126	0.343	1.0000
PEG 5 mg/L – C1	126	-1.212	1.0000
PEG 10 mg/L – C1	126	0.321	1.0000
PAA 1 mg/L – C2	89	-1.291	1.0000
PAA 5 mg/L – C2	89	-0.790	1.0000
PAA 10 mg/L – C2	89	-2.184	0.1897
PVP 1 mg/L – C2	89	0.231	1.0000
PVP 5 mg/L – C2	89	0.371	1.0000
PVP 10 mg/L – C2	89	0.731	1.0000

Table SI 6: summarized statistical information of body length registered at the end of the 21-day chronic toxicity test (Holm method used for p-value adjustment). Each polymer group is compared to its control (C).

Treatment	***df***	***t-ratio***	***p-value***
PVOH 1 mg/L – C1	129	0.443	1.0000
PVOH 5 mg/L – C1	129	-2.656	0.0712
PVOH 10 mg/L – C1	129	-3.459	0.0059
PEO 1 mg/L – C1	129	-0.011	1.0000
PEO 5 mg/L – C1	129	-0.918	1.0000
PEO 10 mg/L – C1	129	-1.206	0.9195
PEG 1 mg/L – C1	129	-1.387	0.8391
PEG 5 mg/L – C1	129	-2.180	0.2175
PEG 10 mg/L – C1	129	-2.051	0.2536
PAA 1 mg/L – C2	89	0.431	1.0000
PAA 5 mg/L – C2	89	0.305	1.0000
PAA 10 mg/L – C2	89	0.079	1.0000
PVP 1 mg/L – C2	89	1.281	0.8145
PVP 5 mg/L – C2	89	1.159	0.6743
PVP 10 mg/L – C2	89	3.284	0.0088

Table SI 7: summarized statistical information of body width registered at the end of the 21-day chronic toxicity test (Holm method used for p-value adjustment). Each polymer group is compared to its control (C).

Treatment	***df***	***t-ratio***	***p-value***
PVOH 1 mg/L – C1	129	0.377	0.9577
PVOH 5 mg/L – C1	129	-3.073	0.0232
PVOH 10 mg/L – C1	129	-2.649	0.0727
PEO 1 mg/L – C1	129	-1.313	0.9577
PEO 5 mg/L – C1	129	-1.284	0.9577
PEO 10 mg/L – C1	129	-0.981	0.9577
PEG 1 mg/L – C1	129	-1.924	0.3935
PEG 5 mg/L – C1	129	-1.168	0.9577
PEG 10 mg/L – C1	129	-1.927	0.3935
PAA 1 mg/L – C2	89	-0.240	1.0000
PAA 5 mg/L – C2	89	-0.229	1.0000
PAA 10 mg/L – C2	89	-0.169	1.0000
PVP 1 mg/L – C2	89	0.0689	1.0000
PVP 5 mg/L – C2	89	0.291	1.0000
PVP 10 mg/L – C2	89	2.112	0.2250

1. Wu, H. Y. *et al.* Eulerian video magnification for revealing subtle changes in the world. *ACM Trans. Graph.* **31**, (2012).

List of Publications

2020:

De Felice, B., **Mondellini, S.**, Salgueiro-González, N., Castiglioni, S., and Parolini, M. 2020. "Methamphetamine exposure modulated oxidative status and altered the reproductive output in *Daphnia magna*." *Science of the Total Environment*, *721*, 137728. 10.1016/j.scitotenv.2020.137728.

This publication is not part of this thesis

Parolini, M., De Felice, B., **Mondellini, S.**, Caprioli, M., Possenti, C. D., and Rubolini, D. 2021. "Prenatal exposure to triclosan induced brain telomere shortening in a wild bird species." *Environmental Toxicology and Pharmacology*, *87*, 103718. 10.1016/j.etap.2021.103718.

This publication is not part of this thesis

2021:

Laforsch, C., Ramsperger, A. F., **Mondellini, S.**, and Galloway, T. S. 2021. "Microplastics: a novel suite of environmental contaminants but present for decades." *Regulatory Toxicology*, 1-26. 10.1007/978-3-642-36206-4_138.

Book chapter. This publication is not part of this thesis

2022:

Mondellini, S., Schott, M., Löder, M. G., Agarwal, S., Greiner, A., and Laforsch, C. 2022. "Beyond microplastics: Water soluble synthetic polymers exert sublethal adverse effects in the freshwater cladoceran *Daphnia magna*." *Science of the Total Environment*, *847*, 157608. 10.1016/j.scitotenv.2022.157608.

2024:

Mondellini, S., Schwarzer, M., Völkl, M., Jasinski, J., Jérôme, V., Scheibel, T., Laforsch, C., and Freitag, R. 2024. "Size dependent uptake and trophic transfer of polystyrene microplastics in unicellular freshwater eukaryotes." *Science of the Total Environment*, *929*, 172470. 10.1016/j.scitotenv.2024.172470.

Boccia, P., **Mondellini, S.**, Mauro, S., Zanellato, M., Parolini, M., and Sturchio, E. 2024. "Potential effects of environmental and occupational exposure to microplastics: an overview of air contamination." *Toxics*, *12*(5), 320. 10.3390/toxics12050320.

This publication is not part of this thesis

2025:

De Felice, B., **Mondellini, S.**, Bertazzo, M., Parolini, M., and Caloni, F. 2025. "Novel miniaturized exposure to evaluate the toxicity of Enniatin B1 on *Daphnia magna*." *Environmental Toxicology and Pharmacology*, 104748. 10.1016/j.etap.2025.104748.

This publication is not part of this thesis

Bostrom A., van den Broek, K.L., Böhm, G., Grunzer, M., Felipe-Rodriguez, M., Fian, L., Stevens, S., Abdeljaoued, A., Budhiraja, V., Carratta, G., **Mondellini, S.**, Özdamar, A.,

Suffill, E., Pahl, S., and Doran, R. 2025. “Scientis’ mental models of microplastics.” *Microplastics and Nanoplastics* 10.1186/s43591-025-00141-w.

This publication is not part of this thesis

Leah M. Thornton Hampton, Briggs Wyler, D., Carney Almroth, B., Coffin, S., Cowger, W., Doyle, D., Hataley, E.K., Hutton, S.J., Mair, M.M., Miller, E.L., Monclús, L., Sharpe, E.E., Samreen, S., Towsif Ahmed, K., Allamby, Q.P.V., Antonio Vital, A.L., Asnicar, D., Bare, J.L., Barrick, A., Berreman, K., Bertrand, L., Boone, V., Bour, A., Brehm, J., Carrasco-Navarro, V., Cook, T., Covernton, G., Cubanski, P., Da Silva, P.M.C., de Souza Leite, L., Gene, S.M., Hermabessiere, L., Hooge, A., Iwasaki, Y., Klasios, N., Knauss, K.M., König Kardgar, A., Kropf, P., Kudu, I.B., Kukkola, A., Laforsch, C., Kennedy, S.B., Leusch, F.B.L., Wei Li, L., Lu, H., Mahan, J., Md Saif, U., **Mondellini, S.**, Norman, J.P., Pandelides, Z., Petersson, T., Philibert, D.A., Kvist, E., Ramsperger, A.F.R.M., Rigutto, G., Ritschar, S., Sandgaard, M.H., Schmitt, J., Schott, M., Schwarzer, M., Seabrook, K.J., Seifried, T.M., Sepahi, R., Siña, M., Testoff, A.N., Vercauteren, M., Wardlaw, C.M., Yeh, A., Zajac-Fay, R., and Mehinto. A.C. 2025. “The Toxicity of Microplastics Explorer (ToMEx) 2.0.” *Microplastics and Nanoplastics.* 10.1186/s43591-025-00145-6.

This publication is not part of this thesis

2026:

Mondellini, S., Schwarzer, M., Kiene, M., Olthof, G., Ghosh, D., Agarwal, S., Löder, M.G.J., Wagner, M., and Laforsch, C. 2026. “Same but different: The response to petroleum and biodegradable bio-based microplastics deviates between *Daphnia magna* clones.” *Ecotoxicology and Environmetnal Safety 312*. 10.1016/j.ecoenv.2026.119934.

Schwarzer, M., **Mondellini, S.**, Bangalore-Narayana, V. K., Wieland, S., Ritschar, S., Kiene, M., Brehm, J., Kress, H., Löder, M. G. J., Mair, M. M., and Laforsch, C. 2026. "Effects of petroleum-based and biodegradable bio-based microplastics versus natural control particles on *Paramecium caudatum*, combined with *in situ* Raman spectroscopic detection*." Journal of Hazardous Materials, 141575.* 10.1016/j.jhazmat.2026.141575.

Mondellini S., Schwarzer, M., Schott, M., Kiene, M., Cormier, B., Ghosh, D., Löder, M.G.J., Agarwaal, S., Wagner, W., Laforsch, C. "Extracted chemicals from microplastics exert increased adverse effects on *Daphnia magna* compared to the pristine particles."

Included in this thesis as a manuscript.

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