Ecotoxicological and Microbial Studies on Weathering Plastic

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Dipl. Biol. Christoph Rummel

aus Mainz

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Dekan:	Prof. Dr. Thilo Stehle
1. Berichterstatter:	Prof. Dr. Beate Escher
2. Berichterstatter:	Prof. Dr. Rita Triebskorn

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Summary

Since the beginning of mass production of synthetic polymers, also referred to as plastic, the resulting litter has accumulated in the aquatic environment. As a result of continuous fragmentation of macro debris, this plastic litter has become a soup of micro- to nano-sized particles with currently unknown impact for human and environmental health. For less than a decade, researchers have been investigating the effects of such particles mainly in laboratory test systems. Weathering processes like UV light-induced photo-degradation have the potential to change material properties and thereby the relevant physical behavior of the synthetic material. Such modulating effects are often disregarded in common ecotoxicological exposure scenarios.

The framework of this thesis was the WEATHER MIC project. Within a consortium of researchers from different scientific disciplines, we prioritized research needs for an improved understanding of abiotic and biotic weathering processes acting on aquatic plastic debris, the relevance of those processes for changing the plastic's fate and impact and to overcome currently limited test conditions in the laboratory (*Publication I*, *Publication II*).

Although intentionally added chemicals (*e.g.,* additives) in polymers have been of concern for long, it remained unknown if substances liberated from additive-free microplastics during abiotic weathering (such as photo-degradation) are of ecotoxicological relevance, *e.g.,* by inducing cellular toxicity pathways and stress responses. Therefore, I prepared leachate waters from four UV-weathered polymers, polyethylene (PE), polyethylene terephthalate (PET), polystyrene (PS) and polypropylene (PP), concentrated and dosed them in reporter gene and microalgae assays (*Publication III*, *Manuscript I*). Leachates from all tested microplastics induced oxidative stress responses with elevated activation for the UV-weathered leachates. PE leachates contained diverse alkyl carboxylic acids stemming from the degrading polymer, which explained over 40% of the observed activation of the Peroxisome Proliferation Activated Receptor γ (PPARγ) as evidenced by mixture effect modelling. The investigated plastic leachates had only effects on growth inhibition in the microalgae *Scenedesmus vacuolatus* with low potency for photosystem inhibition. Effect concentrations derived from the growth inhibition of microalgae showed the similar patterns across all leachates and correlated significantly with those from the reporter gene assays. The weathered PE leachates showed higher activity, which could also be caused by the carboxylic acids, given that their algal toxicity also agreed well with predicted baseline toxicity.

Biotic weathering processes start with the formation of a superficial biofilm that changes the fate and effects of plastic debris (*Publication IV*). As this process represents probably the first and longest biological interaction with environmental plastic, it is of utmost importance to understand the development, structure and function of epiplastic microbial communities and their ecological relevance. Before microbes attach to new habitable (plastic) substrates a thin layer of organic matter (OM), a so-called conditioning film, adsorbs to the substrate surface which has implications for subsequent colonization.

By the means of Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) I tested the hypothesis of material-specific conditioning films on plastic surfaces as well as its relevance for subsequent biofilm formation. I demonstrated that the investigated substrates glass, PET and PS showed selective adsorption patterns towards OM. Differences in the OM fingerprint were also detectable between pre-weathered and dark control polymeric substrates. After the adsorption of this first OM layer the material surface properties, such as the surface hydrophobicity, changed. Noteworthy, the material-specific conditioning films did not entirely mask the material properties but preserved the underlying surface characteristics to the outer organic matter-water interface (*Manuscript II*). These observations provided a potential explanation for subsequent material-specific attachment by microbes during the first days of early colonization phase. As the biofilms matured, taxonomic, structural and functional differences disappeared and the communities on different substrates converged to highly similar communities (*Manuscript III*)

In my presented ecotoxicological and microbial studies, I demonstrated that weathering of the persistent plastic material has impacts on its fate and effects. Material-specific implications of OM adsorption and biofilm succession demonstrated that experiments and results have to be set into the context of larger time frames. The fact that polymer leachates generated under accelerated weathering conditions activated certain cellular gene pathways and caused algae toxicity should prompt us to reconsider critically the current exemption of polymers from the REACH registration and evaluation since the material can no longer be regarded as "inert".

This thesis advances our understanding of chemical leaching during polymer photo-degradation and the biological features that plastic surfaces display during the early microbial colonization phase. Future studies should acknowledge the relevance of weathering processes for the interpretation and robustness of effect assessments of plastics in order to ensure human and environmental safety.

Zusammenfassung

Seit Beginn der Massenproduktion von synthetischen Polymeren oder Kunststoffen, umgangssprachlich als Plastik bezeichnet, akkumuliert deren Abfall in der aquatischen Umwelt. Durch kontinuierliche Fragmentierung von Makroabfällen sind manche aquatischen Ökosysteme heute zu einer Suppe aus mikro- bis nanoskaligen Partikeln geworden, deren Auswirkungen auf die Gesundheit von Mensch und Umwelt wir bisher nur schwer abschätzen können. Erst seit weniger als einem Jahrzehnt untersuchen Wissenschaftler*innen die Auswirkungen solcher Partikel hauptsächlich in Labortestsystemen. Verwitterungsprozesse, wie bspw. durch UV-Licht induziertem Photoabbau, können Materialeigenschaften und damit das Verhalten von Kunststoffen in der Umwelt verändern. Diese relevanten Änderungen im physikalischen Verhalten von Kunststoff(partikeln) durch Verwitterung finden in gängigen ökotoxikologischen Expositionsszenarien oft keine Berücksichtigung.

Den Rahmen dieser Arbeit bildete das Projekt WEATHER MIC. Innerhalb eines Konsortiums von WissenschaftlerInnen verschiedener Fachgebiete priorisierten wir Forschungsfragen zu tieferem Verständnis der abiotischen und biotischen Verwitterungsprozesse, die auf aquatischen Kunststoffmüll einwirken, zur Relevanz dieser Prozesse für den Verbleib und die Effekte des Umweltkunststoffes und zur Anpassung derzeitig begrenzter Testbedingungen im Labor (*Publikation I, Publikation II*).

Obwohl gewollt zugesetzte Chemikalien (z.B. Additive) in Polymeren seit langem Anlass zur Besorgnis geben, ist bisher wenig darüber bekannt, ob Substanzen, die während der abiotischen Verwitterung (z.B. durch Photoabbau) aus additiv-freiem Mikroplastik freigesetzt werden können, von ökotoxikologischer Bedeutung sind, z.B. indem sie relevante zelluläre Signalwege und Stressreaktionen induzieren. Daher wurden wässrige Laugungsprodukte von vier UV-verwitterten Testpolymeren Polyethylen (PE), Polyethylenterephthalat (PET), Polystyrol (PS) und Polypropylen (PP) aufkonzentriert und in zellbasierte Reportergen- und Mikroalgen-Biotests dosiert (*Publikation III*, *Manuskript I*). Laugungsprodukte aller Mikroplastiksorten induzierten oxidative Stressantworten mit erhöhtem Effektpotential bei UV-verwitterten Proben. PE-Laugungsprodukte aktivierten darüber hinaus spezifisch den Peroxisom-Proliferator-aktivierten Rezeptor γ (PPARγ). Unter Anwendung eines Mischungsmodells (Eisbergmodellierung) und effektdiagnostischer Bewertung konnte ich über 40 % des beobachteten biologischen Effektes in PPARγ durch einen hohen gemessenen Gehalt an Alkankarbonsäuren erklären, welche als Abbauprodukte aus dem verwitterndem Polymer stammten.

Die überwiegend unspezifische Toxizität der untersuchten Kunststofflaugungsprodukte ging mit geringer Photosynthesehemmung in der Mikroalge *Scenedesmus vacuolatus* einher. Berechnete Werte für die Basis-Toxizität stimmten gut mit den gemessenen apikalen Wachstumsendpunkten für die Alkankarbonsäuren in den Mikroalgen überein und die aus den Mikroalgen abgeleiteten Effektkonzentrationen korrelierten statistisch signifikant mit den Ergebnissen aus den Reportergen-Biotests.

Biotische Verwitterungsprozesse beginnen mit der Bildung eines oberflächlichen Biofilms, der den Verbleib und Auswirkungen von Umweltplastik verändert (*Publikation IV*). Als wahrscheinlich erste und längste biologische Interaktion mit Umweltplastik ist es von größter Bedeutung, die Entwicklung, Struktur und Funktion von epiplastischen mikrobiellen Artengemeinschaften zu verstehen und ihre ökologische Relevanz zu bewerten. Bevor sich Mikroorganismen auf neuen (Kunststoff-) Substraten ansiedeln, bildet sich zunächst eine dünne Schicht aus organischem Material (OM), ein so genannter Konditionierungsfilm. Dieser hat potenzielle Auswirkungen auf die spätere Besiedlung. Mittels Fourier-Transformations-Ionenzyklotronresonanz-Massenspektrometrie (FT-ICR MS) überprüfte ich die Hypothese der Bildung von materialspezifischen Konditionierungsfilmen auf Kunststoffoberflächen und bewertete deren Relevanz für das nachfolgende Biofilmwachstum.

Ich konnte in meiner Arbeit zeigen, dass OM auf den untersuchten Substraten Glas, PET und PS selektiv adsorbiert wurde. Unterschiede im Konditionierungsfilm waren auch zwischen vorverwitterten und dunklen Kontrollproben der jeweiligen Substrattypen nachweisbar. Nach der Adsorption dieser ersten OM-Schicht änderten sich die Materialoberflächeneigenschaften wie z. B. die Hydrophobie der Oberflächen. Bemerkenswert ist, dass die materialspezifischen Konditionierungsfilme die Materialeigenschaften nicht vollständig maskierten, sondern die zugrunde liegenden Oberflächeneigenschaften bis zur äußeren Organik-Wasser-Grenzfläche erhalten blieben (*Manuskript II*). Dieser Sachverhalt lieferte eine mögliche Erklärung für unsere Beobachtungen von anschließender materialspezifischer Anlagerung von Mikroorganismen in den ersten Kolonisierungstagen. Mit zunehmendem Alter des Biofilms verringerten sich strukturelle und funktionelle Unterschiede zwischen verschiedenen Testmaterialien und die taxonomische Zusammensetzung der verschiedenen Substrate konvergierte zu gleichen Artengemeinschaften (*Manuscript III*).

In den vorgestellten ökotoxikologischen und mikrobiellen Untersuchungen konnte ich zeigen, dass abiotische und biotische Verwitterungsprozesse persistenter Kunststoffe Auswirkungen auf dessen

Verbleib und Effekte haben. Materialspezifische Erkenntnisse über Adsorption von OM und Biofilm-Sukzession sollten uns veranlassen, wissenschaftliche Studien zu Plastik-Biofilmen in größeren zeitlichen Rahmen und Kontext zu setzen. Meine Ergebnisse, dass Polymerlaugungsprodukte aus Verwitterungssimulationen bestimmte zelluläre Genantworten und Stressreaktionen aktivierten sowie Algentoxizität induzierte, sollte uns dazu veranlassen die derzeitige Ausnahme von Polymeren von der REACH-Verordnung zur Registrierung und Bewertung erneut zu verhandeln, da das Material nicht länger als „inert" gelten kann.

Meine Arbeiten erweitern unser Verständnis zur Freisetzung von Substanzen während des Photoabbaus von Kunststoffen und über biologische Eigenschaften von Kunststoffoberflächen während der frühen mikrobiellen Besiedlungsphase. Zukünftige Studien sollten die Relevanz dieser Verwitterungsprozessen auf die Validierung und Verlässlichkeit von Kunststofftestst rategien anerkennen, um so die Gesundheit von Mensch und Umwelt zu gewährleisten.

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1. Introduction

1.1 Our Plastic Age

You sit on it, you drink from it and you wear it: Plastic. But how and when did its story of success begin and where has it led us today? In the year 1839 the Chemist Charles Goodyear invented vulcanized rubber which was the first semi-synthetic polymer. At the beginning of the 20th century, the invention of Bakelite marked the beginning of the modern synthetic polymer industry. The material was durable, isolating and heat resistant which is why it rapidly gained popularity in the industry. After the synthesis of further polymers such as Polystyrene (PS) and Polyvinyl Chloride (PVC), Polyethylene (PE) and Polyethylene terephthalate (PET) in the first half of the 20th century, mass production of plastic begun (Thompson et al. 2009). The yearly production volume has reached around 360 million tons in 2019 with tendency to further increase (PlasticsEurope 2019). Although the first detections and incidences between the resulting plastic litter in the environment and wild life were already reported in the '60s and '70s (Carpenter et al. 1972; Carpenter and Smith 1972), a member of the Council of the British Plastics Federation stated back then that "plastics litter is a very small proportion of all litter and causes no harm to the environment except as an eyesore" (Derraik 2002).

Today, global plastic litter input from rivers into the sea were estimated to be in the range of 0.4 – 4.0 million tons/year (Schmidt, Krauth, and Wagner 2017). Despite those numbers, plastic littering, especially in the aquatic environment, has received increased attention only recently. Richard Thompson's paper "Lost at Sea: Where is All the Plastic", published in Science in 2004 (Thompson et al. 2004) has stirred up scientists to search for the missing fraction of our emitted plastic litter. He discovered that a major fraction of large plastic debris has constantly fragmented to smaller pieces down to the micro size with increasing abundance over the last decades (Thompson et al. 2004) (**Figure 1.1 A**). The article has coined the term microplastic that was later defined at the International Research Workshop on the Occurrence and Effects of Marine Debris as the litter fraction of plastic < 5 mm (Arthur, Baker, and Bamford 2009) mostly generated by the fragmentation of larger macroplastic (>5 mm) (see chapter 1.2). Examples of such fragmentation processes are given in Figure 1.1 depicting macro (**Figure 1.1 B-C**) and micro (**Figure 1.1 A**) debris from a recent expedition to the North Pacific Garbage Patch.

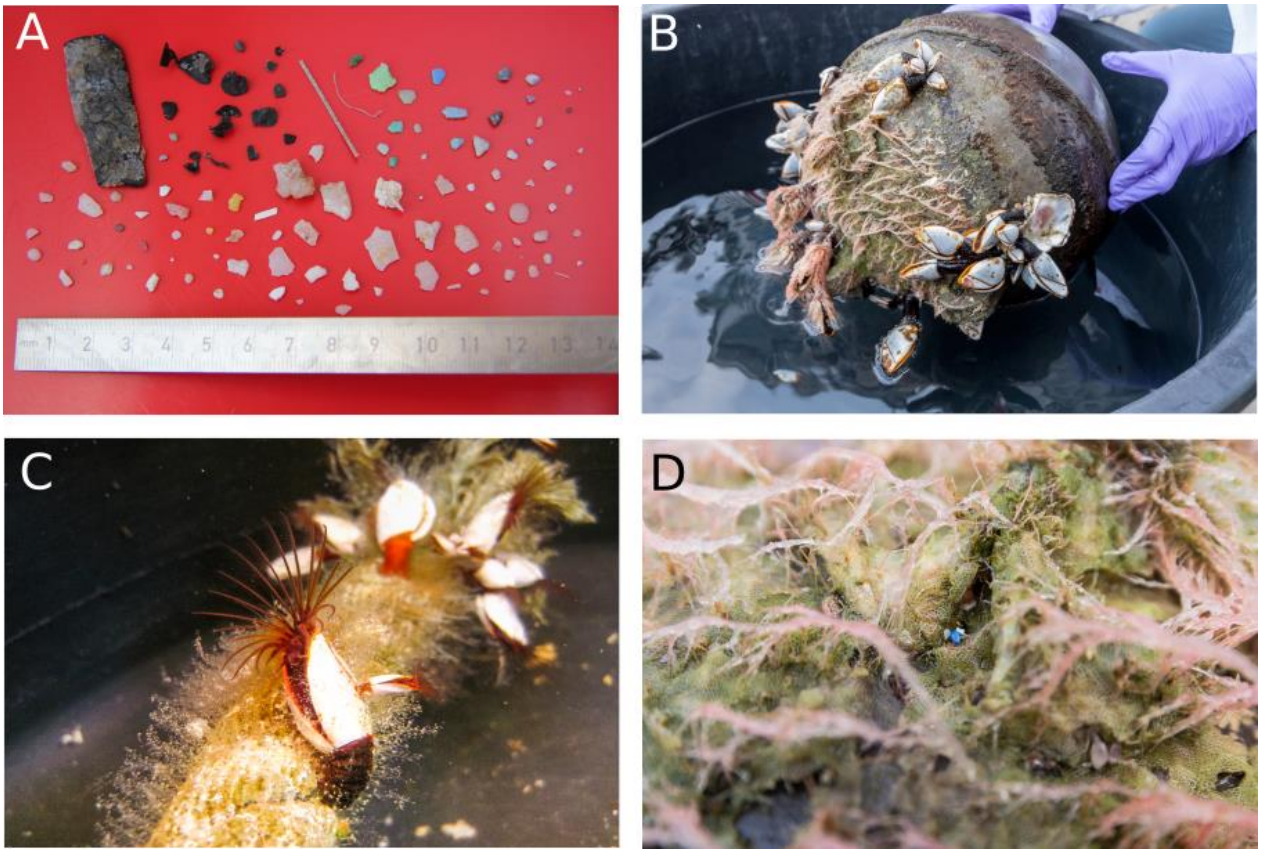


Figure 1.1: Pollution of the aquatic environment by plastic debris. Samples taken during a research cruise (SO 268/3) with RV SONNE crossing the North Pacific Garbage Patch. **A:** Macro- to microplastic different size ranges (source: Annika Jahnke). **B:** Macrodebris (fishing buoy) with fouling community. **C:** fishing tow overgrown with stalked barnacles (Pedunculata). **D:** red-shiny macrophytes on plastic debris (source B-D: Stefan Lips)

The aim to deliver information about the current littering status and effects in the marine, freshwater and terrestrial environment, to present mitigation strategies for industry and policy as well as to raise awareness confronted scientists with the obligation to provide standards and harmonized methods to assess exposure and hazard potential of plastic debris (Hidalgo-Ruz et al. 2012; Hartmann et al. 2019). Understanding the environmental fate and effects of aquatic litter is the prerequisite for an informed risk assessment (Adam, Yang, and Nowack 2019). Over 2000 scientific journal articles (Web of Science search with the keyword "microplastic" on the 28th of December 2020) describe the detection of microplastic in the most remote environments and incidences between organisms and marine debris are proven for more than 700 species (Gall and Thompson 2015). One of the most advantageous features of synthetic polymers, its long durability, has now created a severe pollution issue. This main environmental issue – the persistence of plastic – provides the basic content of this work: the weathering of plastic. Weathering describes the different abiotic and biotic impacts on plastic debris

that causes ageing, degradation, disintegration and final mineralization of the material commonly defined as "the undesirable change [of synthetic polymers] produced by outdoor exposure" (Feldman 2002). Strictly speaking, fragmentation does not belong to "weathering" but is a consequence of weathering. While many scientific studies were undertaken to assess the fate and effects of plastic litter using standard materials and techniques, they often disregarded the influence that weathering may have on the investigated process (chapter 1.3, *Publication I* and *II*). To investigate how weathering changes the transport, fate and toxicity of plastic in the marine environment was the overall aim of the WEATHER – MIC project in which framework this thesis has been carried out. Therefore, the focus of this dissertation are the two main weathering processes: the abiotic and biotic weathering of plastic.

Several environmental factors may cause polymer weathering. The main degradation drivers are solar radiation, heat, moisture and microbial colonization (Pickett 2018) (**Table 1.1, Figure 1 B-D**). Minor impacts will have atmospheric pollutants and dust, thermal cycling, flexing due to wind and electrical loads and others. Most importantly, the degree of impact that a weathering process may have on the polymer depends on the environmental conditions, the polymer chemistry (e.g., polymer type, colorants and additives) and certainly on the investigated criteria of material failure such as tensile strength or elongation at break (Pickett 2018).

To better understand the relevance of weathering plastic in the context of plastic pollution we need to understand the extent of this pollution issue and the environmental problems associated with it. Therefore, the next section gives an overview over the exposure and effects of environmental plastics.

Table 1.1: Summary of different weathering processes and the respective consequences for polymers (adopted from Pickett (2018))

	Environmental variable	Effect on polymer materials
Abiotic weathering	Solar radiation / UV-light	Absorbance of UV light by impurities or polymer's chemical structure itself forming free radicals when C-H bonds break
	Temperature	Increase in reaction rates with temperature once photochemistry was initiated; freeze-thaw cycles may lead to cracking
	Moisture (humidity, condensation, rain)	For some polymers, hydrolysis may be relevant. Rain affects surface appearance and causes washing of degradation products
	Oxygen	Requirement for photo-oxidation
	Mechanical stress	Physical break of polymer chains
Biotic weathering	Microbial growth	Biodegradation at the surface, mineralization of oligo- and monomers, potential interference with abiotic weathering processes

1.2 Exposure of the Aquatic Environment to Plastic

The scientific community has yet not fully agreed on a generally valid definition to categorize plastic debris (but is mainly done by size) (Hartmann et al. 2019). Plastic is subject to ongoing fragmentation into smaller pieces from large marco debris (> 5mm in size), to microplastic (5000 – 335 μm) down to the nano range (< 335 μm) (here definition by Koelmans et al. (2017)). This large size range together with diverse polymer types of different additive formulas and compositions makes it difficult to assess the exposure of a specific fraction of plastics to a variety of organisms that may be more or less prone to be affected by a certain particle type.

Environmental plastic loads are difficult to estimate and emission assumptions are prone to errors. The most reliable number in plastic emission scenarios is the production amount since researcher can retrieve these known and reliable numbers. 275 million metric tons (MT) of plastic were estimated to

be in the oceans in 2010 with emission rates of around 8 million MT of macro- and 1.5 million MT of Microplastic entering the marine systems every year (Jambeck et al. 2015). Even in the unlikely event of drastic emission reductions, Lau et al. (2020) projected 710 million MT of plastic to be present in the oceans by 2040. Lebreton et al. (2017) estimated that 3 – 19 % of this coastal plastic emissions are facilitated by rivers, one of the major transport pathway of land-based sources (Schmidt, Krauth, and Wagner 2017). Interestingly, only approximately 1 % of the global plastic mass estimated to enter the ocean in 2010 could be extrapolated by a global dataset of measured marine plastic debris (van Sebille et al. 2015). That means that the vast majority remains undetected by the current methods applied and its ultimate fate is still under debate. Due to easier accessibility and existing routine methods, researchers have focused mainly on drifting plastic debris at the water surface. However, recent publications suggest the sea floor, deep sea sediments (Bergmann et al. 2017; Kane and Clare 2019; Kane et al. 2020) and the epipelagic and mesopelagic water column (Choy et al. 2019) to be major hot spots of plastic pollution in marine systems.

Microplastic water concentrations may range from 4000 items/m³ (Yangtze Estuary) to below 1 item/m³ (East China Sea, Zhao et al. (2014)) or from 2175 items/kg (Vianello et al. 2013) to 1 item/kg (Dekiff et al. 2014) for sediments. Problematic with such data is the use of a variety of different sampling techniques and detection methods that may eventually resulted in different units and measures (Shim, Hong, and Eo 2017; Prata, da Costa, Duarte, et al. 2019; Muller et al. 2020). Furthermore, sampling procedures and preparations are prone to be affected by cross-contamination which further introduces uncertainties in the empirical data (Rummel et al. 2016; Witzig et al. 2020). To overcome these experimental challenges, researchers have suggested harmonized reporting guidelines just recently to increase robustness, reproducibility and comparability of studies across different microplastic study designs (Cowger et al. 2020).

Conclusively, plastic debris has been detected ubiquitously in freshwater bodies, such as rivers and lakes (Eerkes-Medrano, Thompson, and Aldridge 2015), estuaries (Sadri and Thompson 2014) and the open sea (Cozar et al. 2014). Microplastic, generated as a result of weathering including mechanical impacts such as shear forces or abrasion, is distributed from the water surface (Reisser et al. 2015), through the water column (Choy et al. 2019) down to deep sea sediments (Cauwenberghe et al. 2013). For the assessment of risks posed by plastic debris based on exposure, it is of utmost importance to be aware of the reliability and accuracy of the exposure measurements.

1.3 Effects of Plastic Debris

The public's perception of littering is often an aesthetical concern since it reduces the recreational value of natural water bodies and coastal zones (UNEP 2009). This mainly applies for macro debris but plastic of all sizes from macro- to micro- and nanoplastic may exhibit adverse effects to biota on different trophic levels across all ecosystems, from the terrestrial to the marine environment. Negative effects of plastic debris on biota may range from entanglement to ingestion and may reduce the nutrient and energy budget but may also cause intestinal damage and blockage (Rummel et al. 2016; Zhang et al. 2019; Lei et al. 2018; Jepsen and de Bruyn 2019). Those impacts are frequently reported for macro debris. But uptake of particles in the micro- or nano size range in other organs than the gastrointestinal tract such as the gills or translocation to the circulatory system was also reported for vertebrae and invertebrates in controlled laboratory studies (Watts et al. 2014; Su et al. 2019).

For such effect studies of microplastic, it is important to acknowledge limitations by common laboratory practice (*Publication II*, Potthoff et al. (2017)). The challenge in characterizing the risk of microplastic particles is, that they lay outside the applicability domain for current standardized assessment strategies (Gouin et al. 2019). This applies 1) for the above mentioned environmental concentration assessments by so-far non-standardized exposure assessments (chapter 1.2). But also 2) our current ability to correctly assess adverse effects by microplastic particles in a typical dose-response relationship is hampered by the particles' intrinsic physico-chemical behavior. Are the observed effects specific to a certain particle size class? Is it specific to a certain polymer type or could it be attributed to specific surface characteristics (e.g. surface charge, specific surface area, and surface condition such as topography/roughness)? The versatility of polymer types and their resulting fragments pose problems for scientists to evaluate their impact and relevance for ecosystem functioning (*Publication II*).

Many studies on ecotoxicological effects of microplastic towards test organisms typically apply virgin spherical particles, so-called primary microplastic, of a defined size class (Cole et al. 2013; Lee et al. 2013; Besseling et al. 2014). However, most processes that scientist observed in laboratory studies, will inevitably change upon weathering in natural systems. These changes mainly depend on dramatic changes in physico-chemical properties of plastic surfaces (chapter 2.1, 3.1, 3.2, *Manuscript II*) or plastic particles and thereby interfere with the mechanisms of investigation (Liu, Zhan, et al. 2020). Material changes due to weathering may hamper direct comparison and extrapolation of laboratory studies to *in-situ* observations in natural systems. Furthermore, many experimental studies have applied

microplastic concentrations far above the levels documented for the aquatic environment (Lenz, Enders, and Nielsen 2016). Additionally, weathered microplastic particles occur at a broad size distribution and diverse irregular shapes (Moret-Ferguson et al. 2010). It is worth noting that polymer properties, *e.g.*, of manufactured objects of PE, could already be different from those of the raw pellets (Nowlin 2014). In principle, these assessment challenges are not insurmountable obstacles as long as researchers are aware of the limiting and influencing factors.

An important prerequisite for correctly assessing the effects of particles towards biota is to evaluate the observed toxicological effects in the context of an environmentally realistic scenario in respect to the natural habitat of the investigated organism. Since in natural waters, organisms generally have to cope with natural particulate matter (such as clay or mineral particles) studies should benchmark the observed effects to the presence of these natural particles by the application of adequate controls (Ogonowski et al. 2016; Gorokhova, Ek, and Reichelt 2020). To increase robustness and improve our understanding of particle toxicity in microplastic testing strategies we suggested to consider and report on i) particle surface properties, ii) particle size and shape distribution and iii) bulk parameters (such as density, brittleness or crystallinity) (*Publication II*, Potthoff et al. (2017)).

Summarizing, the ingestion of such plastic debris was detected for a multitude of different species from different trophic levels. Invertebrates, fish, turtles, birds and mammals are reported to ingest plastic litter or to be entangled in (Rummel et al. 2016; Bravo Rebolledo et al. 2013; Goldstein and Goodwin 2013; Gonzalez Carman et al. 2014; Van Franeker et al. 2014; Desforges, Galbraith, and Ross 2015). Further, laboratory studies demonstrated that interactions and toxicological effects of microplastic on biota range from mortality, inflammatory responses, inhibited growth and development, reducing energy, low feeding activity, oxidative damage, immunity and neurotransmission dysfunction or behavioral abnormality for a range of test organisms (Strungaru et al. 2019; Triebskorn et al. 2019).

Adequately assessing the hazard of plastic debris is specially challenging since typical test strategies do not apply and need further adjustments (*Publication II*). One way to estimate the impacts of multiple stressors on the earth system is a framework by Rockström et al. (2009), the so-called Planetary Boundary concept.

1.4 Plastic Pollution as a Planetary Boundary

To evaluate the impact of different anthropogenic stressors for ecosystem functioning Rockström et al. (2009) defined a safe operating space for humanity by "planetary boundaries". The above-mentioned sections support the hypothesis that environmental plastic pollution meets two out of three planetary boundary criteria (defined for chemical pollution (MacLeod et al. 2014)), namely, 1) its global exposure, which is 2) not readily reversible (MacLeod et al. 2014; Persson et al. 2013). Whether it causes an unknown disruptive impact on a vital earth system process would be the third and last criterion. The later condition is most uncertain since per definition it applies to currently unknown impacts where science can provide indications and early warnings. Basically, all planetary impacts were detected and investigated in retrospective processes. Within *Publication I*, we (the WEATHER-MIC consortium) prioritized research needs of weathering plastic within the planetary boundary concept. To reduce uncertainty of the currently unknown impacts of plastic pollution (criterion 3), we conclusively asked for improvement of our understanding of the multiple abiotic and biotic factors influencing the weathering process by characterization of particles over time, including morphology, particle size distribution, and surface properties and their degradation products (Jahnke et al. 2017). This knowledge is a prerequisite for other plastic-related research disciplines such as modelling of transport and distribution of microplastic particles, controlling and monitoring particle concentration in toxicological studies or for interaction with microorganisms (MOs).

1.5 Regulatory of Polymers under REACH

Polymers are generally exempted from product safety assessment and registration requirements within the EU Regulation (EC) No 1907/2006 on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Still, article 138 (2) a) (EC No 1907/2006 REACH) proposes to select sound and valid scientific criteria to report on the risks posed by polymers. This overall risk could include the leaching potential of polymers for unreacted and non-covalently bound monomers and low-molecular weight compounds as criteria in a proposed hazard ranking (Commission 2012; OECD 2019). Analytical studies have detected potential degradation products of different polymers (Gewert et al. 2018; Hakkarainen and Albertsson 2004) which are generally disregarded by the above-mentioned regulations. To my knowledge, a hazard assessment of weathering-induced degradation products has not been reported so far, although transformation/degradation products are commonly of relevance

and concern for the evaluation of chemicals since they may have similar or in some cases higher effect potential than their parent compound (Petrie, Barden, and Kasprzyk-Hordern 2015). Such transformation/degradation products of (industrial) chemicals have to be reported in the Physicochemical Information (7.15) and in the Ecotoxicological Information (9.2 and 9.3.4) within the REACH (EC1907/2006) dossier for registered substances. The relevance of my thesis with respect to the above-mentioned regulatory aspects of polymers under REACH will be discussed in chapter 4.3.

1.6 Aim of this thesis

For the above-introduced fate and effects of plastic debris, weathering processes may play a key role in modifying the material properties in such way, that the original material's entity is masked, modified or severely affected and altered (Liu, Zhan, et al. 2020; Ho et al. 2020). Not least, the ongoing weathering and fragmentation from macro debris to micro- and nano particles should prompt us to investigate these relevant environmental mechanisms that are acting immediately as well as for long periods of time after emission. As a first consequence, researchers should acknowledge this source of uncertainty when, *e.g.*, interpreting laboratory studies using standardized microspheres as representative environmental MP. Second, we should address and overcome our limited understanding of plastic debris in natural systems and waters by specifically studying potential interferences and effects of weathering processes on environmental plastics (*Publication I* and *II*).

Analogous to chemicals where not only the parent compound but also transformation products may have the potential to cause (eco)toxicity (Casellas et al. 2013), also polymers may undergo transformation and degradation upon exposure to abiotic and biotic environmental weathering factors. The mixtures of substances liberated from weathering plastics are called "leachates" and may comprise mixtures of polymer degradation products as well as additives, and potentially their respective degradation products. Whereas leachates from, *e.g.,* municipal landfills or soils are commonly investigated and their (eco)toxicological potential is well accepted, chemical mixtures of leachates liberated during polymer weathering are often disregarded and have not gained much attention in industry and the scientific community. My research questions on the (eco)toxicological relevance of abiotic weathering plastics are presented in section 1.4.1. (**Figure 1.2 A,** "abiotic weathering")

Another aspect, where weathering plastic may alter biological effects is the generation of a biofilm (**Figure 1.1 B-D**). In the environment, basically all surfaces submerged in water are rapidly colonized by such microbial consortia and so they were obviously also detected on environmental plastic debris

(Zettler, Mincer, and Amaral-Zettler 2013). These microbial communities play key roles within our global nutrient cycles (such as carbon or nitrogen cycles) and have the capability to provide various enzymatic pathways relevant for ecosystem functioning. Furthermore, the life history strategy, the pattern like growth, survival and reproduction evolved by natural selection, of living attached at the water-substrate interface facilitated by extracellular polymeric substances (EPS) renders biofilms an important driver of the fate and effects of plastic debris since biofilms form a biological coating. Plastic surfaces change their physicochemical properties as a result of abiotic weathering with potential implications for biofilm attachment. Section 1.6.2 specifies my research questions with respect to biotic weathering (**Figure 1.2. A**, "biotic weathering").

The overall aim and particular challenge of this thesis were to simulate natural weathering conditions in the laboratory and to identify their relevance for our existing knowledge on properties and processes of polymer chemistry and biology. To achieve this goal, I made use of a variety of different biological test systems from cell culture, via whole organism tests (single cell microalgae) to complex natural communities (**Figure 1.2 C**). Employing these test systems, I could gain insights on different levels of biological integrity with increasing complexity (**Figure 1.2 C**). Assessing the impacts of stressors on wildlife at higher levels of biological organization, like populations or communities, will help us to better understand their indirect effects caused, *e.g.,* by species interactions and different sensitivities of life-history traits (Köhler and Triebskorn 2013).

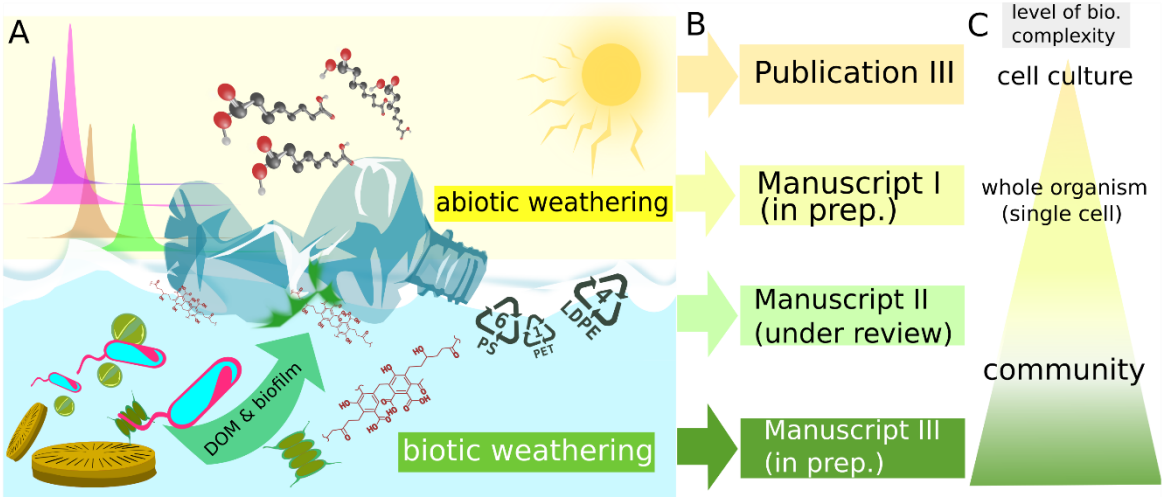


Figure 1.2: The two research domains of this thesis of abiotic weathering by photo-degradation (**A**, top "abiotic weathering") and biotic weathering via microbial colonization (**A**, "bottom" biotic weathering) following the sorption of dissolved organic matter (DOM). These topics are integrated in (**B**) *Publication III* and Manuscripts *I-III* with increasing level of biological complexity (**C**): from *in vitro* cell-culture (reporter gene assays), to single cell whole organism tests (microalgae) to community analyses (natural biofilms).

1.6.1 Abiotic Weathering and Effects of Leaching Substances

As outlined in *Publication II*, properties of plastic are modified by weathering processes. The herein mentioned processes mainly addressed microplastic particle characteristics such as degradation and fragmentation as well as the relevance of the polymer's chemistry. These aspects only concern the bulk polymer fraction (*i.e.*, the particle/debris itself) that stays more or even less intact with ongoing weathering. However, plastic-associated substances have the potential to leach from the polymer (Teuten et al. 2009). These substances can be polymer residues such as mono- and oligomers or chemicals added intentionally (additives such as fillers, UV-stabilizers, plasticizers or processing aids) or unintentionally (unreacted chemicals, impurities, processing byproducts) during manufacturing. Studies have demonstrated the leaching of chemicals from plastic over time with increased leaching probability under the impact of different stressors such as salinity, UV-light and turbulence (Bittner, Yang, and Stoner 2014; Suhrhoff and Scholz-Böttcher 2016; Paluselli et al. 2018).

The former named process of the polymers' leaching potential of ingredients has received relatively high level of attention in industry and the scientific community. Not least food manufacturers and chemical companies who supply food contact materials to the food industry need to comply with the EU's regulation for plastic food contact materials (EU No. 10/2011), which is why the migration of substances (mainly additives) is under scientific scrutiny also in microplastic-related research. One reason for this additive-focused research may be the ubiquitous and daily use of consumer plastics containing a mixtures of additives, partially with known (eco)toxicological effects of the single added substance (*e.g.,* acute toxicity and estrogenic endocrine disrupting activity of phthalates (Chen et al. 2014)). Another reason may be the general opinion that synthetic polymers are inert.

But polymers degrade under the impact of weathering processes and correspondingly release degradation products (Gewert et al. 2018). The emission of degradation products and their biological effect potential, however, has gained little attention. In toxicity tests of microplastic particles, the test organisms are unavoidably exposed to a mixture of particles and dissolved substances leaching from the polymers. In this simultaneous exposure of microplastic particles and dissolved chemicals liberated from the polymer, it remains unclear which stressor contributed to an observed ecotoxicological effect (*Publication II*, Potthoff et al. (2017)). Minor attention so far has been paid to the chemical breakdown and potential toxicity of weathering plastic with focus on the polymer chemistry itself (**Figure 1.3**). I hypothesize that:

- Chemicals leach from synthetic polymers during accelerated weathering (also termed "ad-hoc" weathering applying UV-radiation)
- Chemicals liberated during artificial weathering from mostly additive-free polymers are of ecotoxicological relevance (*e.g.,* by activating certain cellular toxicity pathways and/or algae toxicity)

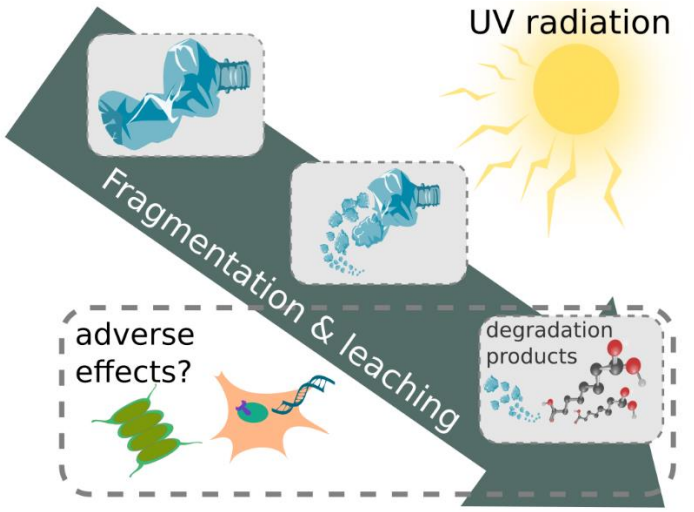


Figure 1.3: Conceptualized fragmentation and leaching of polymers by abiotic weathering conditions such as the UV-radiation applied in these studies. The aim was to identify potential adverse effects caused by polymer degradation products (chapter 2).

To address these hypotheses, we have leached microplastics of different polymer tpyes in artificial seawater and exposed one batch of samples to strong UV-light, whereas the control treatment was kept in darkness. After this accelerated weathering treatment, the leachate waters were concentrated and dosed in cell-based reporter gene and microalgae bioassays to assess their effect potential towards relevant cellular stress responses or algae photosynthesis and growth. A brief introduction to abiotic weathering, the results of *Publication III* (Rummel et al. 2019) and *Manuscript I*, as well as a summary on the main findings are given in chapter 2.

1.6.2 Biotic Weathering and Implications of Microbial Colonization

The second weathering factor acting on plastics and objective of this thesis is a biological process: The colonization of plastic surfaces by microbial communities (**Figure 1.1 B-D, Figure 1.4**). These so-called biofilms colonize almost every submerged surface and build up consortia of bacteria, algae and fungi (and other taxonomic classes) embedded in EPS. Typically they form aggregates, films, mats,

sludges or flocs (Flemming and Wingender 2010). The spatial proximity and variety of different organisms of different trophic levels and ecological functions render biofilms as important drivers of biogeochemical cycles. This role is not least due to their life form and natural habitat at the surface-water interface that leads to highly organized structures and architectures which is essential for their functioning, especially in respect to non-inert substrates (Grimaud 2010). Biofilms are well adapted to harsh conditions such as high-intensity UV-light, high or low temperatures, high alkalinity, acidity or salinity, high pressure or low nutrient availabity. In order to survive these extreme conditions their life strategy as a biofilm is considered to be crucial (Flemming et al. 2016). Their supracellular organization within the EPS matrix facilitates steep gradients (*e.g.* in nutrients, pH or oxygen), high biodiversity, cell-to-cell communication and horizontal gene-transfer (Flemming et al. 2016). Furthermore, the EPS matrix protects biofilms against desiccation, provides digestive capacities and resource capture by sorption. (Flemming and Wingender 2010). The latter process becomes additionally relevant for the sorption of hydrophobic organic compounds as discussed in chapter 4.

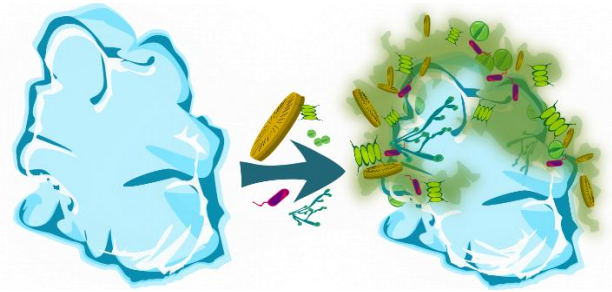


FIGURE 1.4: Conceptualized biofilm formation on a microplastic particle. The growth of microbes on the surface results in a biological coating that has the capacity to interfere with and modify a variety of physico-chemical processes at the water-polymer interface (chapter 3.1). Furthermore, microbial colonization can have important ecological implications (chapter 3.2).

Often these assemblages are termed "fouling communities" which implies their ability to disintegrate and deteriorate the surface they are attached on (F**igure 1.1 B-D)**. In this context, the term "biodegradation" refers to the chemical breakdown of a polymer. Microbes have evolved a plethora of different metabolic capacities and are capable of degrading and mineralizing synthetic polymers to CO_2, H_2O or CH_4 (Shah et al. 2008), thus, contributing to the ultimate fate and removal of plastics in the environment. (Shah et al. 2008). The reason why the superficial colonization of plastic debris is particularly interesting and important from a scientific perspective lies in its interference with a variety of processes not solely related to their ability for biodegradation. The changing interfacial physics and

chemistry and, additionally, biology of plastics during biofilm development is of high importance for their material characteristics (**Figure 1.4**). *Publication IV* (chapter 3.2.2) gives a comprehensive overview regarding the impacts of biofilm formation on the fate and effects of environmental plastics (Rummel et al. 2017).

Examples for the interference of natural systems with the pure physical behavior of plastics are given in studies of, *e.g.,* nanomaterials, where physiological environments favor the rapid formation of protein-corona by adsorption around the nanoparticles. This protein-corona has the potential to mask material properties so that the biological identity is distinct from its synthetic one (Walkey and Chan 2012; Lynch et al. 2007). Studies have demonstrated that the uptake and resulting ecotoxicological effects of nanoparticles towards test organisms can be altered or even alleviated by such adsorption processes (Seitz et al. 2016; Seitz et al. 2015; Nasser and Lynch 2016; Fadare et al. 2020; Fadare et al. 2019). Galloway, Cole, and Lewis (2017) compared this "corona" concept of nanoparticles to micro- and nanoplastic and coined the term 'eco-corona' in accordance with the layers of proteins forming on nanoparticles, the so-called absorbome (Walkey and Chan 2012; Galloway, Cole, and Lewis 2017). Correspondingly, prior to the formation of a biofilm in natural waters, a layer of organic and inorganic molecules adsorbs almost instantaneously to new habitable surfaces such as plastic litter entering the aquatic system. This conditioning film or "eco-corona" formation is under scientific debate to govern biofilm succession and community structure by modifying the surface properties and providing nutrient resources (Schneider and Marshall 1994; Schneider et al. 1994). If various polymer types differ in their related initial conditioning film and subsequent biofilm community structure, it can have important implications for downstream effects of environmental plastic. Identical biological entities, communities and functions would suggest that we could evaluate epiplastic biofilms collectively without further differentiation in their hazard or effect potential. If we face distinct material-specific differentiation of epiplastic communities, this would prompt scientists to consider each polymer type separately. Furthermore, a material-specific community may result in specific functions which could potentially have ecosystem-wide implications. Within my brief review (*Publication IV*), I further elaborated the hypotheses for this thesis that:

- The conditioning film is distinct for different polymer types and weathering treatments
- In dependence of material-specific surface properties, biofilms form plastic-specific communities

I tested these hypotheses by incubating pristine and pre-weathered polyethylene terephthalate (PET), polystyrene (PS) and glass (control) slides in natural stream water (*Manuscript II*, under review) and characterized the quality of the conditioning film by rinsing and concentrating the organic layer followed by high resolution mass spectrometry. The data was compared to surface characteristics of the respective materials. Furthermore, in a short- and long-term incubation experiment, I investigated the structural and functional endpoints of biofilms by next generation sequencing (NGS) techniques, confocal Laser Scanning Microscopy (cLSM) and Pulse Amplitude Modulation (PAM) Fluorometry (*Manuscript III*, in preparation)

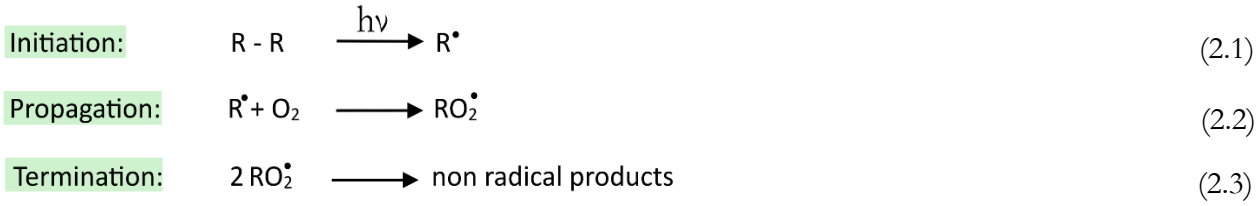
2. Abiotic Weathering

Several different degradation pathways contribute to the weathering of polymers such as chemical, thermal, mechanical, biological and radiolytic or photo-degradation (**Table 1.1**). Three factors can initiate the oxidative degradation of polymers in the presence of oxygen: UV-light, heat or mechanical stress (Izdebska 2016). Since UV-induced photo-degradation is the main driving force of environmental polymer degradation (Andrady 2015b) colleagues and I performed accelerated weathering of polymers within the WEATHER-MIC project focusing on this environmentally relevant degradation process (Gewert, Plassmann, and MacLeod 2015). The next section will give an overview over the chemical reactions and testing methods behind the abiotic polymer degradation, specifically the photo-degradation, followed by my research on the evaluation of the toxic effects of leachates of UV-weathered microplastic particles (*Publication III* and *Manuscript I*).

2.1 The Chemistry of Photodegradation

Industry has undertaken enormous efforts to endure and improve the polymers lifetime and performance over their time of use. Since all materials are subject to weathering they are designed and modified to increase resistance to impact loads that act during their life cycle under normal conditions for their intended use. The outdoor environment is the most severe environment for synthetic polymers (Feldman 2002) since several physical impacts act concurrently and at high dosage that promote polymer degradation.

Pathways of polymer degradation in the marine environment are summarized in a review by Gewert, Plassmann, and MacLeod (2015). In short, polymers with a carbon-carbon backbone such as PE, PP, PS or PVC degrade generally by photo-initiated oxidation in three steps. Initiation (equation (eq.) 2.1): polymer chain scissions caused by UV-light produce free radicals, Propagation (eq. 2.2): these free radicals react with oxygen and form peroxy radicals. This step may promote further degradation via chain scission and crosslinking as well as further radical reactions leading to autoxidation, Termination (eq. 2.3): Inert products are formed by the combination of two free radicals (Gewert, Plassmann, and MacLeod 2015).



*adopted from Geuskens and David (1979)

Initial oxidation occurs in the amorphous regions of the polymer, which are responsible for the material's flexibility, causing disproportionately large changes such as cross-linking and chain-scissions. (Andrady 2015b). As a consequence of cross-linking, reduction in mean molecular weight and loss of additive substances such as plasticizer, the material becomes brittle which may lead to fragmentation upon mechanical stress. Only a thin top-layer of the polymer can be penetrated by UV-light which is why photodegradation processes occur to a depth of 50-100 μm causing crack propagation at the outside (Turton and White 2001; Min, Cuiffi, and Mathers 2020; ter Halle et al. 2016). As a result of photo-oxidation and after the UV-stabilizers and antioxidants (often added during manufacturing which trap free radicals formed by UV-light exposure) have undergone their protective reactions, smaller molecular mass molecules are formed (Gewert, Plassmann, and MacLeod 2015). Furthermore, the material often discolorizes or turns yellow after the degradation and release of the stabilizers (Pickett 2018). In material science, the change of mechanical properties due to weathering can be routinely measured as the tensile strength or elongation at break (Rajakumar et al. 2009). Other endpoints to monitor surface or bulk material functionality and chemistry are, *e.g.,* contact angle measurements, surface charge by zeta potential, absorbance spectra via Fourier-transform infrared (FTIR) or RAMAN spectroscopy and others. Two of these techniques, contact angle measurements and FTIR spectroscopy, find their application in *Manuscript II*.

The ongoing fragmentation caused by the degradation processes described above has led to some aquatic environments nowadays known to be a "plastic soup" (**Figure 1.1**). Despite the problematic disintegration of macroplastic down to micro- and nanoplastic that may cause negative effects upon ingestion and interaction with biota, the other often-overlooked issue is the leaching of substances during weathering. Bejgarn et al. (2015) investigated effects of leachate waters derived from commercial plastic consumer products towards the marine copepod *Nitocra spinipes*. Interestingly, the authors found that some leachate waters increased their ecotoxicological potential with ongoing irradiation time by an artificial weathering treatment indicating enhanced leaching and accumulation

of toxic leachate products (Bejgarn et al. 2015). Contrarily, some of the products became less toxic with prolonged irradiation exposure, probably due to enhanced decay of toxic chemicals liberated from the product. Comparably, Suhrhoff and Scholz-Böttcher (2016) detected enhanced leaching from plastics due to UV-light exposure only for a few chemicals, the majority of analytes remained unaffected by the UV-treatment. A similar effect was described in a recent study by Schiavo et al. (2020) in which the leaching time over 3 weeks with light-dark cycles decreased negative effects towards *V. fischeri*. The effect of artificial solar radiation on leaching DOC from PE and PP varied depending on the material and duration of exposure in a study by Romera-Castillo et al. (2018).

For the above-mentioned studies, chemicals or DOC leaching from commercially available consumer products may be plastic additives, non-covalently bound oligomer or unreacted monomer residues and/or polymer degradation products. Hence, leachate waters are generally a mixture of different chemicals often of unknown composition or even unknown chemical structural information due to the presence of transformation and degradation products. Gewert et al. (2018) identified chemicals that were likely chain scission products from UV-degrading synthetic polymers. For all polymers PE, PP, PS and PET the authors found homologous series of low-molecular weight polymer fragments with oxidized end-groups, mainly dicarboxylic acids (Gewert et al. 2018). It is currently unknown, whether plastics have other ecotoxicological potential than pure leaching of additives. To test this hypothesis, an artificial weathering setup was developed within the WEATHER-MIC consortium and applied in my project studies (Gewert et al. 2018; Oelschlägel, Pfeiffer, and Potthoff 2018).

2.2 Accelerated Weathering and its Application for Ecotoxicological Testing of Microplastic Leachates

To overcome the problematic extrapolation of degradation time scales by natural weathering, researchers have developed alternative weathering testing methods. These ad-hoc weathering techniques apply mostly one specific material stressor in one experiment while several may act in concert at natural exposure. In general, the International Organization for Standardization has set up a large portfolio of ISO protocols to simulate and track the polymer material changes on exposure to damp heat, water spray, salt mist (ISO 4611:2010), exposure to sunlight and climate such as humidity and temperature (ISO 4892 part 1-3 (2013-2016), ISO 877 (2009)). Several researchers have developed different ad-hoc weathering methods. Andrade et al. (2019) highlighted the advantage of adjustable conditions in their set-up and the possibility to scale up volumes. Other researchers induced polymer

ageing by H_2O_2 and Fenton reagent at low pH (Lang et al. 2020). Within our WEATHER-MIC consortium, two different weathering setups were developed. A less extreme weathering set-up was developed by Oelschlägel, Pfeiffer, and Potthoff (2018) by the application of a UV-light source in combination with sample vessels on a rotation table. Furthermore, Gewert et al. (2018) used a strong UV-light source centered in a rotating wheel of quartz glass sample bottles with an additional cooling system.

In chapter 1.6.1, I hypothesized that UV-light caused photo-oxidation of the polymers in artificial seawater and that the chemical mixtures liberated from weathering microplastic are of ecotoxicological relevance (**Figure 1.3**). To investigate the effect of UV-light, as the main driver of environmental abiotic degradation (Andrady 2015b) on the leaching potential of additive-free polymers, we applied the weathering wheel described by Gewert et al. (2018). Four commercially important polymer types (polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), and polystyrene (PS)) were leachated in artificial seawater by project partners at the Department of Environmental Science (ACES). Two positive controls from electronic waste (e-waste) and a computer keyboard, known to contain high concentrations of PCBs, Polybrominated Diphenyl Ethers (PBDEs) and Bisphenol A (BPA) (Morin, Arp, and Hale 2015; Morin et al. 2017) were included to demonstrate the sensitivity of our test battery towards substances liberated from the test material. Leachate waters of dark controls (here after DC) and a strong UV-light exposure scenario (here after UV) corresponding to roughly 1 ½ year of middle European outdoor exposure were concentrated via solid phase extraction (SPE) and dosed in the respective bioassays (**Figure 2.1 a-c**). As a result of this enrichment and dilution in the assay, the effect concentrations are given in the units of the relative enrichment factor (REF). To facilitate a more intuitive interpretation of low and high effect concentrations to be associated with respective low and high hazard potential, I used the inverse effect concentration defined as effect units of bioassays (EU_{bio}).

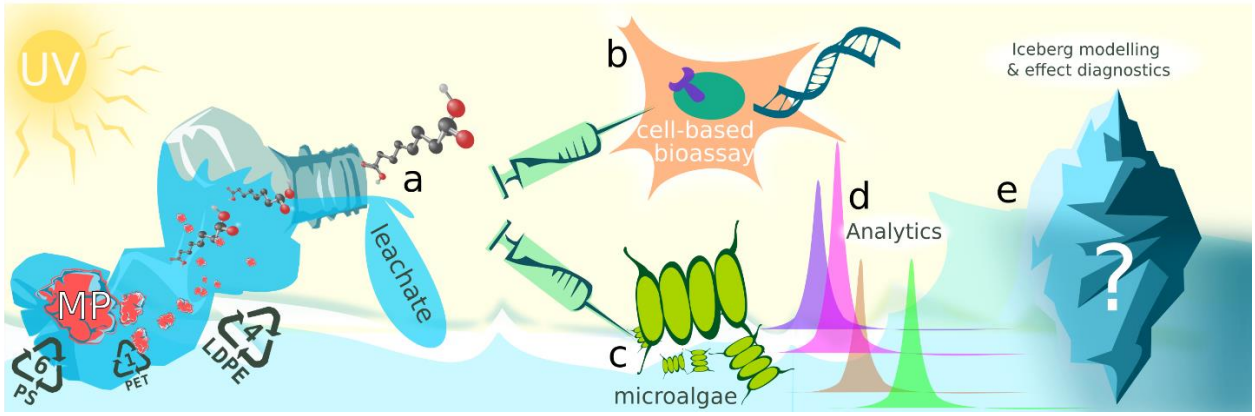


Figure 2.1: Framework of the *in vitro* and *in vivo* studies on the ecotoxicological relevance and identification of the mode of toxic action of microplastic leachates. **a**: Generation of microplastic leachates under UV-light exposure in artificial seawater; **b**: dosing of SPE-concentrated leachates in reporter gene assays; **c**: dosing of the leachates in microalgae assay; **d**: chemical analytics to identify potential polymer degradation products; **e**: effect diagnostics and iceberg modelling to explain the observed effects

2.3 *In vitro* and *in vivo* Bioassays to Identify Mixture Effects and Modes of Toxic Action from Microplastic Leachates

For the study of effects by chemicals liberated during artificial weathering of plastics (**Figure 2.1 a**) we choose a test battery of different reporter gene cell lines (**Figure 2.1 b**). and the microalgae *Scenedesmus vacuolatus* (**Figure 2.1 c**). Using cell lines as an *in vitro* model system to detect chemical mixture effects has several advantageous compared to classical *in vivo* organism tests. Animal husbandry is obsolete and legal and ethical animal issues are not of concern. Cell-based assays are sensitive, cost-and time-efficient alternatives (Escher and Leusch 2011). In short, the recombinant cells carry a vector or plasmid which encodes for a responsive element which is under the control of the receptor of interest. Downstream of this responsive element, or so-called Upstream Activation Sequence (UAS), a reporter gene is cloned. The reporter gene may then encode for luciferase, which can be quantified with luciferin as substrate (*e.g.,* CALUX® assays) or an enzyme such as ß-lactamase which is capable to cleave a fluorescent substrate (*e.g.*, FRET reagent in GeneBLAzer assays) and reporter gene induction can then be directly read out via bioluminescence, absorbance or fluorescence.

By this approach, we aim at identifying the activation of specific cell-biological endpoints and thereby facilitate the identification of certain modes of toxic action (MoA) (Escher and Leusch 2011). The concentrated leachates were dosed in different transgenic cell lines to cover cytotoxicity in all cell lines and the specific effects of i) activation of metabolic enzymes via binding to the aryl hydrocarbon

Receptor (AhR) (Brennan et al. 2015) and the peroxisome proliferator-activated receptor gamma (PPARγ) (Neale et al. 2017; Invitrogen 2007b), ii) specific hormone receptor-mediated effects (estrogenicity, ERα); (Invitrogen 2007a) and ii) adaptive stress responses exemplified by the oxidative stress response (AREc32) (Wang, Hayes, and Wolf 2006; Escher et al. 2012).

In *Publication III*, I gained insight in the MoA of plastic leachates by reporter gene assays. The ecological relevance of such tests, however, increases with the complexity of the applied test system/organism and relation to ecosystems (Köhler and Triebskorn 2013). Ecologically relevant endpoints are photosynthesis and primary production. For the investigation of effects of microplastics towards microalgae in a particle exposure-scenario, we would face the situation that the test organism may be impaired by the physical presence of particles or by secondary effects stemming from leaching chemicals (Prata, da Costa, Lopes, et al. 2019). The first named process may lead to particle adsorption, aggregation or shading effects as discussed by Zhang et al. (2017). Indirect effects towards primary producers by chemicals leaching from microplastic have gained little attention so far. Luo et al. (2019) demonstrated the release of additive compounds into water by leaching microplastic with time and detected decreased quantum yield efficiency at photosystem II at high leachate concentrations for the microalgae *Chlorella vulgaris*. Two sensitive marine cyanobacteria *Prochlorococcus* strains were affected by plastic leachate exposure, with increased transcription of common stress response genes (Tetu et al. 2019). Both studies applied plastic test material where additive leaching could be expected. Therefore testing leachates from additive-free polymers in microalgae represents a tool to identify the MoA on the base of the food chain excluding any effects potentially stemming from leaching substances intentionally added to the polymers (additives). We choose microalgae as a suitable *in vivo* test organism since microalgae (plankton) and biofilm forming organisms (such as protists) may be the first interaction and have the longest contact time between environmental plastics and biota as outlined in *Publication IV*.

To better account for PPARγ and the microalgae results, we tested the hypothesis that dicarboxylic acids, previously identified as degradation products of UV-weathered PE (Gewert et al. 2018), could explain the observed explicit induction of PPARγ or if they caused baseline toxicity in microalgae (**Figure 2.1 d, e**). Therefore, we dosed reference mono- and dicarboxylic acids with a range of carbon chain lengths (C5-C18) into the PPARγ and microalgae assay. For the PPARγ results, the effect concentrations derived from single compound tests were applied to a concentration addition model. The observed bioanalytical effects (here EU_{bio}) were related to the modeled effects based on the sum of each chemical's measured concentration and its relative effect potency (EU_{chem}) (*Publication*

III, Materials and Methods). By this so-called iceberg modelling (Neale et al. 2017; Judson et al. 2016) it was possible to explore how much of the observed effect could be explained by the quantified chemicals (**Figure 2.1 e**).

To investigate the MoA of the carboxylic acids, toxic ratios (TR), as the ratio between the predicted baseline toxicity for the PPARγ cell line, based on the quantitative structure-activity relationship (QSAR) by Escher et al. (2019), and the measured cytotoxicity (IC_{10}) were calculated. Furthermore, I analyzed the respective specificity ratios ($SR_{cytotoxicity}$), the ratio between cytotoxicity and measured effect (EC_{10}) (Escher et al. 2020).

For microalgae, mainly the monocarboxylic acids were active. These remained undetected by the applied high resolution mass spectrometry method and the iceberg modelling was consequentially not feasible. Therefore, I applied the QSAR by Altenburger, Walter, and Grote (2004) to calculate baseline toxicity of the carboxylic acids towards microalgae. This QSAR is based on the hydrophobicity (K_{ow}) of the chemical of investigation. However, at the applied pH in the assay, the carboxylic acids will fully dissociate and be present in their anionic form, for which the cellular uptake is slower and smaller (Fischer et al. 2018). To account for speciation, K_{lipw} of the neutral species of the carboxylic acids was predicted by the logK_{ow}-based QSAR by Endo, Escher, and Goss (2011) and baseline toxicity of the neutral fraction using the ionization-corrected liposome-water distribution ratios [$D_{lip/w}$ (pH 7.4)] were calculated following Escher et al. (2020). By this approach, I calculated TRs also for the microalgae data to further explore the MoA of microplastic degradation products. In a last step, I correlated the cytotoxicity values of the bioassays (inverse IC_{10} as TU_{bio}) to the EU values of the microalgae assay using linear regression to further explore the similarity of the MoA between the different assays.

2.4 Effects of Leachates from UV-Weathered Microplastic in *in vitro* and *in vivo* Test Systems

Applying a test battery ranging from *in vitro* cell-based bioassays to an *in-vivo* whole organism test, I aimed to characterize and to identify the mixture effects and the MoA of leachates from weathering plastics.

Most of the tested leachate samples of PE, PET, PS and PP induced oxidative stress (AREc32) (Figure 1 B in *Publication III*). Here, elevated effects were observed for the UV-weathered samples indicating the generation of small reactive molecules during weathering. For all assays, the positive controls e-waste and keyboard showed clear induction of the respective signaling pathway (Figure 1 A-C in *Publication III*). No induction of the assays AhR and ERα different from the control could be detected for the investigated test polymers. For efficiency and to condense my main findings, the AhR and ERα will not be discussed further in this section.

2.4.1 Specific Activation of the PPARγ by UV-Weathered Microplastic Leachates

PPARγ was predominantly induced especially by PE leachates (**Figure 2.2 A** and Figure 1 C in *Publication III*). Most strikingly, the UV-treated PE (EU_{bio} (PE_{UV}) = 0.50) showed induction levels of PPARγ comparable to the UV-treated positive control keyboard (EU_{bio} ($keyboard_{UV}$) = 0.53) (**Figure 2.2 A**). For PE, the UV-treated samples showed a more than three times higher induction than its corresponding dark control (EU_{bio} (PE_{DC}) = 0.15) which is the most pronounced difference between UV vs. DC treatments in all tested assays. The explicit induction of the UV-treated PE may have indicated the presence of degradation products that were capable of specifically activating PPARγ. Candidates were dicarboxylic acids that were previously identified as chain scission products of degrading PE by Gewert et al. (2018). Since fatty acids (FAs) are natural ligands of the PPARγ (Wang et al. 2014) it is likely that the dicarboxylic acids show similar activity towards this receptor. It is also conceivable that the induction of PPARγ occurred by other known plastic-associated agonists like DEHP (Kambia et al. 2016). The activation potential of active metabolites of DEHP, mono-ethylhexyl phthalate, was previously hypothesized (Lovekamp-Swan and Davis 2003), however its presence in the leachates was unlikely since migration potential of DEHP was marginally affected by UV exposure (Suhrhoff and Scholz-Böttcher 2016) and it is typically not added to the virgin polymer (Narvaéz Rincón and Suárez Palacios 2016).

2.4.2 Non-Specific Toxicity by Plastic Leachates in the Microalgae *Scenedesmus vacuolatus*

For the *in vivo* assay, using the freshwater microalgae *Scenedesmus vacuolatus*, fluorescence and cell number were the two most responsive endpoints to leachates from weathering microplastic (**Figure 2.2 B**, Figure 2 in *Manuscript I*,). Similar to the results of *Publication III*, mainly the two positive controls e-waste and keyboard and the test polymer PE_{UV} displayed ecotoxicological potential to negatively affect microalgae. No specific toxicity, such as inhibition of the photosystem, was induced by substances leaching from weathered PET, PP and PS microplastic and effect concentrations were mainly in the

range of the blanks (Table 1, Figure 2 B in *Manuscript I*). One reason for the relatively low ecotoxicological potential of pre-production plastics may be the absence of any additives. Other studies linked the high toxicity of plastic leachates on freshwater and marine microalgae directly to high additive chemical content (Capolupo et al. 2020) but also enhanced photosynthesis was reported (Chae, Hong, and An 2020). The authors speculated about this hormesis effect that leaching DOC (such as the measured hexabromocyclododecanes, bisphenol A and UV326) might have promoted photosynthetic activity and thereby cell growth (Chae, Hong, and An 2020). Tetu et al. (2019) detected impaired growth, photosynthetic capacity, and genome-wide transcriptional changes by LDPE and PVC leachates for an important primary producer *Prochlorochoccus spec.*

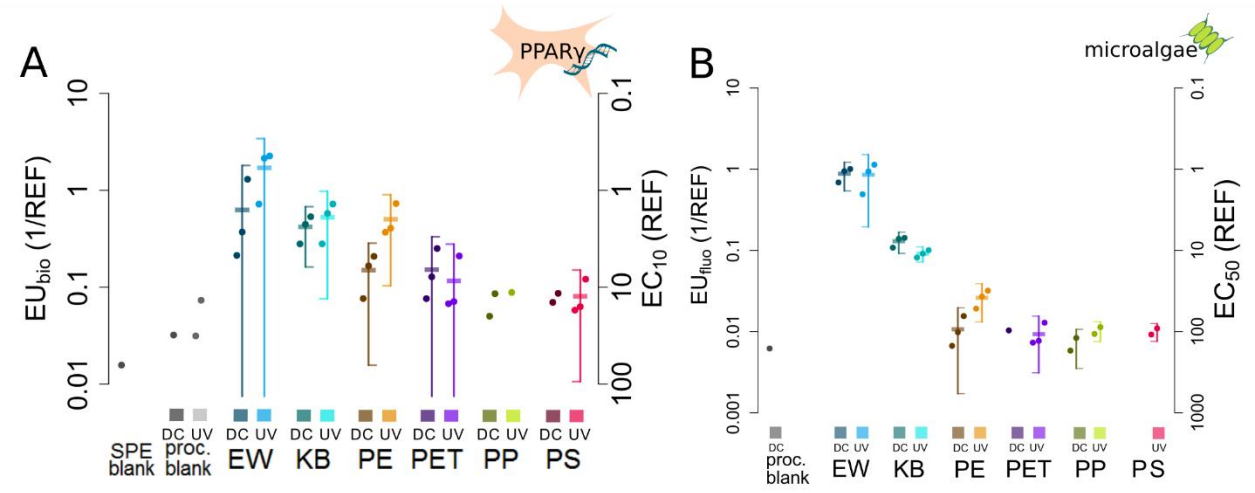


Figure 2.2: Effects of solid phase-enriched microplastic leachates tested in reporter gene and microalgae assays. **A**: Bioanalytical effect units ($EU_{bio,}$) defined as the inverse EC_{10} (1/REF on left y-axis, REF on right y-axis), measured in the cell-based bioassay PPARγ. **B**: Effect units for the microalgae endpoint fluorescence (EU_{fluo}) as the inverse EC_{50} (1/REF on left y-axis, REF on right y-axis). Samples are color coded for SPE blanks, procedural blanks, two positive controls (e-waste (EW) and keyboard (KB)) and the four test polymers PE, PET, PP and PS. Dark controls (DC) and UV-treated (UV) samples are presented juxtaposed in darker and lighter shades of the different colors. If all three replicates resulted in measurable effects, the squares represent the mean, whiskers the upper and lower range of the 95 % confidence interval. The absence of symbols implies effect > highest tested concentration (REF 200 and 197 for the cell and microalgae assay, respectively)

2.4.3 Explaining the Effects of PPARγ by Iceberg Modelling

The $EU_{chem(i)}$ values, derived from single compound testing in PPARγ (Figure 2 in *Publication III*) and the respective measured concentrations (Table S3, SI in *Publication III*) were summed up in a mixture model based on concentration addition. Applying this iceberg model, I could partly explain the

observed effects in the PPARγ bioassay (EU_{bio}) by the mixture effects of the quantified polymer degradation products, the dicarboxylic acids, present in the leachates (**Figure 2.3,** Figure 3 in *Publication III*). They accounted for up to 42 % of the observed EU_{bio} values in the case of PE as indicated by proximity of these samples to the 1:1 line (**Figure 2.3**). Tetradecanedioic acid was the main mixture risk driver of the detected PPARγ induction of the PE extracts, due to three reasons: 1) It was the most potent PPARγ inducer amongst the dicarboxylic acids in the single compound testing (Figure 2, Figure S5, SI in *Publication III*) it was detected at high quantities exclusively in the PE samples with a factor of around three higher liberation for the UV treatment than for the dark control (Table S3, SI in *Publication III*), which is 3) in accordance with the observed bioanalytical effects of the related extracts causing three times higher effects as well (**Figure 2.2 A)**. For PE, the substantial contribution of EU_{chem} to EU_{bio} of, in some cases, over 40 % (**Figure 2.3**) is an important explanatory parameter for the PPARγ gene pathway activation. Since Albertsson, Barenstedt, and Karlsson (1995) identified over 60 PE degradation products, predominantly monocarboxylic acids, we expect those to be present in our PE leachates as well, although they remained undetected by our analytical method.

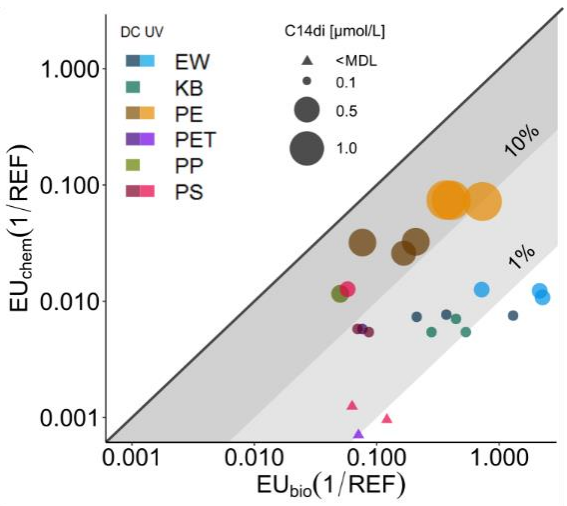


Figure 2.3: The effect units derived from chemical analysis and single compound testing (EU_{chem}) plotted against the bioanalytical effect units (EU_{bio}) for the PPARγ assay (log scale). The 1:1 line indicates that 100 %, dark and light shaded area that 10 % and 1 %, respectively, of the observed effect can be attributed to the analytically determined chemicals. Colors represent the different samples each as the dark control (DC, darker shading) and the UV treatment (UV, lighter shading). The bubble size corresponds to the relative concentration of tetradecanedioic acid, the main driver of the mixture effect. Triangles represent data where tetradecanedioic acid was < MDL.

2.4.4 Identification of the Mode of Toxic Action of Weathered Microplastic Leachates

Leachates induced cytotoxicity (TU_{bio}) in almost all tested assays (Figure S4, SI in *Publication III*). For PPARγ, the investigated PE degradation products, the carboxylic acids, were all baseline toxic indicated by their TRs < 10 (**Figure 2.4 A**). Additionally, carboxylic acids displayed a specific capacity to induce the PPARγ gene pathway that was not influenced by cytotoxicity (**Figure 2.4 A**). Following the proposed classification of Escher et al. (2019), high carbon chain length acids (C10 – C18) appeared to have a moderate specific MoA (SR_{cyto} < 10), while low carbon chain length carboxylic acids (< C10) showed specific toxicity (10 ≤ SR_{cyto} < 100) (**Figure 2.4 A**) that was not influenced by the so-called cytotoxicity-associated "burst" (Judson et al. 2016).

For microalgae, the calculated TRs < 10 for the endpoints fluorescence and cell density were in good agreement with the previously discussed non-specific disturbance of the cell membrane elicited by the acids (**Figure 2.4 B**). Moreover, the microalgae endpoints fluorescence and cell number displayed a very narrow range of TRs indicating low uncertainty between calculated and measured EC_{50} values supporting their baseline MoA (**Figure 2.4 B).** It can be assumed that the critical membrane concentration of 70 mmol/L_{lip} resulting in destabilization of the phospholipid bilayer was reached by the carboxylic acids (Escher et al. 2019) but there were no specific effects induced in algae. Hence, the applied QSAR is potentially not adequate for PS II inhibition since it shall serve solely to calculate baseline toxicity values based on the chemical's hydrophobicity. These observations compare well to the finding that fluorescence and cell number, as apical endpoints, were the most sensitive endpoints in the leachate tests (Figure 1 in *Manuscript I*).

Furthermore, the EU values of fluorescence correlated well to the cytotoxicity values derived from reporter gene assays with regression slopes close to one (**Figure 2.4 C**). The impairment of the photosystem is therefore an indirect effect of baseline toxicity as a similar good correlation to cytotoxicity of reporter gene assays demonstrate (**Figure 2.4 D**).

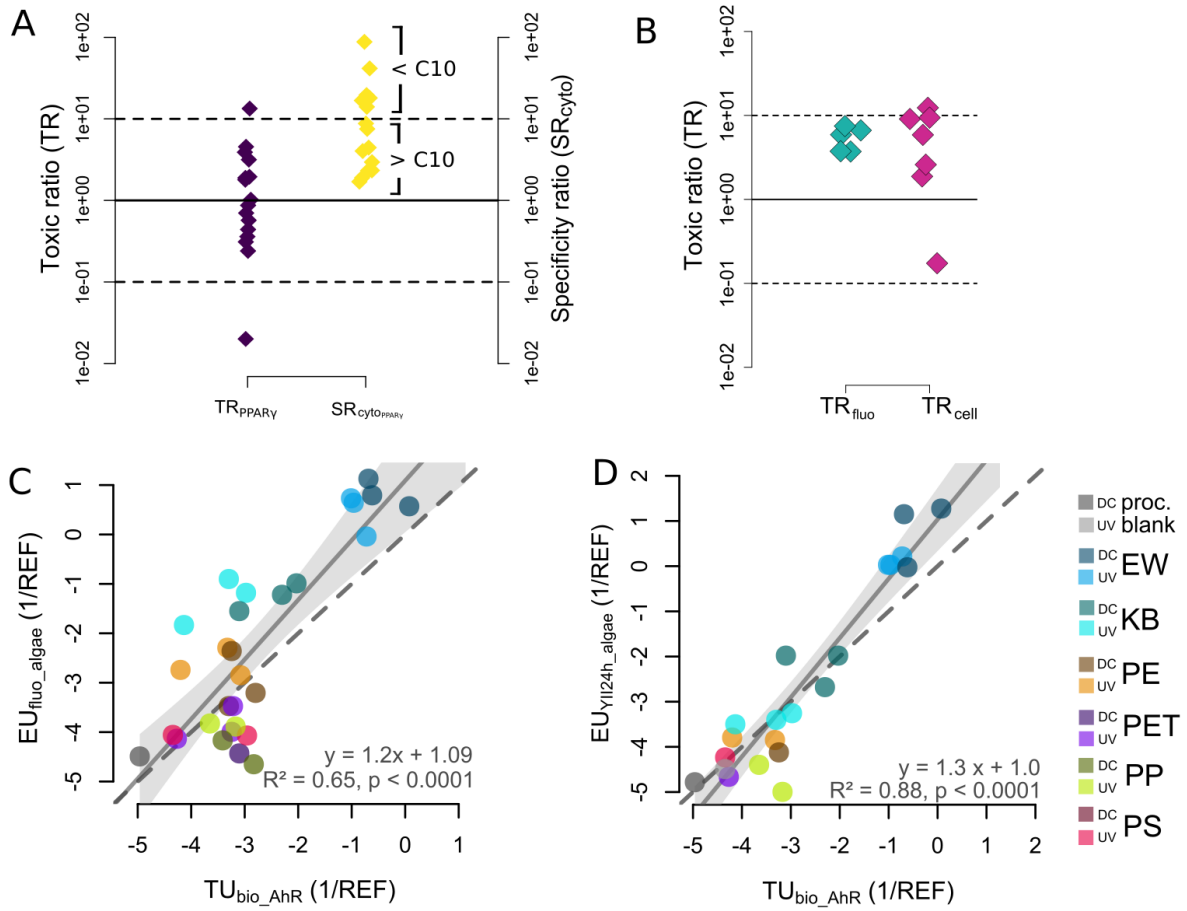


Figure 2.4: Combined effect characterization of the leachates from UV-weathered microplastic by the reporter gene assay PPARγ and the microalgae assay. **A**: toxic ratios (TR) and specificity ratios for cytotoxicity (SR_{cyto}) for reference mono- and dicarboxylic acids (C5-C18) tested in the PPARγ assay. **B**: TRs for the microalgae endpoints fluorescence (fluo), cell number (cell). **C**: statistically significant correlation between cell line and microalgae data could be observed by the linear regression of the apical endpoint fluorescence for microalgae (fluo_algae) as a function of toxic units (TU_{bio}) of the AhR assay (regression parameters, coefficient of determination and p-value are included in the plot) (log-transformed data). **D**: Correlation of TU_{bio} of AhR and the photosynthetic activity at 24h after dosing for microalgae (EU_{YII24h_algae}) (log-transformed data). Dashed line in C and D represents the 1:1 line. Color code for C and D shows the different polymer types with respective dark control (DC) and weathering treatment (UV)

2.5 Conclusions on the Ecotoxicological Effect Potential of Plastic Leachates

There are several aspects highlighting the significance of the above findings: First, the activation of a nuclear receptor (here PPARγ) relevant for the regulation of cellular development and metabolism in higher organisms (Dunning et al. 2014; Berger and Moller 2002) by aqueous plastic leachates

demonstrates that the general (scientific and industrial) opinion that polymers were inert materials (Chow et al. 2018) does not hold true. Second, the pollution of the (aquatic) environment by plastic debris implicates the ongoing exposure of plastic debris to sunlight, which will inevitably lead to its photo-degradation. This may result in even higher emission rates of substances that may induce oxidative stress and have receptor-disruptive potential (here PPARγ). Third, the general opinion with respect to plastics, that leaching of intentionally added chemicals (*e.g.,* plasticizers) solely are causing harm to biota, can be extended by an additional source of substances of concern: the raw polymer itself. The mainly unspecific toxicity of the investigated plastic leachates was reflected in low potency for photosystem impairment in the microalgae *Scenedesmus vacuolatus*. However, baseline toxicity towards microalgae was induced by all leachate types and was in good agreement with the results from the reporter gene assays.

Unraveling the potential effects of weathering plastic and the associated chemicals is a high research priority for planetary health (*Publication I*). Our aim to capture the ecotoxicological potential of the expected mixtures of unknown chemicals leaching from weathering plastic renders *in vitro* assays and whole-organism cell-based assays as practical tools. *Publication III* and *Manuscript I*, as summarized above, were conducted under a bioanalytical and chemical focus of polymers, their leaching behavior and the herewith-connected ecotoxicity. We identified, that weathering played a crucial role for the toxicological profile of plastic leachates causing increased effect potential for almost all of the investigated polymers. However, concurrently to weathering processes that were simulated in the above-mentioned sections and experiments (radiolytic degradation), microbial colonization on plastic surfaces will start from the very beginning once the material has reached aquatic systems. These growing epiplastic biofilms are highly involved in weathering processes of environmental plastics either actively via biodegradation and/or by interfering in abiotic weathering. Therefore, the next section will focus on biotic weathering.

3. Biotic Weathering

The previous chapter was under the focus of abiotic weathering conditions where physical impacts (such as UV-light) caused changes in the polymer with ecotoxicological implications. However, biotic weathering represents an equally important factor modulating the fate and effects of plastics in the environment due to their potential to change the material properties and physical behavior in natural systems. Immediately upon exposure to natural waters and before microbial colonization takes place, new habitable submerged surfaces are instantaneously conditioned by a layer of organic matter (OM) (**Figure 3.1 a**). Then, microorganisms (MOs) start colonizing almost all submerged surfaces, including plastic debris, and build up biofilms (**Figure 3.1 b**). For chronological and mechanistic clarity, the following chapter presents data on the first conditioning of surfaces by OM (chapter 3.1) followed by insights in early biofilm succession (chapter 3.2).

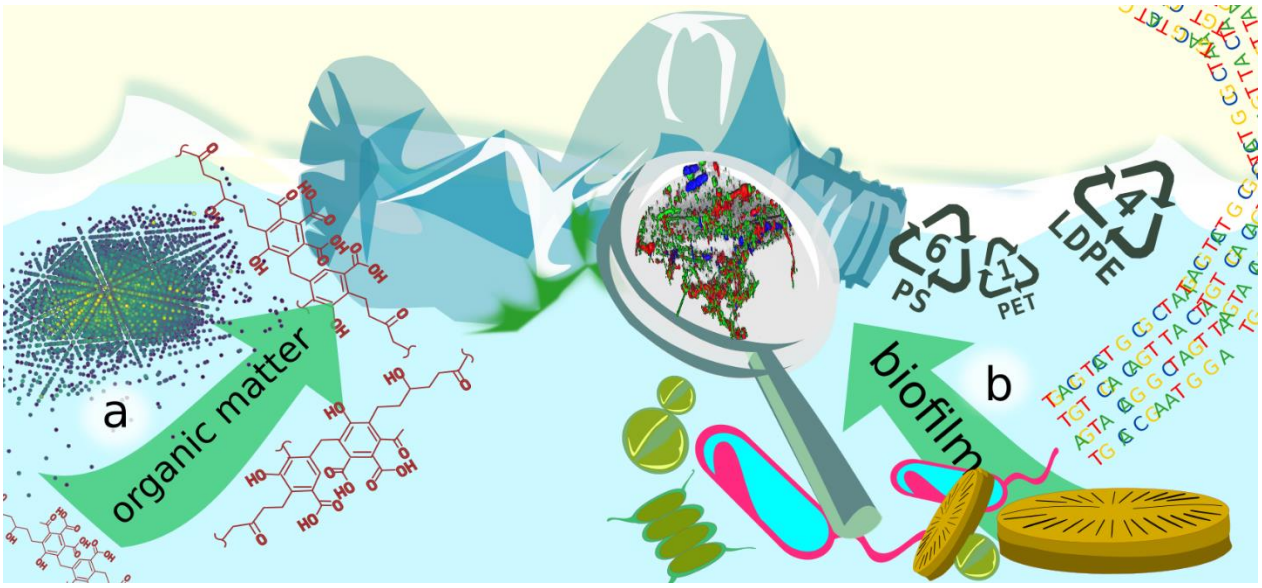


Figure 3.1: The studies presented in chapter 3 investigated relevant early processes of biotic weathering leading to the formation of a biofilm community. **a:** Chapter 3.1 focusses on the adsorption of OM, the so-called conditioning film, on different polymer surfaces as a prerequisite for biofilm formation (*Manuscript II*). **b:** Chapter 3.2 elucidates subsequent early microbial colonization phases based on structural and functional community parameters (*Manuscript II* and *III*).

3.1 The Conditioning Film as a Prerequisite for Biofilm Formation

The following section and studies will further elucidate the concept of the conditioning film and its relevance for biofilm formation on plastic surfaces.

3.1.1 The Conditioning of Surfaces by Dissolved Organic Matter

Dissolved Organic Matter (DOM) is a ubiquitous central component of aquatic ecosystems (**Figure 3.2**). Millions of organic molecules of molecular sizes of up to several kDa differing in structure and composition consist of the elements C, H, O, N, S and P and their quantity and quality reflects biotic and abiotic ecosystem processes (Findlay and Parr 2017) (**Figure 3.2**). DOM is often categorized into two main components, the humic (HA) and fulvic acids (FA) that mainly derive, amongst others, from decaying organic material that was build up in the terrestrial environment via atmospheric CO_2 fixation during photosynthesis by higher plants or via heterotrophic organisms (Bolan et al. 2011). Thus, DOM is a global pool of the above-mentioned elements which is coupled to the biogeochemical cycles via microbial turnover.

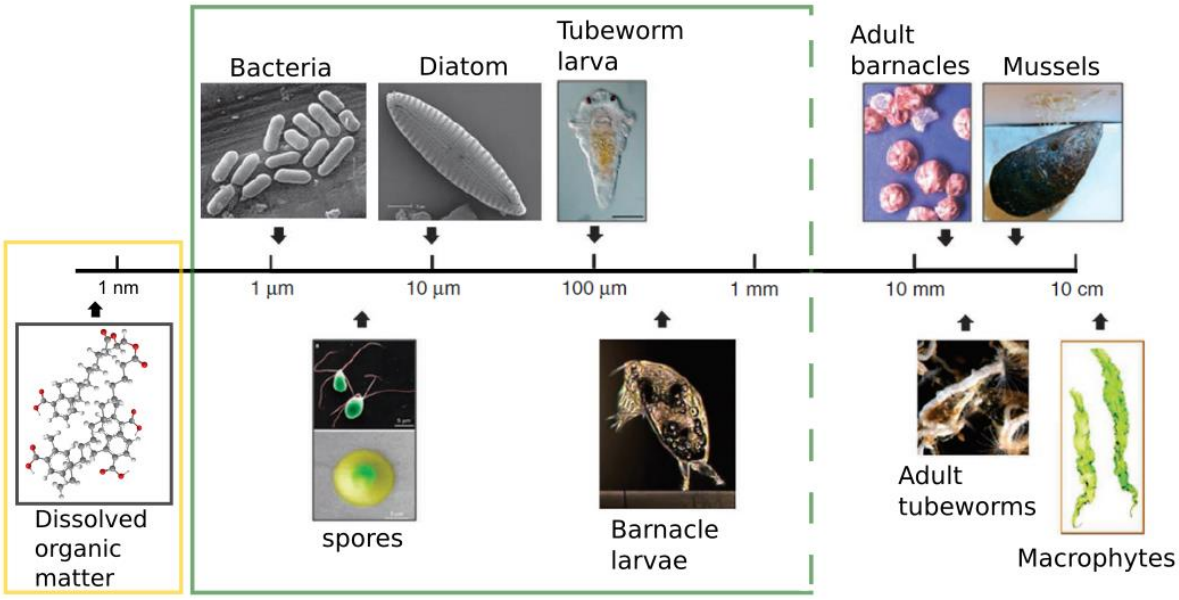


Figure 3.2: Size ranges of different representatives of a fouling community (see fouling community **Figure 1.1 B**) (with permission to publish by Springer Nature a modified version of Callow and Callow (2011)). Yellow box represents the first adsorption of an organic layer (investigated in chapter 3.1 (*Manuscript II*), followed by representatives of a biofilm (green box, with open upper size range due to grazing by micro- and meiofauna, investigated in chapter 3.2. (*Manuscript II* and *III*)).

Microplastic was claimed to substantially contribute to the global carbon pool by leaching DOC (Romera-Castillo et al. 2018). Other studies showed a clear dependence of organic molecules leaching behavior of plastics from a weathering treatment (Lee, Murphy, and Hur 2020). This leached fraction is then available for sorption to other natural substrates such as minerals (Lee and Hur 2020). In those previous studies, the DOM was characterized by a mixture of additives and monomer residues/chain scission products (such as phthalates or BPA). Noteworthy, the authors claimed these organic molecules to be DOM which strictly speaking do not belong to this group of macromolecules stemming from the breakdown of biological matter. Only one study investigated the adsorption of environmental DOM on PS microplastic using two types of DOM, the Suwannee River HA and FA by three-dimensional excitation emission matrix fluorescence spectroscopy (3D-EEM) (Abdurahman et al. 2020). The HA and FA adsorption was characterized by π-π electron donor acceptor and hydrophobic interactions (Abdurahman et al. 2020; Chen et al. 2017).

Material properties may potentially be quickly masked or equalized once submerged in water that contains a cocktail of different organic and inorganic molecules that instantaneously adsorb to the surface. Further evolving the concept of OM adsorption (in analogy to the eco-corona concept), the addition of OM in toxicity tests was demonstrated to alleviate particle toxicity towards the test organism such as microalgae or *Daphnia* (Liu, Jiang, et al. 2020; Saavedra, Stoll, and Slaveykova 2019; Fadare et al. 2020; Fadare et al. 2019). The authors of the previous studies argued, that a corona formation reduced the affinity of the particles to the microalgae cell wall and minimized the adverse effect (Liu, Jiang, et al. 2020; Saavedra, Stoll, and Slaveykova 2019). To extrapolate these results and make projections for plastic-DOM interactions, it is necessary to investigate the sorptive capacity of synthetic polymers towards DOM.

3.1.2 The Conditioning of Plastic Surfaces

In the context of plastic pollution, studies providing insight in the quality and mechanistic understanding of this first OM adsorption were still missing. To investigate the quality of organic matter adsorbed to different polymeric substrates, I measured the fluorescent fraction of surface-associated OM by means of 3D-EEM spectroscopy. As a result, the excitation-emission spectra of the conditioning films derived from PET and PS surfaces displayed a tendency of different DOM qualities indicated by the presence or absence of certain DOM fractions in the 3D EEM spectra (**Figure 3.3**, Figure S1, SI in *Manuscript II*). Differences could even be detected under the influence of an artificial

pre-weathering treatment of the surfaces. This indicated differential sorption properties along the various polymeric substrate types towards OM. The results led to the hypothesis of selective sorption of DOM on different surfaces also under the influence of photo-induced changes of surface characteristics and prompted us to conduct a more refined analysis using Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS) (*Manuscript II*).

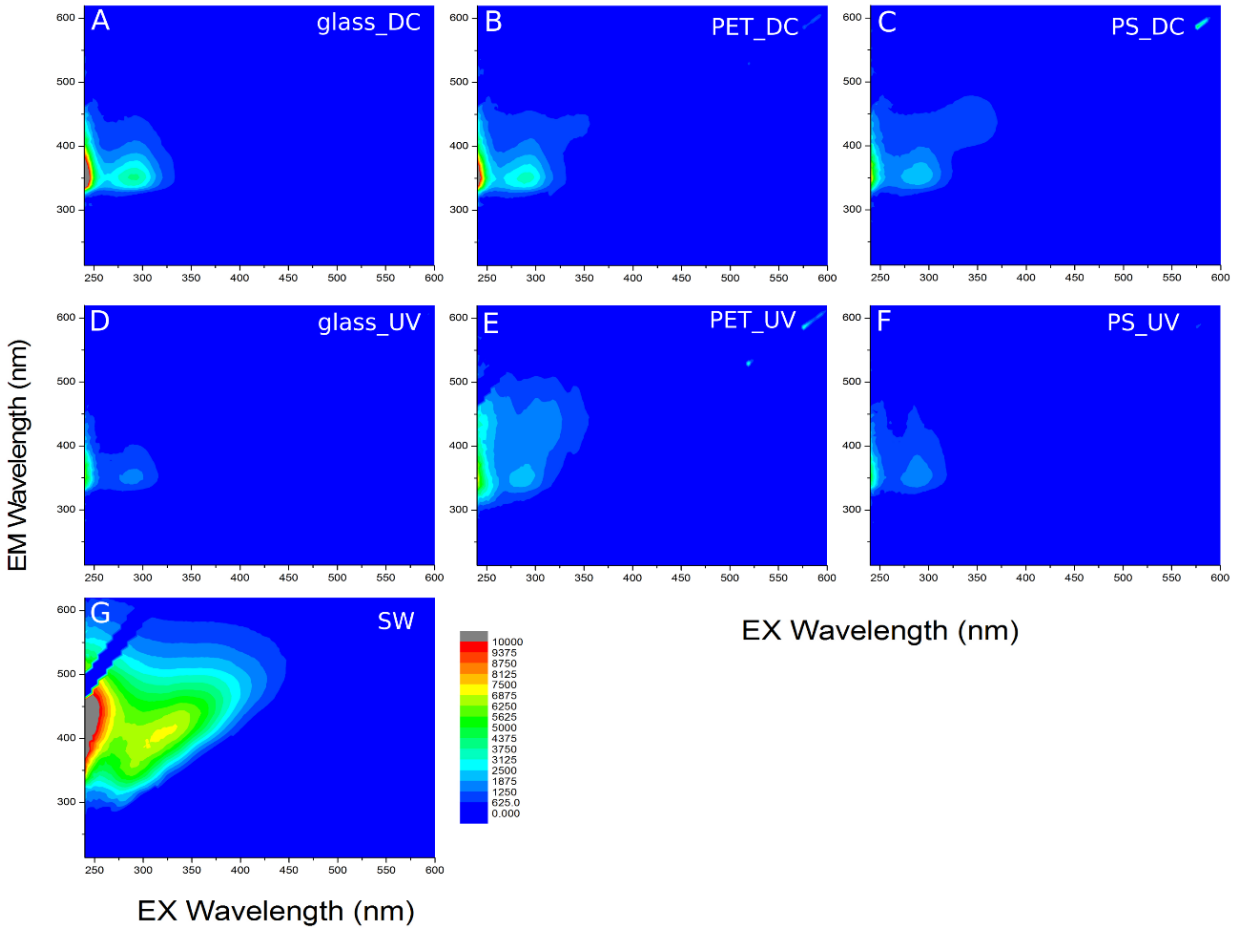


Figure 3.3: Three-dimensional excitation emission matrix fluorescence spectroscopy (3D-EEM) was used to detect any potential differences in the composition of surface-associated DOM on glass (A & D), PET (B & E) and PS (C & F) with respective dark control (DC) and UV-treatment (UV) and the stream water (SW, G) used for incubation. The results led to our hypothesis of selective sorption of DOM to polymer surfaces and formed the basis of my research question addressed in *Manuscript II* to investigate this phenomenon using FT-ICR MS in a refined analysis.

3.1.3 The Quality of the Conditioning Film and Implications for Material Surface Properties

I characterized the DOM composition of stream water (SW) and the fraction that adsorbed to submerged glass, PET and PS sheets by FT-ICR MS. The potential influence of changing surface

properties under the impact of weathering (*i.e.*, UV-induced photo-oxidation) was investigated by including a batch of artificially weathered substrates. As a prerequisite for the testing of dark (DC) and UV-light (UV) treated samples, I verified that artificial weathering caused alterations in the surface hydrophobicity of the materials PET_UV and PS_UV (**Figure 3.4 A**), an increase in the polar share of the surface free energy (SFE) and pronounced changes in the FTIR spectra (Material Properties SI3 in *Manuscript II*). The test polymers were selected based on their importance for commercial use and the availability of pure, additive-free polymer sheets. Glass slides were included to mimic a natural substrate and all substrates were incubated in 0.2 µm filtered SW. After retrieval from the incubation water, the slides were rinsed with water and methanol. The methanol was dried under nitrogen and samples were re-dissolved in MilliQ water, combined with the aqueous fraction and enriched via SPE. The SPE-extracts were measured via FT-ICR MS and signals were assigned to molecular formulas (MFs) allowing for elemental compositions of C, H, N, O, S. MFs were classified according to their degree of saturation (H/C), oxygenation (O/C) and degree of aromaticity (modified aromaticity index by Koch and Dittmar (2006)). The intensity-weighted population density D_k in percent (%) was calculated based on the summarized relative intensities in each compound class following Perminova (2019). Another common way to describe the quality of DOM is to plot the MFs based on their degree of saturation (H/C) and oxygenation (O/C), in so-called van Krevelen plots (Kew et al. 2017).

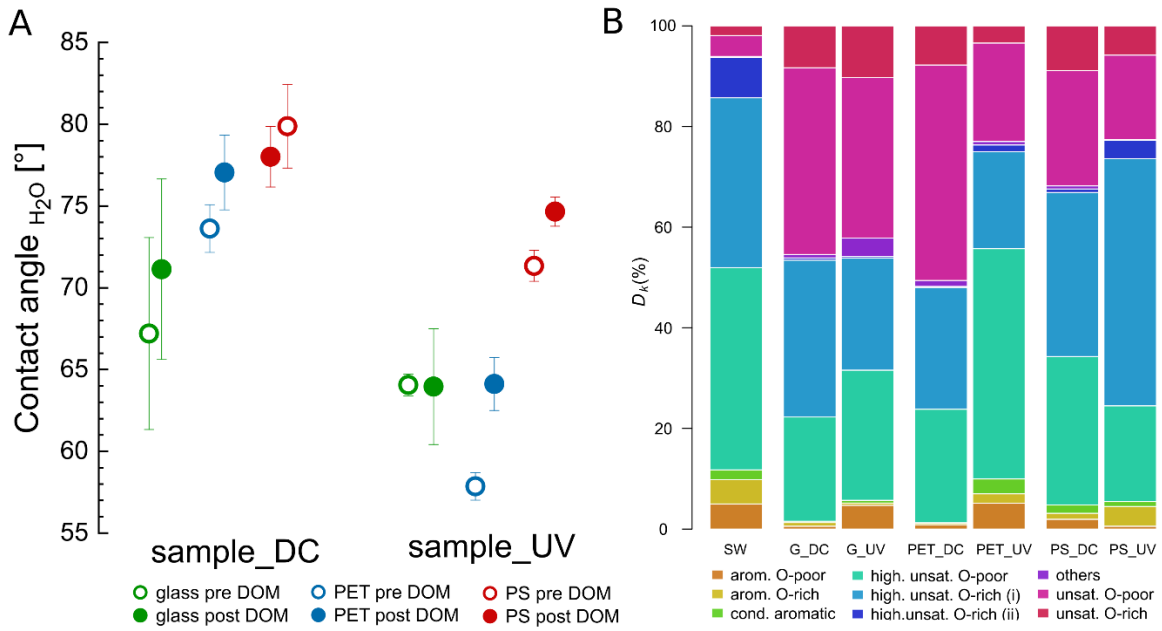


Figure 3.4: A: Surface hydrophobicity measured as contact angles for the dark control (DC) and UV-weathered (UV) substrates each pre- and post-OM adsorption. **B:** Quality of SW-DOM and surface-bound OM fractions based on the summarized relative intensity of each compound class D_k defined by Kamjunke et al. (2019).

SW-DOM selectively adsorbed to different substrates, which could be identified by substrate-specific OM molecular composition (**Figure 3.4 B**, Figure 1 and 2, Figure S 7, S 8, S 9, SI in *Manuscript II*). This adsorption of DOM to the different substrates was generally characterized by a small fraction of "unsaturated O-poor" molecules in SW compared to corresponding largest fractions of "unsaturated O-poor" molecules in all substrate-bound OM samples (**Figure 3.4 B**, Figure 1 A in *Manuscript II*). Substrate-bound OM showed generally larger masses than the original SW (Figure S 10, Table S 2, SI in *Manuscript II*).

The greatest difference in the OM adsorption pattern between DC and UV samples could be identified for PET and PS (**Figure 3.4** and **3.5**, Figure 1 and 2, S 7, S 8, S 9, SI in *Manuscript II*). For MFs co-occurring on PET_DC, PET_UV and SW (facilitated by bioinformatics filtering to identical MFs), more than half of the MFs were enriched on PET_DC generally with more saturated MFs. This separation by the degree of saturation was especially detectable for signals unique to PET_DC and PET_UV (**Figure 3.5 A**). While the difference between PET_DC and PET_UV was mainly driven by the degree of saturation, PS_UV showed a clear trend towards higher oxygenated substances compared to PS_DC (**Figure 3.5 B**).

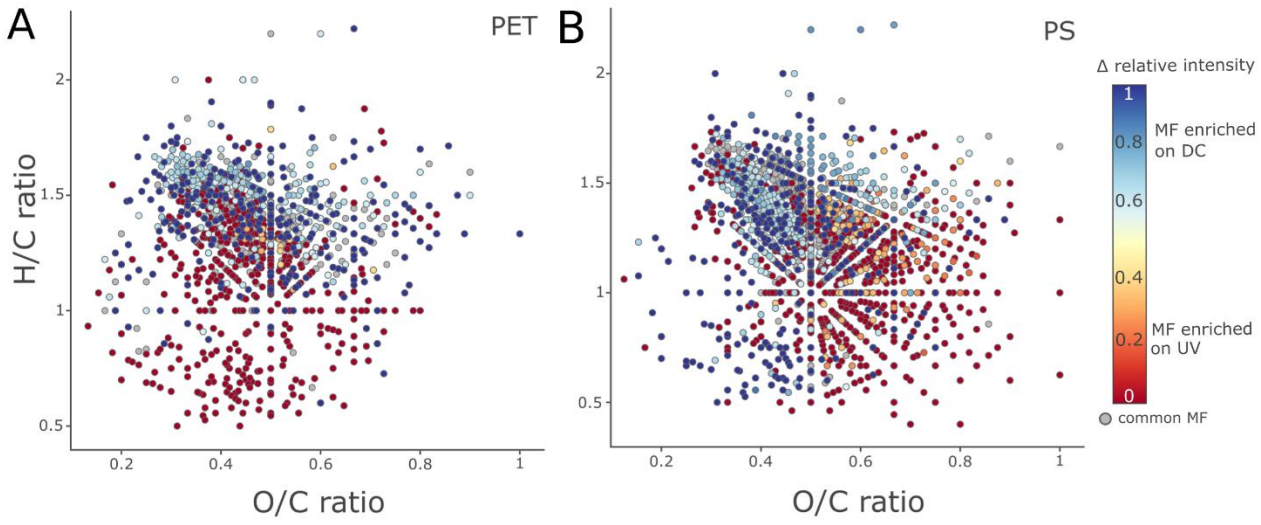


Figure 3.5: Van Krevelen diagrams (H/C over O/C ratios) representing the differences in the relative signal intensities (Δ relative intensity) of adsorbed OM in comparison with dark control (DC) and weathered (UV) substrates PET (**A**) and PS (**B**). Each circle represents a molecular formula (MF), and MFs for each substrate were reduced to those that co-occurred in the incubation stream water. Solid blue and red MFs represent those that were unique to the DC or UV substrate, respectively.

The fractionation of SW-DOM to substrate-associated OM is most likely the result of selective adsorption driven by the physico-chemical properties of the surfaces of the different substrates (**Figure 3.4 A**, Table1, Surface Properties SI 7 in *Manuscript II*,). The most pronounced changes in the material properties such as the contact angle (Table 1 in *Manuscript II*,), the hereof-calculated SFE and carbonyl index (indicative of oxidative reactions) for PET were in good agreement with the identified differences in the relative intensities of adsorbed OM (**Figure 3.4** and **3.5**). The generation of carboxylic acid end groups and moieties during weathering (Horne et al. 2020) may facilitate hydrogen bond formation for interactions with DOM. Similar to our results, Aflori and Drobota (2015) attributed the altered sorption and adhesion behavior of PET towards collagen to surface modifications as a result of UV weathering. Taking into account that weathering induced a decrease in hydrophobicity and changes in the SFE of PET and PS, we can deduce that these changes in material surface properties might have caused substantial differences in the sorption behavior between PET_DC and PET_UV and PS_DC and PS_UV. Furthermore, the OM fingerprint of PS differed substantially from that of PET and glass which may also be attributed to its highly hydrophobic characteristics even for the weathered surface of PS_UV (**Figure 3.4. A**, Table 1 in *Manuscript II*).

Consequences of the adsorption of OM for the material properties were an increase in the contact angles of PET (DC and UV) and PS_UV (increase between 2-6°) while the increase for glass_DC was within the uncertainty range of the standard deviation (**Figure 3.4 A**, Table 1 in *Manuscript II*). Still, distinct material contact angles remained even after the OM layer has formed indicating that no equalizing masking effect that converged the surface properties could be observed. Moreover, surface properties (*e.g.*, contact angle) post DOM incubation were shifted by a similar magnitude to more hydrophobic properties for most of the investigated substrates (**Figure 3.4 A**, Table 1 in *Manuscript II*).

Summarizing, I identified that different polymer surfaces adsorb OM selectively even under the influence of weathering-induced changes of the surface properties. Specific surface properties exhibited by the investigated polymers could have resulted in surface-OM interactions, such as hydrophobic, electrostatic interaction or cation bridging, that were specific for certain fractions of the OM (*i.e.*, here compound classes **Figure 3.5**). These selectively adsorbed OM fractions may display also compound class-specific features, such as charge or hydrophobicity, that caused distinct material properties even after a layer of OM has adsorbed. Hence, the conditioning film seemed to reflect the underlying surface characteristics to a certain extent by passing the material surface properties on to

the outer OM-water interface. The relevance of those findings for the attachment of microbial communities will be investigated in the next section.

3.2 Biofilms

After the conditioning film has formed within seconds to hours, a biofilm community starts to build up on submerged surfaces (**Figure 3.6**). These biofilms serve as an excellent "glue" to mediate the attached life strategy and to provide certain functions to the consortia such as mechanical stability, *i.e.,* giving resistance against currents and shear forces, protection against desiccation, migration of enzymes, the provision of gradients (pH, oxygen content), protection from predation and toxic substances, reserve as a carbon source and cross-feeding between organisms (Romani, Guasch, and Balaguer 2016; Costa, Raaijmakers, and Kuramae 2018). While biofilms may be an aesthetical issue in households (*e.g.,* washing machine, sinks etc.) and be the cause of serious infectious problems in medical applications and devices (implants, tubes, storage vessels) (Habash and Reid 1999; Prabhawathi, Thirunavukarasu, and Doble 2014) they provide key functions in global biochemical cycles (de Carvalho 2018). Most prominent examples of the MOs' key function for global nutrient cycling are their relevance for the carbon, nitrogen and phosphorus turnover (Hutchins and Fu 2017) which manifests in the Redfield ratio (Redfield 1934). Alfred Redfield (1934) recognized the conserved elemental composition of plankton that is similar to the major dissolved nutrients in the ocean (Arrigo 2005).

3.2.1 The Developmental Stages of a Biofilm on New Habitable Surfaces

But how do biofilms develop on submerged surfaces? Figure 3.6 provides an overview of the developmental stages of a biofilm that generally applies to most new habitable surfaces. The scheme is commonly known as the "mushroom" model. Noteworthy, the model by Cogan et al. (2016) was extended by organic molecules adsorbing to the surface prior to microbial attachment (**Figure 3.6 a**) which was investigated in chapter 3.1 (*Manuscript II*). After the conditioning film has formed, MOs come into contact with the surface either actively or by attractive forces and colonize (**Figure 3.6 b, c**). Some MOs will not be able to attach or be repelled (due to repulsive electrostatic forces) (**Figure 3.6 b**) and remain in the pelagic community. The secreted EPS enable adhesion and promote further attachment by other MOs (**Figure 3.6 d, e**). Mainly bacteria are the first colonizers (within hours to days) followed by eukaryotes such as diatoms (**Figure 3.6 f**). A remarkable feature of MOs is their

ability for quorum sensing. This quorum sensing is a form of cell-cell communication enabling synchronized gene expression in response to their population density (Solano, Echeverz, and Lasa 2014). As the biofilm matures it is characterized by a wide range of 3-D structures forming thick, thin, fluffy biofilm matts, films or sludges where parts of it will eventually be pulled off by shear stress (**Figure 3.6 g**). These fragments will then be able to colonize other remote habitats or be available in the water column (partially contributing to so-called eDNA) (**Figure 3.6 h**). Finally, organisms may undergo different transitional or developmental stages, will be released and contribute to the pelagic community again (*e.g.*, spores) (**Figure 3.6 i**).

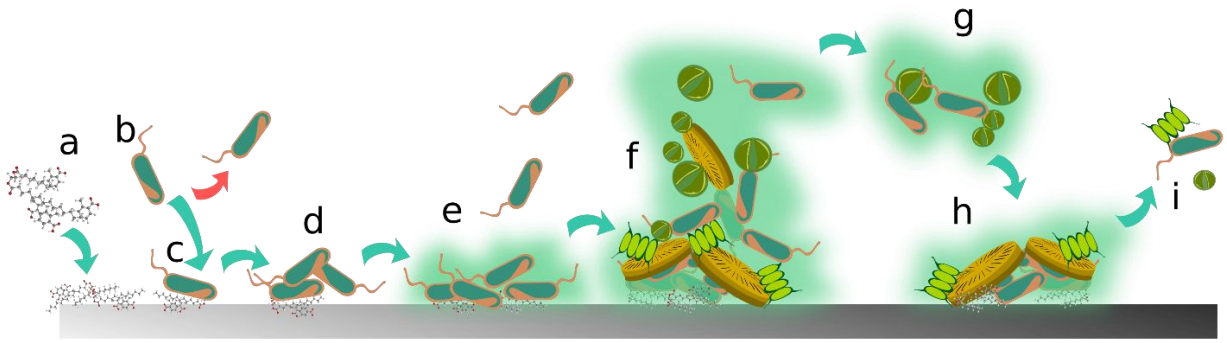


Figure 3.6: Generalized scheme of biofilm attachment, succession and detachment on a new habitable surface ("mushroom" model). **a:** Adsorption of a conditioning film; **b:** Planktonic phenotype; **c:** Newly attached cells and cell that was "repelled"; **d:** Maturing biofilm / biofilm succession embedded in EPS; **e:** Mature biofilm / climax community; **f:** Mature biofilm structures often known as the "mushroom" model; **g:** Detached biofilm aggregates; **h:** Reattached biofilm aggregates; **i:** Newly dispersed cells giving rise to planktonic stages (modified version from Cogan et al. (2016)).

3.2.2 Relevance of Biofilm Formation for Weathering of Plastic and its Fate and Effects in the Environment

Why are biofilms on plastic debris relevant for weathering processes of plastic? Since biofilms represent a biological coating of plastic debris in the aquatic environment their influence on physico-chemical processes must not be disregarded. These epiplastic communities have the capacity to impact a variety of different pathways relevant for the fate and effects of plastic debris (*Publication IV*) (**Figure 3.7**).

With the formation of a biofilm on plastic surfaces, the attached fouling organisms may cause an increase in density of the particle and decrease in buoyancy (Lagarde et al. 2016). The smaller the particle is, the faster it can reach its critical sinking density (Chubarenko et al. 2016; Fazey and Ryan

2016). Concurrently, microplastic becomes sticky due to the EPS matrix, which promotes the formation of hetero-aggregates, including microplastic, microbial communities and detritus (Long et al. 2015) leading to downward transport and sedimentation but also consumption and limited light availability may cause re-surfacing to the water surface (**Figure 3.7**).

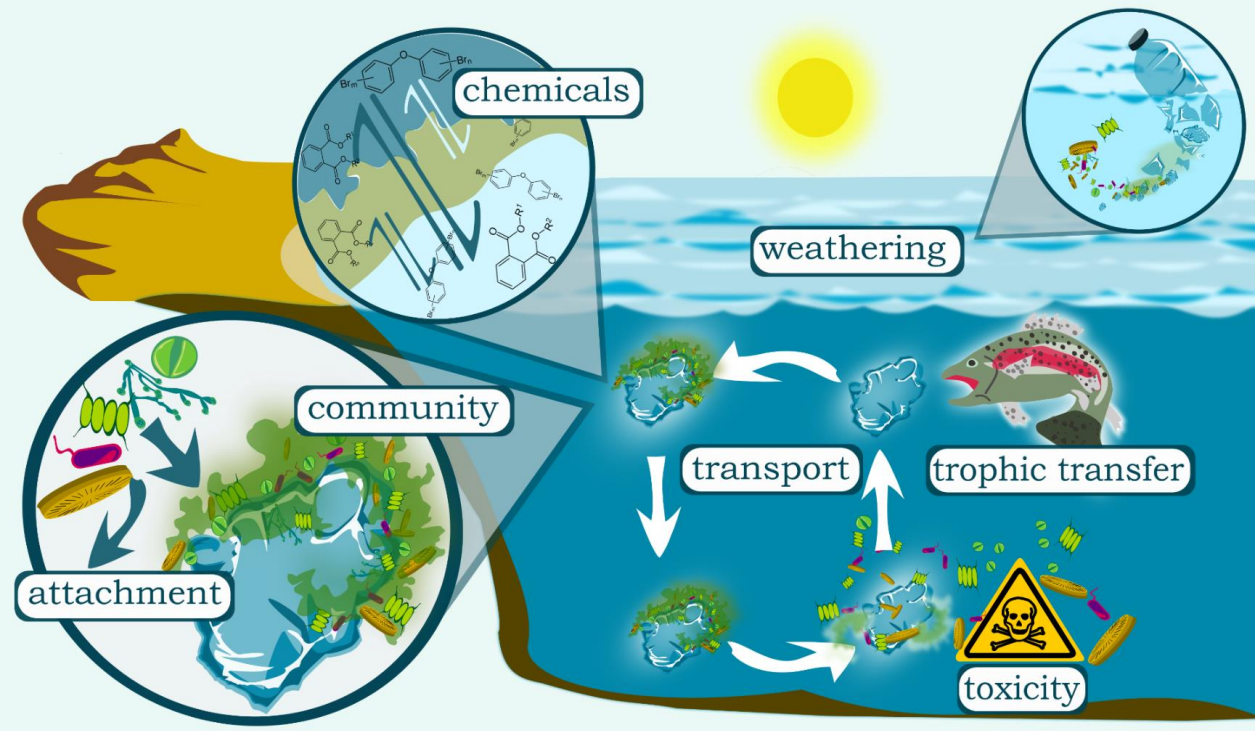


Figure 3.7 Key processes of the fate and potential effects of MP in the aquatic environment that are modified by biofilm formation. Biofilms on submerged surfaces are the result of selective **attachment** of MOs, facilitation and interspecific competition in the microbial **communities**. **Weathering** processes may favor biofilm growth due to increased surfaces available for settling which in turn may shield plastic debris from UV light. However, biofilms have the capacity to biodegrade the polymer. Further, vertical **transport** and the uptake and release of plastic-associated **chemicals** are influenced by biofilm formation on plastic debris. Biological implications of biofilm formation include effects on **trophic transfer** of MP and associated contaminants, community structure of microbial assemblages and potential **toxicity** to grazers.

The transport of hydrophobic organic contaminants (HOCs) between plastic debris and water may be affected by biofilms due to its sorptive properties on the one hand and its ability to metabolize the HOCs on the other (Wolfaardt et al. 1994; Headley et al. 1998; Writer, Ryan, and Barber 2011; Ding et al. 2015). Since the capacity of synthetic polymers to sorb HOCs is of high relevance for the environmental risk assessment of microplastic, the question arises whether thermodynamic and kinetic processes will be influenced by a superficial organic phase consisting of water, lipids and proteins

acting as both a potential sorptive phase (Flemming 1995) and a barrier for diffusive uptake and release of chemicals (Seidensticker et al. 2017). Furthermore, EPS is a diverse biological matrix containing polysaccharides, proteins, lipids and other biopolymers such as humic acids (Flemming and Wingender 2010; Stewart et al. 2013), which may contribute to the sorptive capacity of the biofilm-coated microplastic (Wang et al. 2016) and hetero-aggregates (**Figure 3.7**).

Biological effects of the biofilm formation on plastic debris may comprise the preferential ingestion by primary consumers due to the higher nutritional quality (Carson 2013), or altered uptake and susceptibility of organisms to ingest plastic (particles) caused by heteroaggregation (Campos et al. 2013). The available reports on non-biofilm algae suggest effects of nanoplastic exposure on planktonic microalgae, such as inhibition of photosynthesis, promotion of reactive oxygen species (Nolte et al. 2017; Bhattacharya et al. 2010), growth inhibition (Mao et al. 2018) and reduced chlorophyll-a content (Besseling et al. 2014).

As demonstrated in *Manuscript II*, surface properties of polymers, potentially including those of microplastic, will inevitably change upon conditioning film or eco-corona formation (Ramsperger et al. 2020) Furthermore, weathered surfaces may display a modified topography, increase in surface roughness and changed chemistry (Andrady 2015a; Fotopoulou and Karapanagioti 2015; Cooper and Corcoran 2010; Feldman 2002) (Table 1 in *Manuscript II*). These processes may increase adhesion of MOs (Donlan 2002), carrying capacity of microplastic towards biofilm mass and, ultimately, composition and structure of the microbial communities (Kerr and Cowling 2003; Cazzaniga et al. 2015). Furthermore, successive fragmentation into smaller particles with high surface-to-volume ratio is an important prerequisite for biodegradation.

Although in the environment weathering processes act mostly in parallel, the abiotic (photo-) degradation is often a prerequisite for MO-facilitated biodegradation. Before MOs are able to use polymers as a carbon and/or energy source and finally mineralize synthetic polymers to CO_2 or CH_4 and H_2O, long polymer chains need to be broken down into smaller oligo- or monomers before they can pass the cell membrane. In this context, biodegradable polymers or enhanced degradable polymers are of relevance (Zeng et al. 2016). A variety of bacterial strains were identified to be capable of degrading conventional synthetic polymers (Ghosh, Pal, and Ray 2013), however, in the so-called fouling community, fungi and their ability to mechanically disintegrate plastic by penetrating the polymer with fungal hyphae and their capacity to release degrading enzymes (Sanchez 2020) are currently understudied. Biofilms may not only promote biodegradation of plastic in the environment

but they can also interfere with the abiotic photolytic degradation since they carry light-absorbing pigments reducing the light transmittance of up to 99 % (Weinstein, Crocker, and Gray 2016).

Biofilms have the capacity to actively deteriorate the material surface they inhabit (Amobonye et al. 2021). A prerequisite is, that biofilm-forming organisms first have to adhere and establish a biofilm community to facilitate potential subsequent biodegradation. Microbiologists are currently investigating communities present on plastic surfaces and the underlying factors determining community structure and succession patterns. Zettler, Mincer, and Amaral-Zettler (2013) introduced the term "plastisphere" implying that plastic-associated communities are distinct from the surrounding surface water. Knowledge about the community structure and the underlying forces driving these assemblages at each succession stage will help us to elucidate the impact of plastic pollution on aquatic microbial load and diversity (Oberbeckmann, Loder, and Labrenz 2015). Uncoupling the processes of biotic weathering such as early MO attachment and influencing abiotic weathering processes was a crucial step for my following studies.

As outlined in chapter 3.1, abiotic weathering may affect the material surface properties which may alter the range and diversity of MOs that are able to adhere. Therefore, it is important to highlight that the aim of the following study was not to characterize biodegradation as such but to elucidate the effects of different polymer substrate types that where artificially pre-weathered by photo-oxidization to intentionally modify their surface properties (see section 3.1) and to investigate the influence of these changing material properties on subsequent biofilm formation.

3.2.3. Biofilm Formation on Weathered Plastic Surfaces

In the previous chapter 3.1, we saw that the conditioning film differed between the investigated substrates. As outlined in chapter 3.1.1, this may have consequences for the physico-chemical behavior and biological interaction of polymer surfaces. Within *Publication IV*, I prioritized the research needs to deeper investigate factors that may drive microbial community structure. Many researchers have hypothesized a certain plastic core-community, the 'plastisphere' (Zettler, Mincer, and Amaral-Zettler 2013) , however underlying mechanisms shaping such plastic-specific or even material-specific communities are unknown. One explanation for this detection of material-specific microbial communities may be the relevance of material surface properties. If a certain material displays unique surface properties (surface charge, surface roughness or topography), does this select for organisms capable to adhere?

To address this question, I explored the bacterial and eukaryotic community formation on glass, PET and PS substrates (each as UV and DC treatments) by applying a set of measurement techniques capable to detect changes of ecologically relevant endpoints. In a similar setup as described in chapter 3.1, I investigated early biofilm formation in a short term three-day (sampling at day 1 and 3) and in a long term 32-day (sampling at day 1, 3, 7, 12, 20, 32) incubation experiment (provided in *Manuscript II* and *III*, respectively). The substrate slides were incubated in SW, retrieved on the specified sampling days and passed on to the respective measurements. Biofilms, that include primary producers, are of extraordinary relevance as a nutrient source at the base of the food web. Confocal laser scanning microscopy (CLSM) was used to elucidate different biomass proportions of bacteria, algae and EPS fractions with concurrent three dimensional structure information via staining and image analysis techniques (carrying the unit biovolume [μm^3] derived from z-stacked pixels, so-called voxels) (Neu and Lawrence 2014). The autotrophic community facilitates CO_2 fixation and oxygen provision. Pulse Amplitude Modulation (PAM) fluorometry (Brooks and Niyogi 2011) was applied to measure a key function of aquatic biofilms, the photosynthetic capacity. I hypothesized that the aforementioned differences in the surface properties and conditioning film on plastic surfaces may be reflected in different biofilm biomass and photosynthetic capacity which was tested by generalized additive mixed models (GAMMs) and the Akaike information criterion (AIC) for model selection. If no changes in the apical endpoints and structural composition would occur, it may still be conceivable that the taxonomical community composition could indicate any differences between the substrates of investigation. Therefore, I applied 16S rRNA gene amplicon sequencing techniques (Illumina sequencing) and ordination analyses (principle coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS)) to gain deeper insight in the biofilm community (*Manuscript II* and *III*). While the aforementioned apical endpoints were investigated solely in the long-term study, the latter-named next generation sequencing techniques were applied in both the short- and long-term experiments,

3.2.4 Structural and Functional Capacity of Biofilms on Weathered Plastic

Biovolumes of bacteria, algae and EPS matrix development over 32 days of incubation did not reveal any clear differences between the substrates glass, PET and PS (**Figure 3.8**, Figure 5 and 6 in *Manuscript III*. Still, the applied generalized additive model resulted in the smallest AIC and therefore in best model fit when including "substrate" as a factor (Figure 5 in *Manuscript III*,). Increasing bacterial growth

could be detected until day 12 which then leveled off to a lower growth rate until day 32. Results by Cheng et al. (2020) indicated a similar trend of high growth rates for bacteria attaching on different plastics until day 30, which then leveled off to a mature climax community. For glass, all investigated biofilm structures (bacteria, algae and EPS) showed similar growth pattern irrespective of the weathering treatment (**Figure 3.8 A**, Figure 6 in *Manuscript III*,). When refining the analyses to detect differences between biofilms grown on UV-weathered and DC substrates, PET_DC displayed a one order of magnitude higher bacterial biomass than PET_UV at early biofilm succession (day 1-3)(**Figure 3.8 B**, Figure 6 in *Manuscript III*). Noteworthy, EPS volumes detected on PET_DC at early biofilm development until day three exceeded those of PET_UV. The above-mentioned higher bacterial biomass on PET_DC compared to PET_UV on day 1 and 3 may be the result of such higher EPS biovolumes that could be detected concurrently for PET_DC at early time points (**Figure 3.8 D**). This might have enabled increased cell adhesion and attachment with potential prolonged implications on higher bacterial abundances during succession (day 12-32) (**Figure 3.8 B**). Furthermore, higher hydrophobicity of PET_DC could have provoked increased attachment efficiency for bacteria compared to the more hydrophilic PET_UV (Table 1, Material Properties SI 3 in *Manuscript II*). Comparable to this result, PS_DC showed similar higher bacterial biomass than glass and its UV-weathered counterpart PS_UV on the first sampling days (day 1-3) (**Figure 3.8 C**, Figure 6 in *Manuscript III*). This difference changed and leveled off during the course of the 32-day incubation. A study undertaken on PE, polylactic acid (PLA) and glass, detected statistically significant lowest bacterial abundance for the glass micro particles. Contrasting to my results, Vosshage, Neu, and Gabel (2018) described a different biofilm composition on polymer substrates (polymethyl methacrylate (PMMA)) compared to glass and polycarbonate (PC) substrates, which had downstream effects on consumption and growth rate by a freshwater gastropod.

At early biofilm development on day 7, highest fluorescence signals measured via PAM fluorometry could be detected for PET, while glass showed lowest fluorescent yield (Figure 5 in *Manuscript III*). However, after 32 days of incubation the mean fluorescent yield and quantum yield of the substrates glass, PET and PS was highly similar (Figure 5 in *Manuscript III*).

Summarizing, structural and functional endpoints were characterized by high variability at the early time points until day 7-12 which then leveled off until a biofilm climax community was reached after 32 days of incubation (*Manuscript III*).

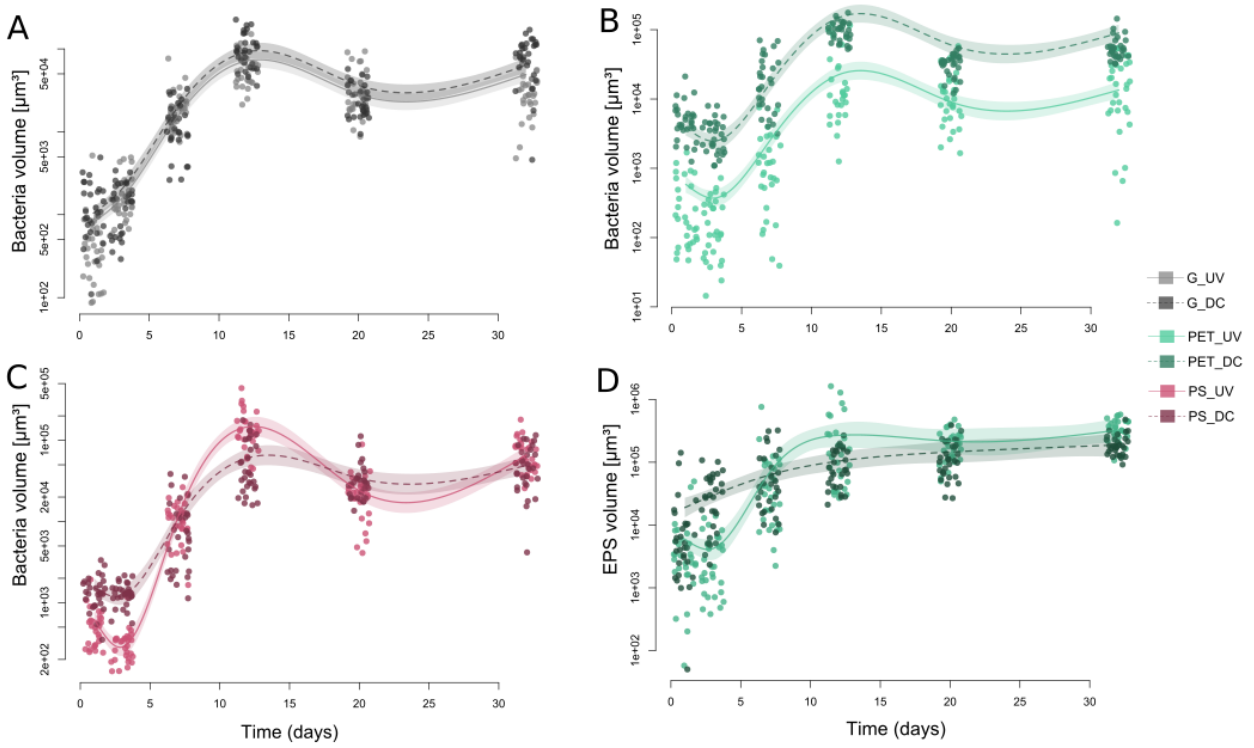


Figure 3.8: Generalized additive mixed models (GAMMs) on the biovolumes [µm³] of bacteria (**A-C**) and extracellular polymeric substances (EPS) (**D**) over 32 days of incubation time including weathering (DC and UV treatment) as a factor. **A:** comparison between bacteria biovolumes on glass_DC and glass_UV with no differences in growth. **B:** PET_DC showed higher abundance of prokaryotes than PET_UV. **C:** bacteria biovolumes of PS_DC and PS_UV with differences at early time points. **D:** EPS volumes measured for the substrates PET_DC and PET_UV. Solid and dashed lines are the predicted means with 95 % confidence bands of the mean as shaded areas and *y*-axis is log-scaled.

3.2.5 The Microbial Communities on Weathered Plastic

The amplicon data of the prokaryotic communities and the afore-mentioned apical endpoints showed considerable similarities indicated by ordination analyses based on dissimilarity measures (**Figure 3.9 A,B**). In two independent studies (the short- and long-term study), early microbial communities until day 3 scattered in their community composition indicated by the large distances between the different substrate communities (**Figure 3.9 A, B**, Figure 5 A in *Manuscript II,* Figure 7 in *Manuscript III*). The pioneer colonizing communities on day one of the short-term study tended to group in a material-specific manner indicated by the close spatial proximity for DC and UV of each substrate type (**Figure 3.9 A,** Figure 5 in *Manuscript II*). The material-specific clustering may be driven by the material's unique surface characteristics which still, as discussed in chapter 3.1, remained after a layer of OM has been adsorbed. This observation highlights a common pattern between material surface properties, OM

signature and the early microbial colonization on different substrates.

The material-specific communities on day one (although not significant but detectable for the most dominant pioneer colonizers (Figure 4 in *Manuscript II*)) may have been controlled indirectly by the surface's physico-chemistry that passes on its features to the outer OM-water interface forming a distinct conditioning film of adsorbed OM (Table 1 in *Manuscript II*). The presented data supports this hypothesis since the material properties were still not similar after OM adsorption (no equalizing masking effect) and OM signatures appeared to be material-dependent. The herein proposed process of early biofilm formation on polymer surfaces is supported by studies with similar results but on non-polymeric materials (Schneider and Marshall 1994; Bos, van der Mei, and Busscher 1999; Gubner and Beech 2000).

No clear material-specific grouping at early time points could be observed for the long-term study (**Figure 3.9 B**, Figure 7 in *Manuscript III*). In this long-term study, I applied a similar batch of UV- and DC- treated polymer substrates, however, analyzing the quality of the OM of the conditioning film was not feasible and therefore conclusions could not be drawn. Interestingly, for the short- as well as for the long-term study, PET communities (DC and UV) were located most distant from glass and PS samples (**Figure 3.9 A, B**). Generally, the communities converged to highly similar community structures indicated by overlaying samples in the ordination analysis (**Figure 3.9 B**) which compared well to the measured apical endpoints such as biomass and photosynthetic capacity (**Figure 3.8 A-C**).

Incubation time was the main driver of biofilm diversity in both studies which is in accordance to other studies (Harrison et al. 2014; Li et al. 2017; Pinto et al. 2019). Harrison et al. (2014), Li et al. (2017) and Pinto et al. (2019) reported highly comparable results of community successions with strong convergence within a few days, weeks or months. This highlights the importance to understand successional processes of biofilms especially with respect to the evaluation of incubation studies with a single sampling point as it is often the case in sequencing studies (Wright, Langille, and Walker 2020). Those may be prone to overestimate the substrate effect for mature biofilms, since a single sampling event represents only a snap shot and may eventually capture communities that have not reached a climax community (Kirstein et al. 2018; Ogonowski et al. 2018).

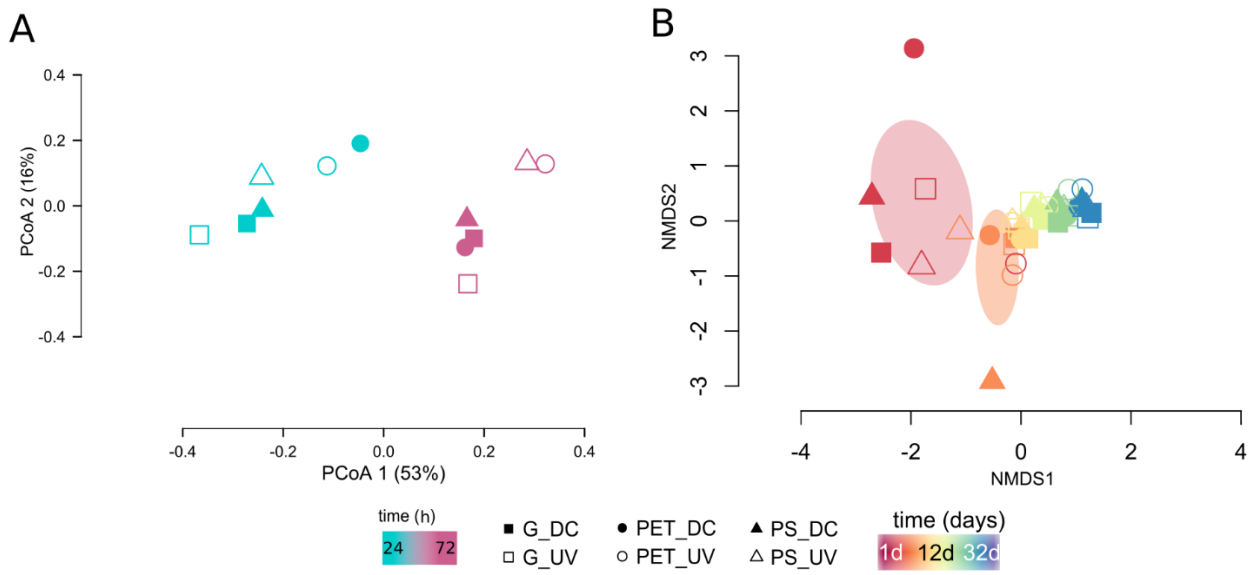


Figure 3.9: Principal Coordinate Analysis (PCoA) (**A**) and non-metric multidimensional scaling (NMDS) plot (**B**) of prokaryotic communities based on 16S rRNA gene amplicon sequencing. **A**: Short-term experiment that was conducted in parallel to the study investigating the adsorption of OM described in chapter 3.1; **B:** Long-term study over 32 days of incubation. Symbols represent the different substrates each as dark controls (solid symbols) and UV pre-weathered samples (empty symbols). Color code represents the sampling time.

3.3 Conclusions on the Conditioning Film and Early Biofilm Formation on Weathered Plastic Surfaces

In *Manuscript II*, I incubated various substrates of glass, PET and PS in natural stream water. To investigate the conditioning film, I rinsed the surfaces, enriched the rinsing water and measured the conditioning OM substances via FT-ICR MS. By this approach, I could demonstrate the selective sorption capacity of different polymer types and weathering state towards OM. As a consequence of this selective fraction from incubation DOM, the underlying surface properties were not completely masked and equalized across different substrates. Moreover, the adsorbed OM layer preserved the materials' surface properties to some extent to the outer OM-water interface which in turn seemed to govern early microbial colonization. The early colonization phase was highly divergent indicating, that different surface characteristics, which remained distinct after the OM layer has formed, could have caused this diversity. As a potential result of the taxonomical differences, I observed structural and functional differences within the same early colonization phase. Those differences became less pronounced with ongoing biofilm succession and the prokaryotic communities as well as structural and functional endpoint measures converged indicated by similar biomasses and photosynthetic

capacities. This convergence led to highly similar communities on different substrates after 32 days of incubation.

The above studies demonstrate that freshwater communities of different substrates may not necessarily differ between polymer types as scientific studies often suggest. As a consequence, the ecological relevance of epiplastic biofilms and their potential to modulate ecosystem-relevant processes may highly depend on the system of investigation (such as potentially trophic state, geography, environmental factors).

4. Implications

In my thesis, I presented data on the two main aspects of abiotic and biotic polymer weathering processes and how they alter the polymer's properties, fate and effects. While polymers are generally regarded as inert, we identified in chapter 2 that substances liberated from additive-free pre-production polymers during leaching have the capacity to induce certain cellular toxicity responses. Most importantly, the observed effects were elevated when the polymer leaching was promoted by artificial UV-light weathering conditions that corresponded roughly to over one year of middle European outdoor exposure. These results are of utmost relevance for a critical evaluation of the hazard potential of environmental plastic since recent test strategies focus mainly on particle toxicity.

In chapter 3, I provided relevant insights in the material behavior under natural conditions. When exposed to (natural) water and biological activity starts on polymer surfaces, the material properties will inevitably change with material-specific implications. The first layer of OM displayed unique features for different surfaces and signatures of OM were presumably the result of selective adsorption processes. This conditioning affected the material properties, such as surface hydrophobicity, to a certain extent. Still, surfaces remained distinct for the investigated materials even after a layer of OM has formed. Community composition changed over time with heterogenous taxonomic and functional capacities during the first few days of succession that converged with time to a homogenous (in structure and function) biofilm community across different substrates.

4.1 Ecotoxicological Relevance of Weathering Plastic

The data and results of chapter 2 have to be seen in a wider context of microplastic research. The general focus of this emerging research field comprises mainly the detection of macro, micro- and nanoplastics in the environment to be able to assess the exposure of ecosystems and biota to this stressor that we have been emitting for the last 60 years. In parallel, scientists test the hypotheses that this anthropogenic debris causes harm to the exposed organisms in the aquatic environment. For plastics, human and environmental health concerns were expressed especially due to the application of hazardous substances during production and as additives contained in plastic articles. This is still the general public and scientific opinion with respect to the evaluation of a polymer's hazard potential. I could demonstrate that chemicals liberated from UV-weathered microplastics induced oxidative stress (*Publication III*) across all tested polymer types. Furthermore, the induction of the receptor-

mediated assay PPARγ indicated alterations in metabolic gene pathways by plastic leachates. Subtypes of the PPAR members, including PPARy, are known to regulate genes involved in glucose and lipid metabolism (MacNaul and Moller 2003) which raises concern about potential adverse downstream effects of its activation by plastic degradation products. Therefore, my results stand in contrast to the prevailing opinion of the inertness of raw polymer materials (*i.e.,* the pure carbon chains) and provide evidence that transformation and degradation of this material under "short" lifetimes is of ecotoxicological relevance (here 1 ½ year outdoor exposure equivalence).

Another focus of the studies in chapter 2 was to assess the impacts and ecotoxicological relevance of microplastics beyond the perspective of the mere polymer particle toxicity. Studies describing particle toxicity towards test organisms are important but may eventually not cover all potential toxicity pathways elicited by plastic (Pirsaheb, Hossini, and Makhdoumi 2020). Nor does our approach of testing solely the mixture toxicity of substances liberated from degrading polymers. For a holistic assessment, both aspects of particle toxicity and leaching chemicals need to be covered. Many microalgae studies were unable to find half-maximum effect concentration values for microplastic particles due to the high concentrations needed to induce significant toxicity and such values may vary depending on the characteristics of specific microplastics (Prata, da Costa, Lopes, et al. 2019). Although (Prata, da Costa, Lopes, et al. 2019) comprehensively reviewed the effects and impacts of microplastic towards microalgae, the authors entirely disregarded the hazard posed by plastic leachates to cause algae toxicity. The detected baseline toxicity towards microalgae by plastic leachates should prompt researchers to deeper explore the hazard potential of plastics under different exposure routes than the so-far classical particle-exposure scenarios. Although baseline toxicity is the minimum toxicity any chemical can exhibit, it does have environmental relevance because baseline toxicity is concentration-additive and all chemicals act together in baseline toxicity. To my knowledge, Zimmermann et al. (2020) was the first study that addressed several exposure scenarios in a comprehensive study by investigating separately i) plastic particles with additives, ii) the particles without extractable chemicals, iii) the extracts of the plastic itself and iv) water soluble plastic migrates in an ecologically relevant test setup. The authors demonstrated that extractable chemicals in the investigated plastic type polyvinylchloride (PVC) were the main driver of toxicity towards *D. magna*, however this was not the case for other polymer types such as polyurethane (PU) and polylactic acid (PLA) (Zimmermann et al. 2020).

The relevance of transformation products of chemicals liberated during our applied weathering treatment could not be addressed adequately in our studies (*Publication III*, *Manuscript I*). Long-term

studies with different exposure periods would help to understand the formation of potential transformation products. Conclusions could be drawn from the e-waste sample for which it was known to contain additives. Already dark control samples induced cellular stress responses and effects of the e-waste leachates were elevated due to the UV-treatment. Presumably, for e-waste not only degradation products but also transformation products of leaching additives could have caused the observed effects. This assumption should be investigated in future studies.

Another important finding of my thesis, with respect to the ecotoxicological relevance of biotic plastic weathering, is the adsorption of OM as outlined in chapter 3.1. So far, several studies demonstrated the altering effect of OM adsorption in microplastic studies (Mei et al. 2020). In many cases, the negative effects towards test organisms were alleviated in the presence of OM potentially due to changed surface characteristics upon adsorption (Schur et al. 2021; Cedervall et al. 2007; Fadare et al. 2020). Contrarily, increased negative effects were also reported which was attributed to increased uptake through elevated aggregation processes facilitated by a corona-formation (Nasser and Lynch 2016) or altered surface characteristics that favored, *e.g.,* cellular uptake (Ramsperger et al. 2020). In those studies, various types of OM, such as HAs and FAs as well as proteins, were applied. The contrasting results derived from those studies highlight the importance to further investigate the quality of adsorbed OM to increase our mechanistic understanding of specific organism-particle interactions. The applicability of my detected material-dependent OM adsorption still needs to be demonstrated for particles in the micro- and nano-range in future studies. Still, the impact of weathering processes to modify the physico-chemical interactions of plastic surfaces highlights the necessity to better understand weathering-induced changes and concurrent plastic-OM interactions. A call for research on such modulating effects by OM adsorption in toxicity testing policies was just raised recently (Nasser, Constantinou, and Lynch 2020).

In *Publication III* and *Manuscript I* (chapter 2), I provided evidence for potential ecotoxicological implications of non-compounded plastic materials under relevant environmental radiolytic impacts (*i.e.,* UV A+B irradiance). With the here presented studies, I could substantially improve our understanding that:

- Chemicals leached from synthetic polymers during accelerated weathering
- Chemicals liberated during artificial weathering from mainly additive-free polymers were capable to activate certain cellular (stress) responses and to cause toxicity in microalgae

4.2 Ecological Relevance of Biofilms

I have investigated the conditioning films and biofilm development on different plastic surfaces as the first biological signature that environmental plastic debris may display. From an ecological perspective it is important to know whether epiplastic biofilm communities select for specific communities and which underlying mechanisms may drive this phenomenon. Identical biological entities, communities and functions would suggest evaluating epiplastic biofilms collectively without further differentiation in their hazard or effect potential according to, *e.g.*, polymer type. In contrast, distinct and material-specific differentiation of epiplastic communities would rather prompt us to consider each polymer type separately.

My presented studies reveal that the very first interaction between plastic surfaces and organic molecules from surrounding natural water followed material-specific adsorption patterns (*Manuscript II*). These differences likely depended on varying surface characteristics as measures of different physico-chemical surface properties have indicated (*Manuscript II,*). Surfaces with either mineral or organic coatings can have profound effects on the interaction between bacteria and the substrate (Scholl et al. 1990; Fletcher 1996). Interestingly, I detected the following attachment and early succession of microbial communities to be a highly divergent process within a short time frame of days. They were presumably the result of stochastic attachment processes and to a certain extent driven by specific organism-surface interactions. The detected convergence of the prokaryotic community within 32 days until highly homogenous assemblies may indicate i) decreasing relevance of surface properties for secondary colonizers and ii) the predominance of highly abundant generalists at later biofilm stages. These insights in taxonomic structures were complemented by functional parameters of the maturing biofilms (*Manuscript III*). Most importantly, results from CLSM and photosynthesis measurements supported our previous observation of community convergence on taxonomic levels by similar photosynthetic activity and biomasses for mature biofilms (*i.e.,* here after 4 weeks of incubation) on glass, PET and PS substrates (*Manuscript III*). The divergent taxonomic patterns during early colonization phase compared well with the small but distinguishable differences in functional capacities for the first few days of succession. However, these differences disappeared over time as well indicating that mature biofilms on polymeric substrates did not display any altered capacity to contribute to ecosystem services as other natural (here glass) substrates would do. In fact, the availability of additional anthropogenic surfaces (*i.e.,* aquatic plastic debris) then needs to be related to

naturally occurring surfaces (*e.g.,* mineral particles) to be able to adequately assess downstream ecological relevance of biofilm formation on plastics.

Contrarily to my findings, researchers continue to detect plastic-specific prokaryotic (core-) communities in natural waters (Kirstein et al. 2018; Kirstein et al. 2019). My data indicated that such observations may certainly depend on the respective time frame researchers look at. This dynamic fits to the hypothesis by Amaral-Zettler, Zettler, and Mincer (2020) that there might be some polymer-specific communities in early attachment and succession, which will eventually converge over time. Furthermore, a potential explanation for these contrasting results may be given by Oberbeckmann, Kreikemeyer, and Labrenz (2017). The authors observed material-specific prokaryotic communities only under certain nutrient-limited conditions. They hypothesized that potentially the more nutrients were available, the quicker a conditioning film and primary biofilm could develop, the faster a secondary, less substrate-specific, biofilm could be established (Oberbeckmann, Kreikemeyer, and Labrenz 2017). There is increasing evidence that environmental factors and not the plastic substrate type has major impact on the microbial community composition (Wright, Langille, and Walker 2020; Oberbeckmann, Kreikemeyer, and Labrenz 2017).

The above discussion fits well into the more holistic perspective that effects and fate of plastic marine debris may vary considerably in different parts of the global ocean (Amaral-Zettler, Zettler, and Mincer 2020). Supposed that plastic communities vary temporally and geographically then there will be variation in the transport potential of invasive, harmful or pathogenic species, their interaction with plastic-associated organic pollutants, additives and metals (Masó et al. 2003; Zettler, Mincer, and Amaral-Zettler 2013; Amaral-Zettler, Zettler, and Mincer 2020). Bryant et al. (2016) calculated for the North Pacific Garbage Patch that the biomass of pelagic microbial communities exceeded the epiplastic autotrophic biomass by roughly three orders of magnitude. Epiplastic communities could be of environmental importance since net oxygen production and respiration rates were significantly higher than those of the pelagic community (Bryant et al. 2016). However, the evidence for the ecological relevance of plastic-inhabiting communities contributing to biogeochemical cycles still has to be provided (Schmitt-Jansen et al. 2020).

In this work, I revealed some material-specific implications of different polymer types with respect to OM adsorption and biofilm succession and demonstrated that experiments and results have to be set into the context of larger time frames. In specific, I demonstrated that:

- The conditioning film differed between polymer types and weathering treatments.
- Mature freshwater biofilm communities were highly divergent in the early colonization phase and that their taxonomical, structural and functional capacity converged with time.

4.3 Regulatory Implications of Weathering Plastic

First concerns about accumulating plastic waste have been expressed in the 80's (see first detections of plastic debris by Carpenter and Smith (1972)) and the plastic industry reacted by starting to recycle plastic materials. Additionally, the long history of, *e.g.,* bisphenol A, today known as an endocrine disruptor and phased out in the EU as Substance of Very High Concern (SVHC), is probably the most well-known example for problems that lawmakers and regulators face with a polymer's safety assessment (Halden 2010). The reason for such late changes in regulation is the lack of knowledge and precaution when placing substances and products on the market.

For intentionally added microplastics, so-called primary microplastic, the European Chemicals Agency (ECHA) has undertaken steps forward to restrict their use in consumer products of any kind just recently. Microplastics have now been added to the registry of restriction intentions and opinion drafts by the European Chemicals Agency's (ECHA) Committee for Risk Assessment (RAC) and Socio-economic Analysis (SEAC) are available to the public (ECHA 2020). The reason for this quite fast legislative step forward lays in the RAC's acknowledgement of plastic pollution as a global phenomenon, the polymers' persistence and adverse effects so that risks are currently not adequately controlled (ECHA 2020) (see chapter 1.4 Plastic as a Planetary Boundary). This is positive for the environmental agencies, the public and the environmental health. However, while yearly emission rates to the oceans of such "primary" microplastics are estimated to vary between 0.8 – 2.5 million tons/a (Boucher and Friot 2017), emissions from mismanaged waste are estimated to represent the major fraction of plastic input to the sea of around 4.8 – 12.7 million tons/a (Jambeck et al. 2015). Therefore, it is of utmost importance to include polymers as such in the discussion of the approval process.

Today, polymers are generally (with some exceptions) exempt from registration (TITLE II) and evaluation (TITLE VI) under Regulation (EC) No. 1907/2006 REACH (REACH article 2, paragraph 9). Still, the regulation for Classification, Labelling and Packaging (CLP) applies for polymers. What needs to be registered by any manufacturer or importer in the European Union is the monomer substance (if certain criteria on percent monomer units in a polymer or the 1 ton/a quantity is exceeded). For additives, the general REACH regulation applies if they are added in concentrations above 2 % w/w and an annual quantity of above 1 ton/a (CIRS 2011). Noteworthy, additives that are

used to preserve the stability of the polymer and impurities are declared as part of the polymer and do not need to be registered separately under REACH (CIRS 2011). Although exempted from registration under REACH, a report prepared for the European Commission estimated that between 30 % and 50 % of all registered polymers may have properties that would require classification as hazardous for human health and/or the environment (OECD 2019; Commission 2012). The regulatory framework includes mechanisms to determine the eligibility of polymers for the exemption of registration mainly based on their unique physico-chemical properties that are generally different from non-polymer chemical substances (Henry et al. 2018). They are based on the general consensus by the OECD expert group on polymers that polymers of low concern (PLC) are those "deemed to have insignificant environmental and human health impacts" (OECD 2009; Henry et al. 2018).

With regard to my results of chapter 2, the regulation does not cover the potential of polymer degradation and herewith-connected relevance for human and environmental safety. I demonstrated that pristine polymers without (to my best knowledge) further blending and compounding, as it would generally not be the case for articles and consumer products, may already exhibit certain ecotoxicological potential towards *in vitro* and whole organism cell-based bioassays (*Publication III, Manuscript I*). A few standardized protocols exist to evaluate the leaching and ecotoxicological potential of solids and polymers in particular. Furthermore, accelerated ageing and subsequent testing guidelines for plastics, polymers and composites are available, mainly designed to ensure that the materials and products meet their expected functionality and durability over their lifetime (*e.g.,* in the automotive industry) (published by DIN, ISO, ASTM etc.). However, this does not necessarily cover human and environmental safety assessments. The here presented data on the ecotoxicological potential of polymer leachates, especially with respect to the investigated environmentally relevant weathering processes, raises concern about the current EU's environmental impact assessment. Therefore, we should undertake all necessary steps to critically question the current practice and reconsider the exemption of polymers under REACH.

5. Recommendations for Future Work

The scientific publications and manuscripts presented in my thesis demonstrate that weathering processes have the potential to alter the (eco)toxicological and ecological fate and impacts of aquatic plastic debris. Abiotic and biotic degradation pathways played a crucial role for the material properties of synthetic polymers and their interactions with the physical and biological environment. Polymers can no longer be regarded as an inert material and correspondingly a strong argument can be made that laboratory results should be complemented by or at least set into more environmentally relevant scenarios. This may comprise the acknowledgement of weathering-induced leaching and subsequent effect potential by microplastics as well as the surface-modulating effects of OM and changing microbial communities that rapidly build up an eco-coating (eco-corona and/or biofilms). Whether the observed material-specific OM fractionation on different macro-sized polymer substrates applies to micro- and nanoplastic particles as well needs to be addressed in future research since this may have consequences not only for colonization but also for their inherent toxicity.

The fact that polymer leachates generated under accelerated weathering conditions activated certain cellular gene pathways and showed algae toxicity should prompt us to critically reconsider the exemption of polymers from the REACH registration and evaluation. Future studies should complement the presented observations by including a higher number of market-relevant polymer types. Furthermore, I could not assess whether negative effects by leachates generally increase or may also decrease with ongoing artificial weathering time. Under prolonged weathering conditions, the emitted degradation products of concern might be subject to further photo-degradation (decreased effect potential) or continuously more toxic substances could be liberated and accumulate in the leachate water (increased effect potential).

The material plastic was once invented as a convenient substitute for stone, wood or pottery. It is designed to last for centuries but nowadays this most desired feature, its durability, becomes its major threat. Evidence is accumulating that plastic can affect human and environmental health. Therefore, we have to change our way of product design, rethink plastics in a circular economy and rigorously stop emissions.

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6. Thesis Publications and Manuscripts

Publication I

Reducing uncertainty and confronting ignorance about the possible impacts of weathering plastic in the marine environment

Annika Jahnke†, Hans Peter H. Arp‡, Beate I. Escher†, Berit Gewert§, Elena Gorokhova§, Dana Kühnel†, Martin Ogonowski§, Annegret Potthoff||, Christoph Rummel†, Mechthild Schmitt-Jansen†, Erik Toorman# and Matthew MacLeod*§

† – Helmholtz Centre for Environmental Research - UFZ, Leipzig, Germany

‡ – Norwegian Geotechnical Institute, Oslo, Norway

§ – Department of Environmental Science & Analytical Chemistry (ACES), Stockholm University, Stockholm, Sweden

|| – Fraunhofer Gesellschaft (IKTS), Dresden, Germany

– Katholieke Universiteit Leuven (KUL), Heverlee, Belgium

* corresponding author's email: matthew.macleod@aces.su.se

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Review

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Reducing Uncertainty and Confronting Ignorance about the Possible Impacts of Weathering Plastic in the Marine Environment

Annika Jahnke,[†] Hans Peter H. Arp,[‡] Beate I. Escher,[†] Berit Gewert,[§] Elena Gorokhova,[§] Dana Kühnel,[∥] Martin Ogonowski,[§,⊥] Annegret Potthoff,[@] Christoph Rummel,[∥] Mechthild Schmitt-Jansen,[∥] Erik Toorman,[#] and Matthew MacLeod*[,§]

[†]Department of Cell Toxicology, Helmholtz Centre for Environmental Research-UFZ, DE-04107 Leipzig, Germany

[‡]Department of Environmental Engineering, Norwegian Geotechnical Institute, NO-0806 Oslo, Norway

[§]Department of Environmental Science & Analytical Chemistry (ACES), Stockholm University, SE-106 91 Stockholm, Sweden

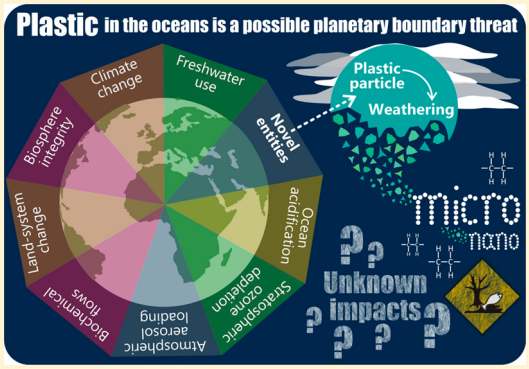
[∥]Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research-UFZ, DE-04107 Leipzig, Germany

[⊥]Aquabiota Water Research AB, SE-115 50 Stockholm, Sweden

[@]Department of Characterization, Fraunhofer Institute for Ceramic Technologies and Systems (IKTS), DE-01277 Dresden, Germany

[#]Hydraulics Division, Department of Civil Engineering, KU Leuven, Kasteelpark Arenberg 40, Box 2448, B-3001 Heverlee, Belgium

ABSTRACT: Plastic in the global oceans fulfills two of the three conditions for pollution to pose a planetary boundary threat because it is causing planetary-scale exposure that is not readily reversible. Plastic is a planetary boundary threat if it is having a currently unrecognized disruptive effect on a vital Earth system process. Discovering possible unknown effects is likely to be aided by achieving a fuller understanding of the environmental fate of plastic. Weathering of plastic generates microplastic, releases chemical additives, and likely also produces nanoplastic and chemical fragments cleaved from the polymer backbone. However, weathering of plastic in the marine environment is not well understood in terms of time scales for fragmentation and degradation, the evolution of particle morphology and properties, and hazards of the chemical mixture liberated by weathering. Biofilms that form and grow on plastic affect weathering, vertical transport, toxicity, and uptake of plastic by marine organisms and have been underinvestigated. Laboratory studies, field monitoring, and models of the impact of weathering on plastic debris are needed to reduce uncertainty in hazard and risk assessments for known and suspected adverse effects. However, scientists and decision makers must also recognize that plastic in the oceans may have unanticipated effects about which we are currently ignorant. Possible impacts that are currently unknown can be confronted by vigilant monitoring of plastic in the oceans and discovery-oriented research related to the possible effects of weathering plastic.



INTRODUCTION

Plastic debris is ubiquitous in the world's oceans, where it is subjected to physical stress, ultraviolet (UV) radiation, fluctuating temperatures, salinity, oxidizing conditions, and colonization by a range of microorganisms, including phytoplankton, bacteria, and fungi. Plastic in the environment is known to fragment into progressively smaller particles. Particles of "microplastic" in environmental samples are typically defined as having a diameter of <5 mm[1] and may originate from a range of plastic materials. Recently, fragmentation into "nanoplastic" (<100 nm in size) has been observed in laboratory systems, and similar fragmentation is also expected to occur in the environment.[2−5] Plastic usually contains chemical additives and reversibly sorbs chemicals from the environment, and there are several possible degradation pathways for plastic polymers in the marine environment that produce a mixture of chemicals that are chain-scission products from the polymer backbone.[6] Weathering plastic is thus causing global-scale exposure of the world's oceans to tiny plastic particles and to the mixture of chemical additives and polymer degradation products that leach from plastic.

The potential impacts of weathering plastic in the oceans pose assessment challenges that are characterized by both uncertainty and ignorance.[7] It is clear that we must assess the risk of impacts that are known or that can be anticipated on the basis of our experience with other pollution problems. The challenge in this context is to conduct scientific studies to reduce uncertainty in risk assessment of the known or anticipated impacts of plastic in the oceans and eventually to

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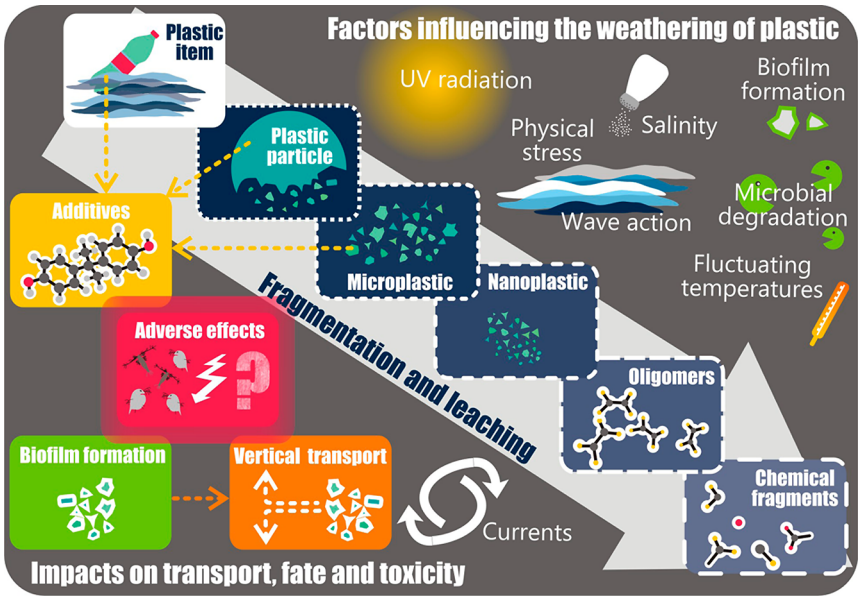

Figure 1. Summary of the factors that influence the weathering of plastic in the marine environment with resulting impacts on transport and fate processes and possible adverse effects.

develop appropriate tools to manage the risks. In the case of weathering plastic debris, the known and anticipated impacts are mostly related to toxicological effects at the individual and ecosystem levels. However, we must also confront the possibility that weathering plastic in the marine environment is having harmful effects about which we are currently ignorant.

The potential for unknown effects of pollutants to have catastrophic consequences has recently been discussed within the planetary boundary framework. The planetary boundary concept introduced by Rockström et al.[8] aimed to define a set of limits within which humanity could operate without disrupting vital Earth system processes that regulate the planet. Chemical agents govern five of the nine planetary boundaries originally defined by Rockström et al., i.e., ozone depletion (halocarbons), climate change (CO_2, CH_4, and other climate-forcing agents), ocean acidification (CO_2), the nitrogen and phosphorus cycles, and chemical pollution.

Recognizing that chemicals defined several of the identified boundaries, Persson et al.[9] proposed that there are more planetary boundaries governed by chemical pollution but that we are currently ignorant of their existence. They defined a set of three conditions that must be simultaneously met for chemical pollution to pose a planetary boundary threat. (1) The pollution must be having an unknown disruptive effect on a vital Earth system process. (2) The disruptive effect must not be discovered until it is a problem on a planetary scale. (3) The disruptive effect must be poorly reversible.

MacLeod et al.[10] defined profiles for pollutants that meet each of these three conditions. Weathering plastic debris is already known to meet two of the three conditions because it is causing global-scale exposure of the oceans (profile 4 for condition 2)[10] and because the exposure is not readily reversible (profile 1 for condition 3).[10] Therefore, plastic in the oceans would fulfill all three conditions to be a planetary boundary threat if it also meets condition 1 because it is causing a currently unknown disruptive effect on a vital Earth system process (profile 1 for condition 1).[10]

Fortunately, no serious disruptive effects of plastic have so far been observed. However, the quantity of plastic waste available to enter the oceans could increase by up to an order of magnitude between 2015 and 2025.[11] Therefore, there is a need to study the fragmentation, biofilm growth, and sedimentation processes that plastic undergoes to improve our understanding of the ultimate fate and effects, in terms of distribution, persistence, ingestion, trophic transfer, and adverse effects and toxicity. An overview of these processes is presented in Figure 1, which depicts the fragmentation and leaching of a plastic item to macroscopic plastic particles, microplastic, nanoplastic, oligomers, and chemical fragments, as a result of diverse stresses from weathering in the marine environment, e.g., UV radiation, biofilm formation, and physical stress through turbulence. The same environmental exposure processes, together with ocean currents, determine the geographical distribution and sinking behavior of plastic, and thus the location and timing of environmental and ecological exposure to weathering plastic. Improving our understanding of these processes will contribute to reducing uncertainty in risk assessment of plastic debris for known or suspected end points. At the same time, however, we must be conscious that plastic in the oceans is a potential planetary boundary threat and be vigilant about searching to discover effects. Below, we review the current understanding of exposure and effects of weathering plastic in the world's oceans and identify research priorities.

EXPOSURE OF THE GLOBAL OCEANS TO WEATHERING PLASTIC

Current research on plastic particles in estuarine, harbor, and sea environments is focused on their origin and distribution patterns on shorelines,[12,13] in subtidal sediments,[14] and in surface waters.[15] Only recently has more focus been directed toward the water column and open sea sediments.[16−19] Generally, it is expected that plastic becomes more brittle with physical aging and weathering[20−22] and thus is more prone to fragmentation over time.

Weathering by physical stress caused by wave action, abrasion by other particles, stones, and sediment, temperature fluctuations, UV-initiated degradation, microbial degradation, and biofilm formation will change the surface and structural

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properties of plastic material.[23−26] Some of these processes are responsible for creating "secondary microplastic" from large plastic debris, such as bottles and other plastic litter. Biofouling has been found to increase the effective density of floating microplastics, which is one of the mechanisms through which plastic debris with a density lower than that of seawater sinks and eventually is deposited on the seabed.[27,28] Particles formed by weathering processes may also aggregate with phytoplankton[29] and natural inorganic particles such as clays that have higher sedimentation rates.[16] Plastic particles consumed by copepods and other zooplankton that produce fast-sinking fecal pellets would have higher rates of sedimentation and burial.[19,30] The spatial variability and seasonality in plankton communities can thus affect the horizontal and vertical distribution of small plastic particles.[19] However, research on these weathering processes is still scant, as highlighted in the conclusions of a recent "State of the Science" report published by the U.S. Environmental Protection Agency.[31]

A few modeling studies have simulated the dispersion of microplastic particles at sea using three-dimensional (3D) hydrodynamic software,[32,33] and there have been modeling studies of the fate of microplastic in rivers.[34,35] The 3D modeling studies conducted to date treat plastic particles as inert tracers without considering changes in their size distribution, shape, and density due to weathering, aggregation with suspended clays, and biofilm formation. Published data for microplastic particles indicate that their size distribution is approximately log-normal,[36] though information about <300 μm particles in the water column is scarce.[37] However, changes in particle size distribution are not the only important parameters affecting transport that are influenced by weathering. Changes in density, particle shape distribution, surface charge properties, surface roughness, and particle brittleness may also play a role. Therefore, modeling the fate and transport of plastic with high fidelity to the real system cannot be achieved if plastic particles are assumed to be inert tracers.

As a part of weathering processes, biofouling can also enhance the uptake of plastic particles into the food web and slow both leaching of chemicals from the plastic and sorption of chemicals from the ambient water. Moreover, biofouling can affect the density and thus sinking rate of plastic particles, potentially determining the exposure of deep sea and benthic organisms. In the water column or when buried in sediment, plastic particles are not exposed to UV light. Hence plastic degradation in these environments is expected to occur only as a result of microbial degradation.[25] Therefore, to predict the fate and impact of plastic in the whole ocean environment, we need to understand the multiple interactions between weathering and biofilm growth and composition, and their joint effects on plastic density, sinking rate, and the consumption of plastic by filter-, suspension-, and deposit-feeding organisms.

Studying weathering plastic collected from the marine environment requires analytical techniques for identifying the plastic polymer and assessing the degree of weathering it has undergone. The two current state-of-the-art approaches to characterize the polymer type of plastic particles found in environmental samples are Fourier transform infrared spectroscopy and Raman spectroscopy.[37,38] The relative intensity of infrared or Raman signals from carbonyl groups (the "carbonyl index"), tensile properties, and average molecular weight are useful measures of molecular changes that accompany weathering processes.[39] Innovative alternative methods in the literature include pyrolysis coupled to gas chromatography and mass spectrometry (GC/MS),[40] GC/MS analysis of Soxhlet extracts of environmental microplastic particles,[41] and scanning electron microscopy coupled to energy-dispersive X-ray spectroscopy, which recently has been shown to be useful in characterizing microplastic surfaces and providing information about a material's elemental composition as a means of distinguishing microplastic from inorganic materials and biological material.[42]

KNOWN AND SUSPECTED EFFECTS OF WEATHERING PLASTIC IN THE OCEANS

The long-term effect of weathering, on the scale of decades or centuries, is expected to be beneficial because it will ultimately remove plastic from the marine environment by mineralization and transfer to deep, inaccessible sediments. However, there are concerns about the short- and medium-term effects, like leaching of chemical additives from the plastic debris, sorption and subsequent release of organic pollutants, and chemical degradation of plastic polymers into oligomers and chemical fragments that may be persistent, bioaccumulative, and/or toxic. For example, the extent of endocrine disruption effects in fish feeding on polyethylene naturally weathered for three months in San Diego Bay, CA, was higher than that in fish feeding on virgin polyethylene,[43] which was likely due to chemicals that had sorbed to the plastic. Another study using the marine crustacean *Nitocra spinipes* found that the toxicity of the leachate from ground plastic materials obtained mostly from consumer items could either increase, decrease, or remain the same after simulated weathering under a UV lamp.[44]

The size and shape of plastic particles have been shown to modulate their effects in feeding experiments. For example, irregular polyethylene fragments (~1−10 μm) that were produced to resemble weathered plastic showed potential to be more harmful to daphnids than commercially produced microplastic spheres of a similar size.[45] Size-dependent effects of microplastic fed to zooplankton have been observed, with the effects differing among the test species and the physiological responses that were monitored.[46,47] Thus, analytical methods and bioassays that can account for how the size distribution and morphology of plastic change over time and in different environments are required to fully understand and anticipate toxic effects.

The ingestion of microplastic by various animals has been demonstrated, and the potential adverse effects on marine biota have become a cause for concern. It has been proposed that microplastic could physically block the gut, gills, or feeding appendages in fish, zooplankton, and other invertebrates, causing decreased rates of growth and possibly starvation and death.[48] However, the majority of feeding studies have employed unrealistically high microplastic concentrations and used virgin microplastic. Another concern with many published studies is the lack of appropriate controls that measure effects of exposure to naturally occurring particles of a similar size, including inorganic particles and natural polymers (e.g., cellulose or chitin), in addition to the effects of exposure to plastic particles. Therefore, the relevance of many published studies to environmental settings is unclear. Although fragmented microplastic has been shown to be more harmful than virgin microplastic or natural clay to daphnids,[45] experimental studies employing weathered plastic at environmentally realistic concentrations and in combination with the mixture of organisms and detritus commonly encountered in aquatic environments are entirely lacking.

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The presence of organic chemicals sorbed to plastic particles raised concerns about their potential to be a vector for transfer of chemicals into the food web.[49] Plastic has a high sorptive capacity for hydrophobic organic chemicals, and it may contain chemical additives, some of which have been shown to cause endocrine-disrupting effects.[50] However, when the effect of ingestion of plastic on the bioaccumulation of organic chemicals is considered along with other sources of accumulation from passive uptake, respiration, and feeding on natural diet items, it is expected to be negligible.[51,52] Considering that high-trophic level organisms are known to biomagnify persistent hydrophobic organic chemicals from their food, it is plausible that ingested and subsequently egested plastic could even be a sink for these pollutants.[53] However, this proposed "cleaning" mechanism was not large enough to be observable in a laboratory study of elimination of polychlorinated biphenyls from rainbow trout fed a diet that included 40% by weight polyethylene microspheres.[54] Two recent critical reviews conducted by different groups of authors summarized the available scientific evidence and concluded that ingestion of microplastic was not likely to significantly influence the exposure of organisms in the marine environment to hydrophobic organic chemicals.[55,56]

One caveat is that most experiments and modeling to date have been based on partition ratios and kinetic parameters for virgin plastics. Karapanagioti and Klontza[57] compared the partitioning of phenanthrene between saltwater and beached, weathered plastic to the partitioning between saltwater and unweathered polyethylene and polypropylene and found higher partition ratios for the weathered plastic. More studies of the effects of weathering on the sorption and desorption of chemicals should be conducted.

RESEARCH PRIORITIES

More knowledge of the following topics is needed: (i) improved understanding of the multiple abiotic and biotic factors influencing the weathering process (Figure 1) by characterization of plastic particles over time, including morphology, particle size distribution, and surface properties, and their degradation products; (ii) elucidation of the role of weathering on sorption and desorption kinetics and the capacity of plastic to sorb chemicals; (iii) characterization of how weathering affects the spatial and temporal distribution of plastic debris, including microplastic and nanoplastic particles; (iv) identification of the adverse effects and mechanisms by which plastic particles and their degradation products affect biological systems (cell-based, organism, population, and community assays, including different trophic levels); (v) development and validation of standardized test methods suitable for assessing biological effects of plastic particles and chemicals that leach from weathering plastic in model organisms; (vi) elucidation of the role of biofilms in fate processes such as aggregation, sedimentation, and burial, and also on uptake and effects of plastic particles in marine organisms; and (vii) assessing risks related to weathering plastic in the marine environment by combining exposure assessment with effect assessment.

The development of a numerical model for predicting the 3D transport, dispersion, and fate of microplastic particles will facilitate a better understanding of plastic degradation and distribution in the marine environment, from the surface to the sediment bed. This requires the coupling of a particulate transport model (similar to a sediment transport model) with a hydrodynamic model to predict transport pathways and turbulence intensity levels. Missing currently is a model that predicts the evolution of microplastic particle transport properties, mean size or size distribution, and density. These properties could be described with a kinetic model that explicitly accounts for the fragmentation−aggregation−sedimentation processes, which in turn partially determine the persistence and fate of plastic particles, and can be derived in analogy to flocculation models for cohesive sediments (e.g., ref 58). The models should predict where plastic can accumulate below the surface by accounting for underwater currents, sediment resuspension, and other turbulence that may lead to either dilution or enrichment of plastic particles. They should also consider the "biological pump" that affects the sinking and burial of plastic particles that is affected by variability in the abundance of plankton that either colonize the plastic particles or transport them in fecal pellets to the seabed. This information is crucial for designing monitoring programs, identifying vulnerable ecosystem compartments, and developing risk assessment methodology for this emerging class of contaminants. In addition, such models will improve our scientific understanding of the distribution of microplastic by providing a platform for scenario analysis of alternative hypotheses about sources and fate processes that can be compared against field monitoring data.[59]

Research on weathering plastic is needed not only to improve our understanding of the current and potential future threats from marine litter but also to develop solutions. Understanding the degradation of plastic in the marine environment, and how it affects transport and fate, can assist in the design of "green" plastic materials. Furthermore, it can also help in the design of more sustainable management and recycling strategies, by identifying thresholds and providing guidance to avoid the risks from excessive use and emissions of harmful chemicals and plastic materials. By considering the fate of plastic debris in the environment, we can start to address the issues of plastic pollution more holistically from scientific, regulatory, and design perspectives.

Plastic debris in the oceans fits the profile of a planetary boundary threat in at least two of the three categories defined by MacLeod et al.,[10] in that it is causing planetary-scale exposure that is not readily reversible. Thus, plastic products should be candidates for precautionary substitution or phase-out with more benign alternatives, for example, by substituting paper packaging or glass when possible. Plastic waste should be minimized by improving recycling infrastructure and closing material flow cycles. Plastic debris is a planetary boundary threat if it additionally causes a currently unknown disruptive effect on the Earth system. There is no systematic way to overcome our ignorance and discover such an unknown effect, but vigilance through environmental monitoring and scientific study of processes related to weathering of plastic debris may contribute to avoiding transgressing a currently unknown planetary boundary.

AUTHOR INFORMATION

Corresponding Author

*E-mail: matthew.macleod@aces.su.se. Telephone: +4686747168.

ORCID

Matthew MacLeod: 0000-0003-2562-7339

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Notes

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Publication II

From the Sea to the Laboratory: Characterization of Microplastic as Prerequisite for the Assessment of Ecotoxicological Impact

Annegret Potthoff,[†]* Kathrin Oelschlägel,[y] Mechthild Schmitt-Jansen,[‡]
Christoph Daniel Rummel[‡] and Dana Kühnel[‡]

[†] Fraunhofer Institute for Ceramic Technologies and Systems _ IKTS, Department Characterization, Dresden, Germany

[‡] Helmholtz Centre for Environmental Research GmbH _ UFZ, Department Bioanalytical Ecotoxicology, Leipzig, Germany

* corresponding author's email: Annegret.potthoff@ikts.fraunhofer.de

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Invited Commentary

From the Sea to the Laboratory: Characterization of Microplastic as Prerequisite for the Assessment of Ecotoxicological Impact

Annegret Potthoff,† Kathrin Oelschlägel,† Mechthild Schmitt-Jansen,‡ Christoph Daniel Rummel,‡ and Dana Kühnel‡*

†Fraunhofer Institute for Ceramic Technologies and Systems – IKTS, Department Characterization, Dresden, Germany
‡Helmholtz Centre for Environmental Research GmbH – UFZ, Department Bioanalytical Ecotoxicology, Leipzig, Germany

EDITOR'S NOTE:

This is 1 of 15 invited commentaries in the series "Current Understanding of Risks Posed by Microplastics in the Environment." Each peer-reviewed commentary reflects the views and knowledge of international experts in this field and, collectively, inform our current understanding of microplastics fate and effects in the aquatic environment.

ABSTRACT

The presence of microplastic (MP) in the aquatic environment is recognized as a global-scale pollution issue. Secondary MP particles result from an ongoing fragmentation process governed by various biotic and abiotic factors. For a reliable risk assessment of these MP particles, knowledge about interactions with biota is needed. However, extensive testing with standard organisms under reproducible laboratory conditions with well-characterized MP suspensions is not available yet. As MP in the environment represents a mixture of particles differing in properties (e.g., size, color, polymer type, surface characteristics), it is likely that only specific particle fractions pose a threat towards organisms. In order to assign hazardous effects to specific particle properties, these characteristics need to be analyzed. As shown by the testing of particles (e.g., nanoparticles), characteristics other than chemical properties are important for the emergence of toxicity in organisms, and parameters such as surface area or size distribution need consideration. Therefore, the use of "well-defined" particles for ecotoxicological testing (i.e., standard particles) facilitates the establishment of causal links between physical-chemical properties of MP particles and toxic effects in organisms. However, the benefits of well-defined particles under laboratory conditions are offset by the disadvantage of the unknown comparability with MP in the environment. Therefore, weathering effects caused by biological, chemical, physical or mechanical processes have to be considered. To date, the characterization of the progression of MP weathering based on powder and suspension characterization methods is in its infancy. The aim of this commentary is to illustrate the prerequisites for testing MP in the laboratory from 3 perspectives: (i) knowledge of particle properties; (ii) behavior of MP in test setups involving ecotoxicological test organisms; and (iii) accordingly, test conditions that may need adjustment. Only under those prerequisites will reliable hazard assessment of MP be feasible. *Integr Environ Assess Manag* 2017;13:500–504. © 2017 SETAC

INTRODUCTION

Secondary microplastic (MP) is generated under the influence of different abiotic and biotic processes, leading to the fragmentation of larger plastic items to particles less than 5 mm (Arthur et al. 2009; Barnes et al. 2009). Because the presence of MP in the aquatic environment is acknowledged as a global-scale challenge, knowledge on the interaction with biota is essential for environmental risk assessment. To derive effect data for a reliable risk assessment of these materials, extensive testing with standard organisms under reproducible laboratory conditions with well-characterized MP suspensions is needed.

The aim of the present commentary is to illustrate the prerequisites for testing MP in the laboratory from 3 perspectives: 1) the properties of particles, 2) the behavior of MP in test setups, and 3) accordingly, the adjustment of test conditions.

Because MP in the environment represents a mixture of particles that differ in properties, such as size, color, polymer type, and surface characteristics, it is likely that only specific particle fractions pose a threat to single species (Figure 1). Hence it is important to assign hazardous effects to specific

* Address correspondence to annegret.potthoff@ikts.fraunhofer.de
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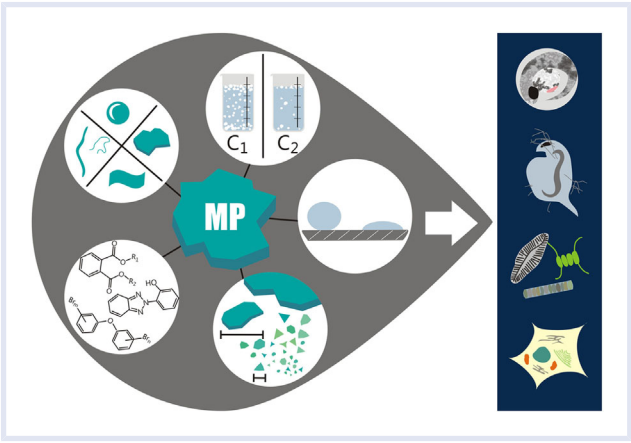

Figure 1. Toward a tiered approach: assessment of MP properties, MP behavior in liquid media, and (eco)toxicological testing. MP = microplastic.

particle properties. As shown recently, the hazard of spherical and irregularly shaped plastic particles of similar size differed when fed to *Daphnia magna* (Ogonowski et al. 2016). Additionally, it is unclear which properties of a particle are most relevant for an ecotoxicological effect. In traditional testing of chemicals, effects are related to the concentration of a substance in the test system or its dose at the biological target site. However, for particles, characteristics other than the concentration, for example the surface area or size distribution, may be the most important influencing factor. In that regard, the use of "well-defined" particles for ecotoxicological testing (i.e., standard particles) is a favorable alternative approach, facilitating the establishment of causal links between physical–chemical properties (chemical composition, size, surface properties, etc.) of MP particles and toxic effects in organisms.

However, the benefits of well-defined particles as used in the laboratory are offset by the disadvantage of the unknown comparability with MP in the environment. Another approach for the investigation of MP particles would be to extract them from environmental samples. However, for various reasons it is impractical to use field particles for ecotoxicological testing because sampling itself is challenging and will probably result in insufficient amounts for toxicological testing. Isolation, clean-up and fractionation methods are scarce; however, separation of plastic particles from inorganic and organic matter is an important prerequisite for toxicological testing. Further, extracted MP itself will usually be a mixture of various polymer types of unknown composition.

In order to draw conclusions for the aquatic environment from laboratory testing, sufficient knowledge about particle properties and their behavior in experimental setups is required. Only under those prerequisites reliable hazard assessment of MP will be feasible.

MICROPLASTIC PARTICLES: SAMPLE PREPARATION IN LIQUID MEDIA

To test the potential hazard of MP for marine and freshwater organisms, well-defined particles have to be transferred into the test system, that is, dispersed in an appropriate liquid (water or test medium). This is a crucial step because their specific properties such as wettability, density, sedimentation or floatation behavior pose challenges on the dispersion process. Although for conventional chemicals as well as for nanomaterials, extensive experience and in some cases standardized test guidelines for the preparation of test solutions or suspensions exist, these procedures cannot directly be converted into operating procedures for MP particles (Hartmann et al. 2016). The specific differences in sample preparation for toxicological tests among conventional chemicals, nanomaterials, and MP particles are pointed out in Table 1.

Homogeneous dispersion of the test substance in the test medium is an important requirement for toxicological testing. For chemicals with low solubility, usually solvents are used to facilitate dispersal in the liquid phase. However, similar to the dispersion of nanomaterials, the preparation of a homogeneous suspension of MP particles is difficult and requires additional effort such as energy input (e.g., ultrasound) and the use of dispersant aids to prevent particles from agglomeration and sedimentation by colloid–chemical stabilization. For example, hydrophobic nanomaterials do not directly disperse in a polar medium such as water. Therefore, substances such as proteins or soy lecithin have been used to improve the dispersal (e.g., Meißner et al. 2010). This procedure requires a careful evaluation of the impact of the dispersant aid itself on the test organisms. While it is easy to track toxicological effects of the dispersant aid by appropriate experimental design (i.e., using solvent controls), unknown effects due to modified bioavailability of

Table 1. Comparison of relevant properties for dispersal in liquid media: Chemicals, inorganic particles or nanomaterials, and plastic particles

Type of substance	Density	Wettability	Release of constituents	Aging
Chemicals	Known, irrelevant	—	—	Yes[a]
Inorganic particles Nanomaterials or nanoparticles	> Density of water	Mostly high, depending on material type	Possible, depending on material type	Yes[b]
Plastic particles	≈ Density of water	Mostly low	Possible for additives	Yes[c]

[a] Transformation of chemicals due to processes such as photo-oxidation.
[b] Physical and chemical properties such as agglomeration behavior, dissolution, surface oxidation.
[c] Physical and chemical properties such as density, crystallinity, size.

particles may occur, specifically particle uptake and distribution in organisms. For MP particles, similar approaches are feasible, such as the use of artificial dispersant aids (surfactants) or extracellular polymeric substances of microorganisms that act as natural dispersant aids. The use of extracellular polymeric substances such as algae exudates to foster submersion of MP in laboratory test systems is considered favorable because it resembles the natural situation (Lobelle and Cunliffe 2011) in which biofilm formation may eventually lead to negative buoyancy of low-density particles.

However, a specific feature of some synthetic polymers is their low density (e.g., LDPE), which results in particles floating on top of a test suspension. This specific behavior leads to serious problems during toxicity testing because the bioavailability of MP for test organisms residing in the water column will be limited (Rehse et al. 2016). Accordingly, the relevant concentration to which organisms are exposed to MP is largely unknown, irrespective of the immense importance of this information for hazard assessment. In that regard, the different salinities of fresh- and saltwater and accordingly of the test media used for test organisms from the respective habitats, play an important role. The density of MP in some cases is very similar to the density of water. The lower density of freshwater or media with low salinity may foster submersion of MP particles, whereas particles in seawater may tend to float on the surface. The change in density due to biofouling is attributed to a mass increase by the attachment of microorganisms. This issue underlines the importance of MP particle characterization in liquid media.

Another challenge from the perspective of both particle characterization and toxicology is the release of auxiliary substances from the particles into the surrounding media. For nanomaterials, mostly ions are released, such as Zn^{2+} or Co^{2+} from zinc oxide (ZnO) or tungsten carbide–cobalt (WC-Co) nanoparticles, respectively (e.g., Kühnel et al. 2012). For MP, the leaching of soluble additives shows an equivalent dissolution effect, and impact on organisms has been demonstrated by Bejgarn et al. (2015) and Li et al. (2016). However, exposing organisms to a mixture of particles and dissolved substances results in difficulties in the evaluation of toxicity tests because the bioavailability and uptake kinetics of particles and plastic-derived chemicals into organisms may differ. At simultaneous exposure it remains unclear to which extent the particles and the dissolved chemicals contribute to a certain toxicological effect. The testing of particle-free solutions of plastic leachates may help to untangle the particle effects from those of released additives.

Moreover with regard to sample preparation, challenges are as basic as concerning the use of pipettes usually made of plastic, which may lead to either plastic cross-contamination of the initial sample or sorptive losses of the MP sample to the plastic material of equipment in the lab. Whereas plastic pipettes can easily be replaced by glass pipettes, the situation is more difficult for plastic tubes in measurement devices. Because any unwanted interaction between particles and laboratory material will influence the analytical results, careful control for interferences is warranted. First of all, the amount of MP in the measured sample needs to be tracked to account for losses. Hence, the calculation of the MP recovery rate during the preparation of suspensions and subsequent analyses is considered an important issue to be addressed in the future.

To date, no guidelines or standardized procedures regarding MP handling for preparation of samples and their dispersion for toxicity testing exist. As is known from risk assessment of other types of particles, those guidelines are required as a prerequisite for understanding the results obtained from ecotoxicological testing.

HOW TO IMPROVE THE CHARACTERIZATION OF MIRCOPLASTIC PARTICLES

Until now, a variety of MP characteristics are under discussion regarding their importance and impact on the environment. While chemical analyses by infrared or Raman spectroscopy are mostly applied for polymer type identification (Kappler et al. 2016), integral parameters for other MP characteristics usually are not addressed:

1) As is known from medical applications such as bone substitution development, the specific surface area of particles and the roughness of surfaces have an impact on interactions between MP and microorganisms (e.g., Li et al. 2012). A common method for surface characterization is the analysis of the specific surface area according to Brunauer et al. (1938) by gas adsorption, normally using N or Kr. Together with particle size, information on surface area and porosity is derived. The roughness is measured by microscopic methods such as laser scanning microscopy.
2) Functional groups at polymer particle surfaces determine the surface charge properties, which can be characterized by zeta potential measurements. As is known from other materials such as inorganic particles, the interaction of particular or dissolved organic or inorganic material with the surface leads to a significant change in surface charge (e.g., Meißner et al. 2010). Analytical methods such as electrophoresis are often limited to small (micron-sized) particles; the challenge is to establish and validate methods suitable for micron- to millimeter-sized particles.
3) The analysis of particle size distributions is very diverse. Established methods such as laser diffraction cover a wide range of particle sizes. The current analytical challenge is the characterization of broad size distributions that cover an expected nanoscaled fraction (Lambert and Wagner 2016) up to particles in the millimeter range. An appropriate method of sample preparation is required and needs to be established.

From investigations of other types of particles such as inorganic nanomaterials or functional coatings, the importance of these physical-chemical parameters for interaction between MP samples and organisms is known (Krug 2014).

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MODIFICATION OF MP PROPERTIES DUE TO WEATHERING PROCESSES

Specific consideration for the investigation of the hazard posed by MP must be given to weathering processes. Weathering leads to the fragmentation and degradation of large plastic items down to molecular fragments under the influence and the continuous changes of biological, chemical, and physical parameters (Jahnke et al. 2017; ter Halle et al. 2016). As a consequence, MP present in the environment has to be perceived as a mixture of particles with diverse characteristics. The most relevant weathering processes and the relevant MP properties are summarized in Table 2. For their interpretation these parameters must be considered interdependent; it is not possible to change a single parameter without influencing the others.

The wide range of influential properties of the material makes hazard assessment of weathered MP particles a major challenge. It has been demonstrated that mimicking weathering processes may have an impact on the outcome of toxicological investigations of MP particles (Ogonowski et al. 2016). During abrasion processes, the fragmentation of particles leads to an increase in the surface-to-volume ratio. As is known from the nanomaterial risk assessment, particle size distribution and surface area are often linked to ingestion and toxicological effects.

In natural surroundings such as the marine environment, particles of various sizes exist, yet the smallest sizes (nanoplastics) are assumed to be present though they are not yet detectable with current sampling strategies. A proper interpretation of test results requires the analytical coverage for achieving detailed knowledge on the size distributions of the investigated material. Therefore it is necessary to fractionate the pristine or weathered particles, either by sedimentation and filtration processes or by floatation in combination with sieving. The obtained suspension contains the desired size fraction but an unknown mass concentration of MP particles. For toxicological testing of well-defined plastic particles, the determination of the relevant exposure concentration is a fundamental prerequisite. Methods such as the analysis of the total organic C (TOC) content may help to solve this issue because it is an integrated measure of the total MP particle concentration.

Depending on the test system, different characteristics of the MP fractions may be relevant. Daphnids, for instance, may be sensitive to a specific size fraction of particles; they ingest particles by filter feeding. In general, different size fractions of MP may exert different physical effects on organisms, either by attachment to the organism's surface or upon ingestion. For biofilm communities, on the other hand, particle size may be less relevant, but surface properties such as roughness and surface charge may have a higher influence on growth. For the attachment and stabilization of biofilms, extracellular polymeric substances play an important role. These are stabilized by weak physical–chemical interactions such as hydrogen bonding or van der Waal interactions, and therewith depend on the surface charge (Flemming and Wingender 2010). The resulting differences in composition and density of biofilm on MP may induce significant changes in the physicochemical properties of MP, making it less hydrophobic and more buoyant (Lobelle and Cunliffe 2011).

The aim of future research efforts should be to characterize the influence of individual MP parameters affected by weathering on the potential toxic effects in organisms.

RECOMMENDATIONS AND OUTLOOK

Because plastic litter has emerged as a major pollution issue in both marine and freshwater systems, the assessment of its potential environmental hazard is essential. However, state-of-the-art and common practical procedures for this assessment as available from toxicity testing of chemicals may not be applicable for MP particles. The investigation of particles collected from the environment is limited by sampling efficiencies and characterization of the collected particles. Additionally, the diversity of MP in environmental samples in terms of, for example, polymer type, size, and shape, may not result in clear correlations of the relevant MP characteristics responsible for a defined effect. To derive a causal link between MP key characteristics and a biological effect, the use of well-defined particles and their weathering products under laboratory conditions is recommended but poses various challenges.

Table 2. Overview of the influence of biological, chemical, and physical weathering on fundamental MP properties

Processes during weathering	Relevant MP particle parameters modified by weathering
Biological (such as colonization by biofilms, microbial degradation, interactions with dissolved and particulate biological matter; incorporation into fecal pellets)[a]	Density, surface properties such as charge and specific surface area
Chemical or physical (e.g., UV radiation, salinity, changes in temperature, interactions with dissolved organic matter such as humic acids, sorption)	Crystallinity, surface properties such as roughness and charge, brittleness, particle size distribution, concentration of dissolved matter (e.g., additives), molecular weight, polymer functional groups, tensile strength, etc.
Mechanical (abrasion, wave action, currents, interactions with other particles such as clay)	Particle size distribution, particle shape distribution, roughness, specific surface area, surface charge

MP = microplastic.
[a] Source: Cole et al. 2016.

In any case, an appropriate analysis of the properties of the investigated MP particles retrieved from the field, pristine MP or laboratory-aged MP, is crucial. During all steps of sample preparation and subsequent analyses, attention should be paid to plastic cross-contamination from equipment and instruments. Analytical methods required to describe relevant particle properties are not yet established. In conclusion, in addition to the knowledge about the chemistry of the polymer, we suggest a set of minimum criteria, which should be considered for a meaningful testing of the environmental hazard of MP particles:

1) Particle surface properties (area, roughness, charge)
2) Particle size and particle shape distribution
3) Bulk parameter (density, brittleness, crystallinity).

Novel methods or the adaptation of existing methods are needed to address the specific properties of MP particles. Applying these novel or optimized methods will help us to understand the weathering change of MP behavior in the environment and to find correlations to the weathering processes in the lab. An improved physical–chemical characterization of the particles is the key to understanding the relevant differences between well-defined (either pristine or weathered in the lab) and environmental MP. The next steps for investigations include the evaluation of the relevance of each single parameter, among them leaching and formation of degradation products, for the interpretation of ecotoxicological data.

Experiences gained from the application of standard operating procedures, such as Organisation for Economic Co-operation and Development (OECD) guidelines for testing nanomaterials, indicated that adjustments of the test conditions to specific characteristics of MP may be needed. Until standard test protocols such as OECD guidelines or International Organization for Standardization (ISO) standards for MP are in place, structural approaches for dispersion and handling as proposed for testing nanomaterials (e.g., Potthoff et al. 2015) may be adopted for MP to form the basis for a risk assessment of MP particles in the aquatic marine environment.

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Publication III

Effects of Leachates from UV-weathered Microplastic in cell-based bioassays

Christoph D. Rummel[†*], Beate I. Escher[†II], Oskar Sandblom[‡], Merle M. Plassmann[‡], Hans Peter H. Arp[¥T], Matthew MacLeod[‡] and Annika Jahnke[†]

[†] Department of Bioanalytical Ecotoxicology and Department of Cell Toxicology, Helmholtz Centre for Environmental Research - UFZ, Permoserstr. 15, DE-04318 Leipzig, Germany.

[II] Center for Applied Geoscience, Eberhard Karls University Tübingen, Environmental Toxicology, Hölderlinstr. 12, DE-72074 Tübingen, Germany.

[‡] Department of Environmental Science and Analytical Chemistry (ACES), Stockholm University, Svante Arrhenius väg 8, SE-114 18 Stockholm, Sweden.

[¥] Department of Environmental Engineering, Norwegian Geotechnical Institute (NGI), Sognsvn. 72, NO-0855 Oslo, Norway.

[T]Department of Chemistry, Norwegian University of Science and Technology (NTNU), NO-7491 Trondheim, Norway.

* corresponding author's email: christoph.rummel@ufz.de

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Effects of Leachates from UV-Weathered Microplastic in Cell-Based Bioassays

Christoph D. Rummel,*[,†] Beate I. Escher,[†,‡] Oskar Sandblom,[§] Merle M. Plassmann,[§] Hans Peter H. Arp,[∥,⊥] Matthew MacLeod,[§] and Annika Jahnke[†]

[†]Department of Bioanalytical Ecotoxicology and Department of Cell Toxicology, Helmholtz Centre for Environmental Research-UFZ, Permoserstraße 15, DE-04318 Leipzig, Germany

[‡]Center for Applied Geoscience, Eberhard Karls University Tübingen, Environmental Toxicology, Hölderlinstraße 12, DE-72074 Tübingen, Germany

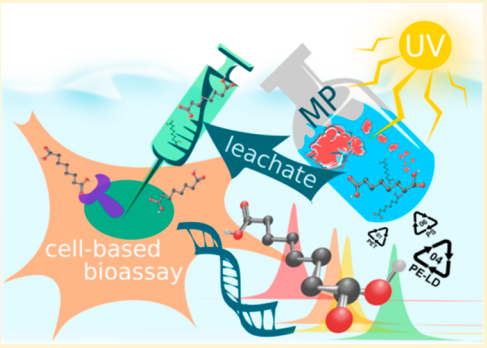
[§]Department of Environmental Science and Analytical Chemistry (ACES), Stockholm University, Svante Arrhenius väg 8, SE-114 18 Stockholm, Sweden

[∥]Department of Environmental Engineering, Norwegian Geotechnical Institute (NGI), Sognsvann 72, NO-0855 Oslo, Norway

[⊥]Department of Chemistry, Norwegian University of Science and Technology (NTNU), NO-7491 Trondheim, Norway

Supporting Information

ABSTRACT: Standard ecotoxicological testing of microplastic does not provide insight into the influence that environmental weathering by, e.g., UV light has on related effects. In this study, we leached chemicals from plastic into artificial seawater during simulated UV-induced weathering. We tested largely additive-free preproduction polyethylene, polyethylene terephthalate, polypropylene, and polystyrene and two types of plastic obtained from electronic equipment as positive controls. Leachates were concentrated by solid-phase extraction and dosed into cell-based bioassays that cover (i) cytotoxicity; (ii) activation of metabolic enzymes via binding to the arylhydrocarbon receptor (AhR) and the peroxisome proliferator-activated receptor (PPARγ); (iii) specific, receptor-mediated effects (estrogenicity, ERα); and (iv) adaptive response to oxidative stress (AREc32). LC-HRMS analysis was used to identify possible chain-scission products of polymer degradation, which were then tested in AREc32 and PPARγ. Explicit activation of all assays by the positive controls provided proof-of-concept of the experimental setup to demonstrate effects of chemicals liberated during weathering. All plastic leachates activated the oxidative stress response, in most cases with increased induction by UV-treated samples compared to dark controls. For PPARγ, polyethylene-specific effects were partially explained by the detected dicarboxylic acids. Since the preproduction plastic showed low effects often in the range of the blanks future studies should investigate implications of weathering on end consumer products containing additives.



INTRODUCTION

Pollution of the aquatic environment by plastic debris has become ubiquitous over the last decades and fits the profile of a planetary boundary threat.[1] Plastic material in the environment is impacted by weathering processes such as UV light-induced degradation, mechanical stress, temperature and salinity changes, as well as biological influences exerted by superficial biofilms and fauna.[2−5] Weathering causes fragmentation, leading to formation of microplastic (<5 mm),[6] and to the liberation of additives, related degradation products, and products of polymer chain-scission reactions as free chemicals.[2]

Many studies have investigated the potential effects of microplastic by addressing the physical presence and impact of the particles themselves. Negative effects on organisms from different trophic levels such as algae, daphnia, and fish have been reported for laboratory studies using pristine microplastic particles.[7−9] Furthermore, plastic debris has the potential to serve as a source and sink of persistent organic pollutants (POPs)[10] which may facilitate the transport of such substances, often referred to as the "vector effect".[11] Depending on the polymer's intended use, additives such as UV stabilizers and flame retardants are added to preproduction polymers during manufacturing.[12] Once released to the environment, plastic debris may act as a source of these additives and hence has the potential to negatively impact organisms.[13] The high sorptive capacity for hydrophobic organic contaminants such as Polychlorinated Biphenyls (PCBs)[14,15] and Polycyclic Aromatic Hydrocarbons (PAHs)[16] renders polymers also a sink for these compounds.

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Considered in total, it is apparent that under the influence of environmental weathering, plastic materials, including the polymer carbon backbone chains and associated chemicals, will ultimately release a complex mixture of chemicals that includes many unknown degradation products. Unraveling the potential effects of weathering plastic and the associated chemicals is a high research priority for planetary health.[1]

Previous studies have described acute toxicity of leachable fractions of various plastic types toward *Daphnia magna*[17,18] and the marine copepod *Nitocra spinipes*.[19] Li et al. (2016)[20] revealed larval toxicity and settlement inhibition to the barnacle *Amphibalanus amphitrine* during a 24 h exposure scenario in leachates from seven recyclable commercial plastic products. Cytotoxic end points like cell growth, survival and colony-forming capability were negatively affected by plastic leachates from biomedical devices tested in the human cell line L929 after 1 h of exposure.[21] Coffin et al. (2018)[22] detected estrogenic effects and binding to the arylhydrocarbon receptor (AhR) by chemicals leached from virgin, weathered and field-collected in situ plastic samples from the North Pacific Gyre.

The most important abiotic degradation process for plastic in the environment is UV radiation-initiated autocatalytic radical oxidation.[2,23] Recently, Gewert et al. (2018)[24] identified a set of low molecular weight polymer chain scission products liberated from commercially important polymers exposed to UV light. They were mainly dicarboxylic acids, but also included other oxidized end-groups. Toxicological studies on the chemicals leaching from plastic often lack related chemical analyses as well as consideration of UV light-induced changes of the polymers' chemical composition, a so-called fingerprint, compared to the pristine material.

To improve our understanding how weathering can influence MP-induced effects, we aimed in this study to identify potential activation of cellular signaling pathways by leachates that were generated as a result of artificial UV light-induced weathering of four commercially important polymers (polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), and polystyrene (PS)) in artificial seawater (ASW). To demonstrate the sensitivity of our test battery toward substances liberated from the test material, two positive controls from electronic waste and a computer keyboard known to contain pollutants and/or additives were included. A test battery of four cell-based bioassays was chosen to cover relevant biological end points. They were selected based on the available analytical data of e-waste as a positive control for which high concentrations of, among others, PCBs, Polybrominated Diphenyl Ethers (PBDEs), and Bisphenol A (BPA) were measured in previous studies.[25,26] Another material selection criterion referred to prominent plasticizers and additives often added to preproduction polymers in the plastic industry to customize the material for its intended use.[12]

Concentrated leachates were dosed into cell-based bioassays covering (i) cytotoxicity; (ii) activation of metabolic enzymes via binding to the AhR[27] and the peroxisome proliferator-activated receptor gamma (PPARγ);[28,29] (iii) specific, receptor-mediated effects (estrogenicity);[30] and (iv) adaptive stress responses exemplified by the oxidative stress response.[31,32] Liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) was used to identify potential degradation products, i.e., carboxylic acids, in the leachates. To better account for PPARγ results, we tested the hypothesis that dicarboxylic acids, previously identified as degradation products of UV-weathered PE,[24] could explain the observed explicit induction of PPARγ, by dosing reference mono- and dicarboxylic acids with a range of carbon chain lengths (C5−C18) into the PPARγ assay. In a last step, we applied a concentration addition model to compare the observed bioanalytical effects to the chemical analytical data.

MATERIALS AND METHODS

Test Material and Chemicals. The polymers PE, PET, PP, and PS, purchased from *Goodfellow* (Hamburg, Germany), were chosen as test polymers due to their high production tonnage in European commerce and industrial importance.[33] According to the distributor's information, these pellets were "additive free", only containing antioxidants and trace levels of an unknown pigment in the case of PS to make it look more glass-like when molded (*Goodfellow*, personal communication). Analytical data on degradation products from the identical material was published by Gewert et al. (2018).[24] The pellet test material was milled to <350 μm by the company Messer GmbH (Bad Soden, Germany) to ensure a high surface-to-volume ratio (Figure S1, Supporting Information (SI)). In order to demonstrate that the method was applicable to detect substances leaching from weathering microplastic in cell-based bioassays, we chose two positive controls: a homogenized sample of shredded electric cable plastic waste (e-waste, EW) sampled at a Norwegian electric cable waste-handling facility[25] and a new computer keyboard (keyboard, KB) likely containing flame retardants. Analytical data on BPA and flame retardants of the e-waste was previously published by Morin et al. (2015)[25] and Morin et al. (2017),[26] respectively. Mono- and dicarboxylic acids (α,ω position) of carbon chain lengths of C5, C7−C12, C14, C16, and C18 were purchased from Sigma-Aldrich (Steinheim, Germany) (detailed information SI Table S1). Methanol (Honeywell, Riedl de Haën, Seelze, Germany), ethyl acetate (Honeywell, Riedl de Haën, Seelze, Germany), and water (Fisher Chemical, Schwerte, Germany) were of LC grade.

Weathering. A detailed description on the weathering setup is given by Gewert et al. (2018).[24] In short, triplicates of 50 g of each test material, suspended in 200 mL (i.e., liquid−solid ratio of four) of ASW (Instant Ocean Sea salt, Blacksburg, Virginia U.S.A.) in quartz glass vessels were weathered by intense UV A+B light irradiation (OSRAM Supratec HTC400−241 R7s UVA/UVB lamp), combined with horizontal rotation of the vessels around the lamp. Six vessels were weathered at a time using a custom-made wheel, which rotates the quartz glass vessels around the UV lamp to ensure equal UV exposure and to provide gentle mixing of the particles (SI Figure S2). The samples were weathered for 96 h. UV treatments (UV) were done in triplicates with corresponding dark controls (DC, identical setup but wrapped in aluminum foil, n = 3) of one polymer simultaneously. During the weathering process the temperature was kept between 20 and 30 °C by an air flow cooling system. The 96-h UV treatment in the rotating vessels simulated about 410 days of Middle European sun exposure.[24] Procedural blanks were generated by completing the weathering protocol with ASW without microplastic. A detailed description of the lamp properties and solar simulation equivalence calculation can be found in the SI (Section S1. Experimental setup).

Solid-Phase Extraction. After weathering, the microplastic/leachate water mixture was filtered over a 40 μm steel filter to remove the particles. The chemicals present in the

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leachate water were enriched on solid-phase extraction (SPE) cartridges (HLB Plus Oasis 225 mg, Waters GmbH, Eschborn, Germany, conditioned with 5 mL of ethyl acetate/methanol (1:1, v:v), 5 mL methanol and 5 mL of Milli-Q water), dried, and stored at room temperature until analysis. Elution was performed using 10 mL ethyl acetate and 10 mL methanol, and the extracts were combined. Additionally, the extracts were filtered (GF/F Whatman) to remove residues of the artificial seawater salt that precipitated during elution. Three SPE blanks using 200 mL of LC grade water that was enriched and eluted were generated to identify potential background effects. Samples were then blown down to dryness under nitrogen, redissolved in 1 mL methanol and stored at −20 °C. An aliquot of 50 μL was taken from each sample, blown down to dryness and stored at −20 °C for chemical analysis. A detailed SPE protocol can be found in the SI (Section S2. SPE protocol).

Cell-Based Bioassays. To measure the activation of xenobiotic metabolism signaling pathways, the AhR-CALUX assay described by Brennan et al. (2015)[27] and performed according to Nivala et al. (2018)[34] and the PPARγ-bla GeneBLAzer assay[28,29] following the method by Neale et al. (2017)[28] were applied. The activation of oxidative stress response was investigated with the AREc32 assay[31] according to Neale et al.[28] and Escher et al.[32] Potential endocrine disruption was measured with the ERα-bla GeneBLAzer assay for estrogenicity[30] according to the procedure described by König et al. (2017)[35] (SI Table S2). Testing the concentrated plastic leachates was conducted as follows: An aliquot of the sample was blown down to dryness and redissolved in the assay medium (DMEM with GlutaMAX or Opti-MEM, respectively, Thermo Fisher, Waltham, U.S.A.) to prevent exposing the cells to solvents. Cells were seeded in 384 well-plates with a Biotek dispenser, samples were diluted and dosed with a liquid handling system (Hamilton Microlab Star, Bonaduz, Schwitzerland) to guarantee precise dosing and repeatability.[28] Directly before dosing and after 24 h of exposure, the confluency of the cells in all wells in the cell plates was measured using an IncuCyte S3 live cell imaging system (Essen BioScience, Ann Arbor, Michigan, U.S.A.).[34] After 24 h the reporter gene product was quantified after adding the appropriate substrates and measuring fluorescence or luminescence using a microplate reader (Infinite M1000 Pro, Tecan, Grödig/Salzburg, Germany). A first high-concentration dosing (of relative enrichment factors of up to 167 of the extracts, see "Data Evaluation") combined with serial dilution was performed for the detection of cytotoxicity and for range finding. This first experiment was followed by another serial dilution (for the leachates) or a linear dilution (for the carboxylic acids) in a noncytotoxic concentration range for confirmation of the first measurement and to increase robustness and statistical power. The generation of the dilution series was performed on a dilution plate followed by the cell exposure, conducted in technical duplicates. If the data sets deviated from each other, measurements were repeated to confirm dose−response curves and to reduce uncertainty. The deviating data were not included in the final evaluation of the dose−response curves.

Instrumental Analysis. The dried aliquots of the concentrated leachates were taken up in 100 μL of methanol/Milli-Q water (1/1) and analysis of dicarboxylic acids was performed using an UltiMate 3000 Rapid Separation Liquid Chromatography system (Dionex, Germering, Germany) coupled to a Q Exactive HF Hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). The method was adopted from Gewert et al. (2018)[24] as specified in the SI (Section S3. Instrumental Analysis). A six point calibration of pure substances in methanol/Milli-Q water (1/1) was used for quantification of dicarboxylic acids, applying TraceFinder 4.1 (Thermo Scientific). Method detection limits (MDL) and method quantification limits (MQL) were based on dicarboxylic acid concentrations detected in the dark and UV-treated procedural blanks. MDL was calculated as the mean blank concentration of a given carboxylic acid (n = 3) plus three times standard deviation. Analogously, the MQL was calculated as the mean plus nine times standard deviation. If peaks were detected but not quantifiable (i.e., < MQL), then half of the MQL was used for further computation as detailed in SI Table S3.[36]

Mono- and Dicarboxylic Acids in PPARγ and AREc32. Following previous analytical results,[24] we measured the effect of mono- and dicarboxylic acids of various chain lengths (SI Table S1) separately in the two most responsive assays, PPARγ and AREc32, to identify their potential to activate these signaling pathways. Chemicals were dissolved directly in assay medium or via a methanolic spike solution to facilitate dissolution at highest medium solubility. Methanol concentration in the assay medium was kept under 0.1%.

Data Evaluation. The sample concentrations in the bioassays were calculated as the product of the enrichment factor of the extraction (EF_{SPE}) and the bioassay dilution factor ($DF_{bioassay}$), which results in the relative enrichment factor (REF) (see eq 1):

$$REF_{Leachate} = EF_{SPE} \times DF_{bioassay}\left[\frac{L_{water}}{L_{bioassay}}\right] \quad (1)$$

The three receptor-based bioassays (AhR, PPARγ and ERα) were run with corresponding reference compounds (SI Table S2) that elicit high responses in the assay to calculate dose−response curves given as percent (%) response relative to the maximum effect of the reference compound (SI Figures S6−S12). Agonistic responses were determined as the effect concentration (EC) causing 10% response (EC_{10}) over the control cells.

For the adaptive stress response, which is based on the regulation of an antioxidant responsive element (ARE) by transcription factors and for which the dose−response curves would not show leveling off, the response is given as the induction ratio (IR) of 1.5, i.e., 50% over the controls ($EC_{IR1.5}$).

Using GraphPad Prism Sofware Inc. (version 8.0.0), cytotoxicity was calculated as percent decrease in cell viability compared to unexposed control cells.[34] According to Escher et al. (2018),[37] all concentrations above 10% decrease of cell viability (inhibitory concentration, IC_{10}) were removed from the analyses of reporter gene activation to circumvent false positive detections due to a so-called cytotoxicity-associated "burst".[38] The slope and the standard error (SE) of the slope for reporter gene activation were calculated using log−logistic and linear models to calculate EC_{10} and $EC_{IR1.5}$ values. Previous studies have shown that 10% induction is statistically significantly different from the control and can thus be interpreted as a sample-specific effect relative to the control.[37]

Statistical Assessment. EC data can be counterintuitive to describe the dependence of low EC levels and large effect

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sizes. Therefore, the above-mentioned EC_{10}, $EC_{IR1.5}$ and IC_{10} values (in the units REF) derived from the bioassays were plotted as the inverse value (in the units 1/REF) on a log scale with effect units (EU_{bio}) (eq 2) used in the case of activation of specific effects and toxic units (TU_{bio}) for cytotoxicity (eq 3). Analogously to the EU_{bio} for unknown mixtures, we define $EU_{bio(i)}$ for a single compound (i) as the inverse $EC_{10(i)}$ derived from the bioassays (eq 4). Bioanalytical equivalent concentrations are presented in Section S4. Bioanalytical equivalent concentrations and in Tables S4−S6.

$$EU_{bio} = \frac{1}{EC_{10}} \text{ or } \frac{1}{EC_{IR1.5}} \quad (2)$$

$$TU_{bio} = \frac{1}{IC_{10}} \quad (3)$$

$$EU_{bio(i)} = \frac{1}{EC_{10(i)}} \text{ or } \frac{1}{EC_{IR1.5(i)}} \quad (4)$$

With the available data, assumptions for a robust linear regression model were violated hampering extended statistical analyses. Therefore, the mean, standard deviation and the 95% confidence interval were calculated for qualitative comparison between the samples in those cases that all triplicates resulted in a measurable effect. If not stated otherwise, then values in the "results" section are the calculated means. Due to the low number of replicates (n = 3), further statistical computation was not meaningful. From single compound EC_{10} data of mono- and dicarboxylic acids a linear least-squares regression was calculated to test for correlation between molecular mass and EU_{bio} using RStudio (version 1.1.456). Validity of the model assumptions was examined using qqplot and checking for normality of the residuals. The difference of the slope from zero was considered significant with α = 0.05.

The EC_{10} values derived from the single compound testing of dicarboxylic acids and the measured sample concentrations of detectable dicarboxylic acids were applied to a mixture toxicity model. Escher et al. (2013)[39] have previously demonstrated that concentration addition applies for the reporter gene assay AREc32 and other end points.[40] Hence, we defined effect units derived from chemical analysis ($EU_{chem(i)}$). $EU_{chem(i)}$ was calculated analogously to toxic units[41,42] as the ratio of measured concentrations c_i of a chemical i and its EC_y value (here: EC_{10}, eq 5). It can be used to explain effects measured in bioassays (here: EU_{bio}) by a certain contribution of n detected chemicals i as the sum of $EU_{chem(i)}$ given as EU_{chem} (eq 6) and to identify the fraction of effect unexplained by the known chemicals (EU_{bio}−EU_{chem}) by so-called iceberg modeling.[28,37]

$$EU_{chem(i)} = \frac{c_i}{EC_{10(i)}} \quad (5)$$

$$EU_{chem} = \sum_{i=1}^{n} EU_{chem(i)} = \sum_{i=1}^{n} \frac{c_i}{EC_{10(i)}} \quad (6)$$

RESULTS

For all assays, the positive controls e-waste and keyboard showed clear induction of the respective signaling pathway (Figure 1A−C), sometimes exceeding 100% effect of the reference compound (SI Figure S7, e.g., AhR sample KB 1−3, KB_DC 1−3). Furthermore, the plastic-free blanks (DC and UV-treatments) resulted in detectable effects for most of the assays for at least one replicate in AhR, AREc32, and PPARγ (Figure 1A−C). For the ERα assay, only e-waste and keyboard resulted in measurable EC_{10} values (Figures S3 and S10). Due to the absence of detectable effects for our test material, the ERα assay was excluded from the subsequent discussion. Cytotoxicity was observed for some of the test polymers (Figure S4).

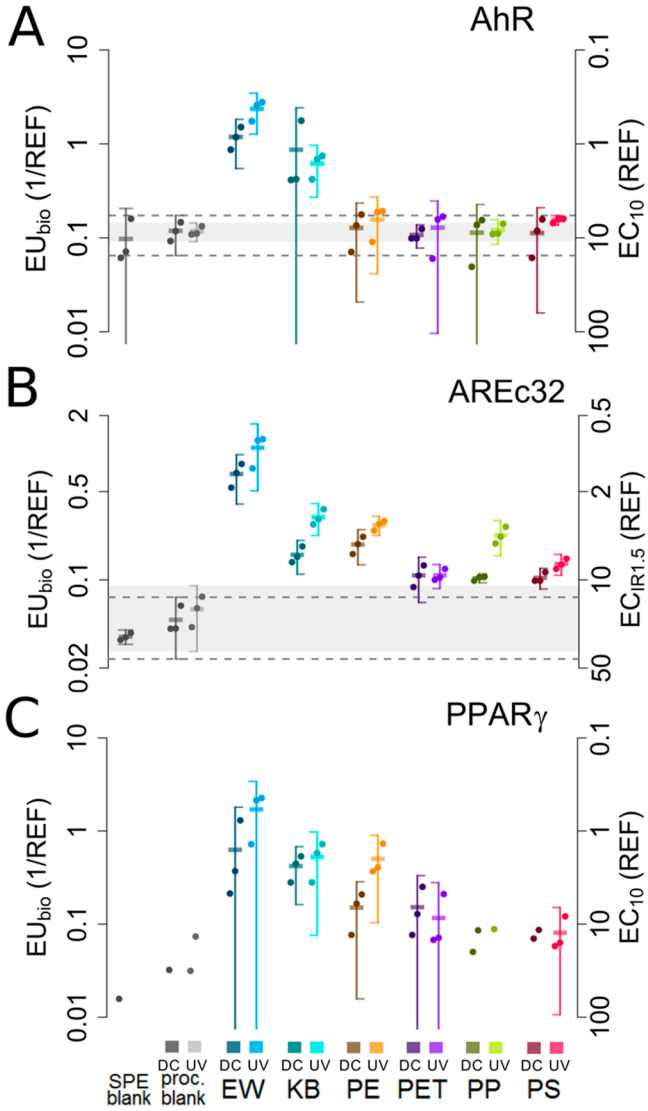

Figure 1. Bioanalytical effect units (EU_{bio}, eq 2) defined as the inverse EC_{10} (1/REF on left y-axis, REF on right y-axis), measured for SPE blanks, procedural blanks, two positive controls (EW and KB) and the four test polymers PE, PET, PP, and PS in the cell-based bioassays AhR (A), AREc32 (B) and PPARγ (C). Dark controls (DC) and UV-treated (UV) samples are presented juxtaposed in darker and lighter shades of the different colors. The squares represent the mean, whiskers the upper and lower range of the 95% confidence interval. The dashed lines and the shaded area represent the minimum and maximum ranges of the 95% confidence interval of the procedural DC and UV-treated blanks to highlight differences from the respective control. For PPARγ, no prediction intervals were included because only one to two replicates resulted in measurable EC_{10} values.

AhR. The AhR signaling pathway was clearly activated by the positive controls, with effects by more than a factor three higher for e-waste (EU_{bio} (EW_{DC}) = 1.19, EU_{bio} (EW_{UV}) = 2.37) than for keyboard (EU_{bio}(KB_{DC}) = 0.87, EU_{bio} (KB_{UV}) =

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0.62) (Figure 1 A, Table S4, and SI Figure S7). All test polymers showed low activation of the AhR that did not differ from their corresponding DC or UV procedural blanks indicated by the overlapping 95% confidence band. EU_{bio} of the procedural blanks (EU_{bio} ($blank_{DC}$) = 0.12, EU_{bio} ($blank_{UV}$) = 0.12), and the test polymers (EU_{bio} (all test $polymers_{DC}$) = 0.11−0.13, EU_{bio} (all test $polymers_{UV}$) = 0.12−0.16) were more than a factor of 10 lower than the EU_{bio} values of the DC and UV-treated e-waste. All three replicates of the SPE blanks showed induction of AhR with the lowest mean EU_{bio} of all tested samples in this assay (EU_{bio}(SPE blank) = 0.097) (Figure 1 A). All samples caused cytotoxicity with pronounced effects of the e-waste leachate (DC and UV) (SI Figure S4A).

AREc32. The AREc32 assay, responsive to many chemicals that cause oxidative stress,[39] was activated by all blanks (SPE and procedural blanks), though only with low EU_{bio} (EU_{bio} (SPE and procedural blanks) = 0.04−0.06, Figure 1B, Table S5, and SI Figure S8). The SPE water blanks were at the lower end of the procedural blank levels. All positive controls and the test polymers induced oxidative stress that was above the 95% confidence interval of the respective blanks. Highest effects could be observed for e-waste (EU_{bio} (EW_{DC}) = 0.69, EU_{bio} (EW_{UV}) = 1.11). With the exception of PET, all UV-treated samples showed generally higher EU_{bio} values than their corresponding dark controls. The most pronounced difference between the treatments could be observed for the keyboard (EU_{bio} (KB_{DC}) = 0.16, EU_{bio} (KB_{UV}) = 0.31) and PP (EU_{bio} (PP_{DC}) = 0.10, EU_{bio} (PP_{UV}) = 0.23) where effects for UV treatments were more than a factor two higher than the dark controls, and confidence bands did not overlap. No cytotoxicity was detected for the blanks (SI Figure S4B) even at the highest tested REF of 167 (SI Figure S8). For PP and PS, cytotoxicity could only be measured for UV-treated samples (SI Figure S4B).

PPARγ. Most strikingly, the UV-treated PE (EU_{bio} (PE_{UV}) = 0.50) showed induction levels of PPARγ comparable to the UV-treated positive control keyboard (EU_{bio} (KB_{UV}) = 0.53) (Figure 1C, Table S6, and SI Figure S9). For PE, the UV-treated samples showed a more than three times higher induction than their corresponding dark controls (EU_{bio} (PE_{DC}) = 0.15) which is the most pronounced difference between UV vs DC treatments in all tested assays. Only one SPE blank showed a low EU_{bio} (SPE blank) = 0.016. One dark control and two UV-treated procedural blanks showed activity of EU_{bio} ($blank_{DC}$) = 0.032 and EU_{bio} ($blank_{UV}$) = 0.031−0.073. No EC_{10} value could be determined for several samples of PP and PS. E-waste displayed the strongest activation of the PPARγ signaling pathway across all samples (EU_{bio} (EW_{DC}) = 0.63, EU_{bio} (EW_{UV}) = 1.70). The remaining test polymers resulted in EU_{bio} values that were in the upper range of the procedural blanks.

Analytics. No monocarboxylic acids could be detected with the LC-HRMS setup applied. Furthermore, not all dicarboxylic acid standards were ionizable and thus, only octanedioic acid, nonanedioic acid, decanedioic acid, undecanedioic acid, dodecanedioic acid and tetradecanedioic acid could be analyzed in the leachates (SI Table S3). Their MDLs and MQLs are listed in SI Table S3. Since no recovery experiments targeting these compounds were conducted, the given concentrations should be regarded as semiquantitative. Dicarboxylic acids could be detected above the MDL in the e-waste (UV and DC), the keyboard (DC), PE (UV and DC), PET (DC), PP (DC and UV), and PS (DC and UV). They were quantifiable only in the e-waste (DC and UV), PE (DC and UV), PP (DC), and PS (UV) leachates with dodecanedioic and tetradecanedioic acid as the most frequently quantified dicarboxylic acids (SI Table S3). PE showed the highest concentration of tetradecanedioic acid with differences between DC and UV treatment up to a factor three (PE_{DC} = 0.47−0.60 μM, PE_{UV} = 1.34−1.39 μM).

Mono- and Dicarboxylic Acids. The investigated mono- and dicarboxylic acids were inactive in the AREc32 assay (SI Figure S11) and the AREc32 was therefore not further considered. With increasing chain length (i.e., molecular weight M), the carboxylic acids showed linearly increasing $EU_{bio(i)}$ (decreasing $EC_{10(i)}$) in the PPARγ signaling pathway (Figures 2 and S12). However, a slope that is statistically significantly different from zero was only observed for the monocarboxylic acids (F = 15.39, df = 8, R^2 = 0.66, p < 0.01). The monocarboxylic acids resulted in $EU_{bio(i)}$ values of one and two orders of magnitude higher than the corresponding dicarboxylic acids (SI Table S7). The slope of the dicarboxylic acids was mainly driven by the high $EU_{bio(C14di)}$ of tetradecanedioic acid (M = 258.4 g/mol) to induce PPARγ while short-chained dicarboxylic acids showed low activation of PPARγ (Figure 2).

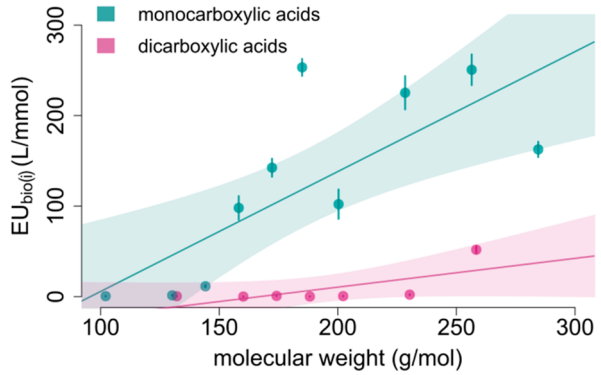

Figure 2. Effect units ($EC_{bio(i)}$ = $1/EC_{10(i)}$) elicited in the PPARγ assay by mono- and dicarboxylic acids of increasing chain length (C5, C7−C12, C14, C16, C18). Monocarboxylic acids showed a significant correlation between molecular weight and target activation (R^2 = 0.66, p < 0.01), which was nonsignificant for the dicarboxylic acids (solid line represents the respective regression line with the shaded area as the 95% confidence band).

Iceberg Modeling. The $EU_{chem(i)}$ values, derived from single compound testing in PPARγ and the respective measured concentrations were summed up in a mixture model based on concentration addition (eq 6) (SI TableS3). They accounted for up to 42% of the observed EU_{bio} values in the case of PE as indicated by proximity of these samples to the 1:1 line (Figure 3). The positive controls showed high EU_{bio} but associated low EU_{chem} which located them more distant from the 1:1 line. The smallest percentage of effects explained by the iceberg model with simultaneous frequent detection of dicarboxylic acids could be observed for the e-waste and keyboard with partly under 1% and 2%, respectively. The concentrations of tetradecanedioic acid increased linearly with increasing EU_{bio} (SI Figure S5).

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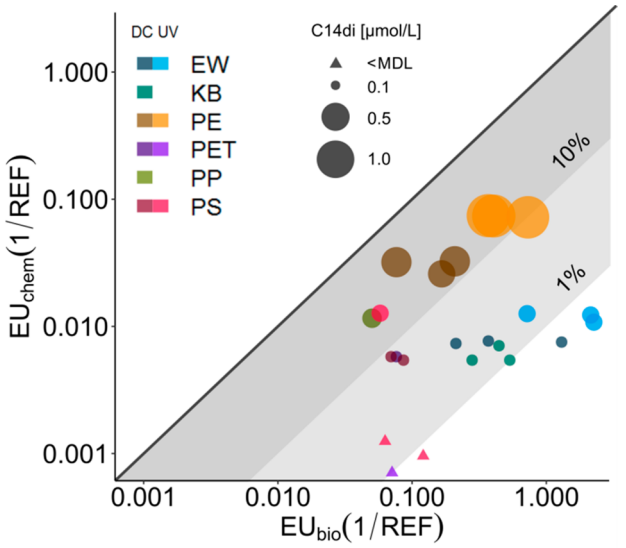

Figure 3. EUs derived from chemical analysis and single compound testing (EU_{chem}) plotted against the bioanalytical effect units (EU_{bio}) for the PPARγ assay (log scale). The 1:1 line indicates that 100%, dark and light shaded area that 10% and 1%, respectively, of the observed effect can be attributed to the analytically determined chemicals. Colors represent the different samples each as the dark control (DC, darker shading) and the UV treatment (UV, lighter shading). The bubble size corresponds to the relative concentration of tetradecanedioic acid, the main driver of the mixture effect. Triangles represent data where tetradecanedioic acid was < MDL.

DISCUSSION

AhR. The low EU_{bio} values of the SPE blanks suggest a limited effect of the sample processing (enrichment, elution, and concentration) on the induction of AhR since two out of three replicates were located at the lower end of the procedural blanks. The induction of AhR by the plastic-free blanks may stem from impurities in the ASW or from the UV-weathering experiment (Figure 1A). According to the manufacturer, the investigated preproduction resin pellets were largely additive-free. A nonsystematic search for typical additives in the full scan data showed the presence of selected additives above blank level only for the e-waste and the keyboard. Therefore, the test polymers were expected to result in low induction in this assay, which was confirmed by their EU_{bio} values that were in the range of the confidence intervals of the corresponding blanks. Potential plastic additives such as brominated flame retardants[43] are known AhR inducers,[44] however, these are often added during processing and not as primary ingredient[12] and hence were likely absent in the leachates of our test polymers. The low induction of the xenobiotic metabolism by our test polymers may further indicate the absence of the most prominent phenols and plasticizers used as monomers and additives in synthetic polymers such as BPA, *n*-nonylphenol and diethylhexyl phthalate (DEHP) since these are known agonists of the AhR.[45,46]

Most importantly, the positive controls e-waste and keyboard provided a proof-of-concept that our test system was capable of detecting effects of chemicals liberated from (weathering) plastic in cell-based bioassays. The concentration of BPA in the e-waste that was tested here was 188 ± 125 mg_{BPA}/kg.[25] If we assume chemical equilibrium between the e-waste and the ASW (Section S5. Mass balance model and eq S3), we can apply a simple two-phase mass-balance model (eqs S3 and S6) to estimate the aqueous leachate concentration of BPA as 2.0 ± 1.3 µmol/L. This rough estimation is only a factor two higher than the BPA leachate water concentrations measured at Norwegian landfills of up to 0.9 µmol/L,[25] demonstrating the environmental relevance of our positive control as a worst case scenario of plastic leachates. Applying the iceberg model for a single compound (here BPA) (eq 5) we can derive an $EU_{chem(i)}$ value of 0.02 (AhR $EC_{10(BPA)}$ = 0.1 mM) which corresponds to a marginal effect contribution of BPA for e-$waste_{DC}$ between 1.3 and 2.3% and e-$waste_{UV}$ with 0.7−1.2%. That means that the mixture effect of many other chemicals in the e-waste account for the observed biological response. Several brominated flame retardants were also reported in this e-waste sample[26] that could have contributed to the observed effects. An important fact throughout all assays is that e-waste showed high EU_{bio} values of around 1 or sometimes higher which means that the experimentally generated leachate water was diluted for the targeted effect range while sample EU_{bio} values below 1 indicate enrichment.

In contrast to our AhR results, Coffin et al. (2018)[22] detected, although statistically not significantly, higher AhR induction by leachates from UV-irradiated consumer plastic than for untreated consumer plastic. Their observations were supported by chemical analyses that suggested enhanced desorption of AhR-active substances such as PCBs and BPA due to the UV treatment.[22]

AREc32. The EU_{bio} values of the SPE blanks were in the lower range of the procedural blanks (DC and UV), indicating, similarly to the AhR results, a certain background effect of the ASW and the experimental procedure on the induction of AREc32 (Figure 1 B). In return, it could also mean that the SPE processing may already introduce AREc32-activating substances either from the LC grade water, the SPE cartridges or the processing itself. Previous studies reported similar $EC_{IR1.5}$ values for solid-phase extracted ultrapure water samples of REF > 20 (EU_{bio} < 0.05) in the AREc32 assay.[32] Our presumably low background contamination is supported by the absence of cytotoxic effects for all blanks (Figure S4B).

Substances that stem from degrading plastic may potentially have caused the oxidative stress response in this assay. The apparent influence of the UV treatment on the ARE induction may indicate that substances were liberated at higher levels from the test polymers and the positive controls during artificial UV irradiation than from the dark controls. This UV-dependent effect is in accordance with a leaching study by Bandow et al. (2017)[47] that detected more explicit leaching of both inorganic and organic compounds in UV-A-irradiated than in merely thermo-oxidized polymer samples.[47] Small reactive molecules have the capacity to induce oxidative stress.[48] Gewert et al. (2018)[24] tentatively identified low-molecular weight fragments with oxidized end groups as degradation products of PE, PET, PP, and PS, applying the identical UV aging setup used here. We hypothesize that these degradation products may potentially be responsible for the observed oxidative stress response. There exist mechanisms for potential cross-talk between the AhR and ARE signaling pathway,[49] however, bifunctional inducers such as certain dioxins or PAHs that are capable of simultaneous activation[50] were probably absent in our test polymer leachates since AREc32 was clearly activated but not AhR. Interestingly, the induction of AREc32 of DC samples indicates that even under dark conditions chemicals that cause oxidative stress are liberated from the test polymers.

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PPARγ. On the one hand, the few measurable EC_{10} values of the PPARγ blanks led to some uncertainty when comparing our test polymers to the blanks as done previously for AhR and AREc32. On the other hand, the low detection frequency of the blanks stands for low background contamination of our method and increases the robustness of the response caused by the investigated polymers. The polymers PE (DC only), PET, PP, and PS showed induction ratios comparable to the upper range of the blanks and hence did not allow us to distinguish between samples and blanks. Contrarily, the explicit induction of the UV-treated PE may indicate the presence of degradation products that are capable of specifically activating PPARγ. Candidates are dicarboxylic acids that were previously identified as chain scission products of degrading PE by Gewert et al. (2018).[24] Since fatty acids (FAs) are natural ligands of the PPARγ[51] it is likely that the dicarboxylic acids show similar activity toward this receptor. It is also conceivable that the induction of PPARγ occurred by other known plastic-associated agonists like DEHP.[52] Lovekamp-Swan and Davis (2003)[53] hypothesized that the active metabolite of DEHP, monoethylhexyl phthalate, activates the PPARγ. The fact that the migration potential of phthalates from a PE-based end consumer product (i.e., a shopping bag) was marginally affected by artificial UV exposure[54] renders this class of substances less plausible candidates for the observed induction of PPARγ. Furthermore, it is unlikely that plasticizers like DEHP were added to the PE virgin pellets, as these are typically added to melted virgin pellets during molding.[55]

Mono- and Dicarboxylic Acids. FA derivatives are known ligands of the PPAR family.[51,56−60] Our observed positive correlation between FAs of different carbon chain lengths and their potential to activate PPARγ is supported by observations by Wolf et al. (2008)[61] who described a similarly increasing induction capacity of perfluoroalkyl acids toward PPARα with increasing carbon chain length, up to C9. It should be noted that FAs can act as ligands for all three subtypes α, δ, and γ[62] with PPARγ showing the most restricted FA binding profile.[59]

The effect correlation with carbon chain length and the discrepancy between mono- and dicarboxylic acids may mainly be driven by toxicokinetic processes since (1) a linear relationship between lipid permeability and carbon chain length was observed for monocarboxylic acids,[63] (2) dicarboxylic acids have shown lower abilities to permeate lipid bilayer membranes than their corresponding monocarboxylic acid,[64] and (3) long-chain FAs may be more resistant to metabolism.[65] A toxicodynamic explanation for our observations may be a more effective activation of PPARγ by long-chain FAs.[66] Similar to our finding, Intrasuksri et al. (1988)[65] detected higher PPAR induction potency for FAs in decreasing order from oleic acid (C16) > octanoic acid > octanedioic acid. The neutral form can only passively permeate through the membrane which is impermeable for the anionic form of the fatty acids.[67]

Iceberg Modeling. For the iceberg modeling, we need to consider the analytical results. The frequent but low detected quantities of dicarboxylic acids of different carbon chain lengths throughout the blanks may be regarded as background contamination of unknown source in our experimental setup or the laboratory itself (SI Table S3). We accounted for this background by setting the calculated MDLs and MQLs as quality criteria. While the dicarboxylic acids found in the e-waste and keyboard may stem from impurities and additives, their presence in the PS remain unexplained.

Applying the iceberg model, the observed effects in the PPARγ bioassay (EU_{bio}) were partly explained by the mixture effects of the quantified polymer degradation products, the dicarboxylic acids, present in the leachates. Tetradecanedioic acid was the main mixture risk driver of the detected PPARγ induction of the PE extracts, due to three reasons: (1) It was the most potent PPARγ inducer among the dicarboxylic acids in the single compound testing (Figure 2), (2) it was detected at high quantities exclusively in the PE samples with a factor of around three higher liberation for the UV treatment than for the dark control (Table S3), which is (3) in accordance with the observed bioanalytical effects of the related extracts causing three times higher effects as well (Figures 3, and S5).

For PE, the substantial contribution of EU_{chem} to EU_{bio} of, in some cases, over 40% (Figure 3) is an important explanatory parameter for the PPARγ gene pathway activation. Since Albertsson et al. (1995)[68] identified over 60 PE degradation products, predominantly monocarboxylic acids, we expect those to be present in our PE leachates as well, although they remained undetected by our analytical method. Presumptively, they were not ionizable by our method since derivatization is often a prerequisite for chemical analysis of FAs.[69] It is hence very likely that the identified compounds did not cause the effect alone, but that the mixture effect of all chemicals that are present in the leachate is relevant.[28] It should be noted that the identified dicarboxylic acids, as potential products of UV-weathered PE, could account for a certain effect contribution in PPARy. They could not explain the observed induction of AREc32. We observed higher induction of AREc32 by the UV-treated samples (Figure 1B), still, the mono- and dicarboxylic acids, were largely inactive when tested as single compounds in the AREc32 assay (SI Figure S11). As a consequence, unknown substances might be responsible for the effects in AREc32 which is supported by UV-independent induction of AREc32 by the dark treated samples. The distance of e-waste and keyboard from the 1:1 line in the lower 1% area (Figure 3) indicates that unknown compounds accounted for a larger fraction of sometimes over 99% of the observed effects.

Implications. This study investigated the influence of UV-induced weathering on the liberation of unknown chemical mixtures from largely additive-free preproduction pellets and their effects in cell-based bioassays, addressing a range of cellular response pathways. Compared to measured concentrations of plastic debris in an urban river of up to 0.121 g/L[70] our applied plastic mass concentration for the leaching experiment was 250 g/L. In many cases our UV-treated positive control e-waste resulted in EU_{bio} values >1 (1/REF). That means that the generated leachate water tested in the bioassays was diluted to target the observed effect range. Accounting for this dilution our observed effects were at concentrations of two to three orders of magnitude above high-end plastic concentrations in the environment. Our intention was to reflect the extreme case, to aim for measurable effects. Still, we could address environmental concentrations in the case of the e-waste for which environmental leachate water concentrations showed high levels of contaminants[25,26] as demonstrated for BPA. Under environmental conditions, substances leaching from plastic material may undergo transformation or microbial degradation. These processes will impact their fate and ecotoxicological relevance, but were not subject of this study. Generally, the observed effects of our test polymers were in the lower range compared to our contaminated positive controls. Therefore, future studies

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should focus on more realistic end consumer products, usually containing additives, and their relevance for the aquatic environment to act as a source of leaching and degrading compounds potentially of concern.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b02400.

Additional information includes details on (S1) the experimental setup, (S2) the SPE protocol, (S3) instrumental analysis, (S4) the bioanalytical equivalents, and (S5) the mass balance model; (Tables S1−S7) specifications on the mono- and dicarboxylic acids, the bioanalytical test battery, all results of the assays AhR, AREc32, PPARγ, the concentrations of dicarboxylic acids detected in the leachates, and the test results of mono- and dicarboxylic acids for PPARγ; (Figures S1−S5) the test material, the weathering setup, the bioanalytical effect units measured in ERα and bioanalytical toxic units for all assays, and the correlation of tetradecanedioic acid and the EU_{bio} of PPARγ; (Figures S6−S12) the concentration−response curves for the reference compounds of the different assays, for the leachates tested in AhR, AREc32, PPARγ, and ERα and the carboxylic acids tested in AREc32 and PPARγ (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49 341 235 1512; e-mail: christoph.rummel@ufz.de.

ORCID

Christoph D. Rummel: 0000-0001-5829-3908
Beate I. Escher: 0000-0002-5304-706X
Merle M. Plassmann: 0000-0003-3042-187X
Matthew MacLeod: 0000-0003-2562-7339

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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Publication IV

Impacts of Biofilm Formation on the Fate and Potential Effects of Microplastic in the Aquatic Environment

Christoph D. Rummel[†]*, Annika Jahnke[‡], Elena Gorokhova[§], Dana Kühnel[†] and Mechthild Schmitt-Jansen[†]

† Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research-UFZ, Permoserstr. 15, DE-04318 Leipzig, Germany

‡ Department of Cell Toxicology, Helmholtz Centre for Environmental Research-UFZ, Permoserstr. 15, DE-04318 Leipzig, Germany

§ Department of Environmental Science & Analytical Chemistry (ACES), Stockholm University, Svante Arrhenius väg 8, SE-106 91 Stockholm, Sweden

* corresponding Author: christoph.rummel@ufz.de

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Impacts of Biofilm Formation on the Fate and Potential Effects of Microplastic in the Aquatic Environment

Christoph D. Rummel,*[,†] Annika Jahnke,[‡] Elena Gorokhova,[§] Dana Kühnel,[†] and Mechthild Schmitt-Jansen*[,†]

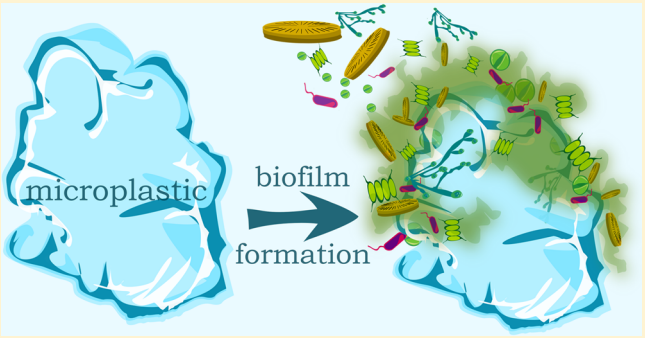
[†]Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research-UFZ, Permoserstrasse 15, 04318 Leipzig, Germany

[‡]Department of Cell Toxicology, Helmholtz Centre for Environmental Research-UFZ, Permoserstrasse 15, 04318 Leipzig, Germany

[§]Department of Environmental Science & Analytical Chemistry (ACES), Stockholm University, Svante Arrhenius väg 8, 106 91 Stockholm, Sweden

Supporting Information

ABSTRACT: In the aquatic environment, microplastic (MP; <5 mm) is a cause of concern because of its persistence and potential adverse effects on biota. Studies of microlitter impacts are mostly based on virgin and spherical polymer particles as model MP. However, in pelagic and benthic environments, surfaces are always colonized by microorganisms forming so-called biofilms. The influence of such biofilms on the fate and potential effects of MP is not understood well. Here, we review the physical interactions of early microbial colonization on plastic surfaces and their reciprocal influence on the weathering processes and vertical transport as well as sorption and release of contaminants by MP. Possible ecological consequences of biofilm formation on MP, such as trophic transfer of MP particles and potential adverse effects of MP, are virtually unknown. However, evidence is accumulating that the biofilm−plastic interactions have the capacity to influence the fate and impacts of MP by modifying the physical properties of the particles. There is an urgent research need to better understand these interactions and increase the ecological relevance of current laboratory testing by simulating field conditions in which microbial life is a key driver of biogeochemical processes.



INTRODUCTION

In the aquatic environment, plastic litter has emerged as a major pollution issue, because it is only slowly degradable,[1,2] is ubiquitously present in our rivers and seas,[3,4] may represent a hazard to wildlife,[5] and may be a potential planetary boundary threat.[6,7] Current investigations of the fate of marine plastic debris include various surveys that aim to develop an understanding of its distribution from beaches and shorelines to remote islands or the great ocean gyres,[8] as well as downward transport, from the sea surface through the water column[9] to bottom sediments.[10] Also, plastic contamination in freshwaters is currently gaining attention.[11]

Apart from the aesthetical issues of littering, adverse effects on wildlife are obvious for large plastic debris, i.e., macroplastic (>5 mm).[12,13] During its residence in the environment, large plastic debris becomes brittle and undergoes fragmentation due to weathering forces generating so-called microplastic (MP; <5 mm).[14,15] While large plastic debris may have adverse effects on fish, birds, and other top consumers in aquatic environments,[5,13] the size of the MP makes it suitable for ingestion by smaller organisms at lower trophic levels.[16] Although no studies have so far reported any ecologically plausible adverse effects of MP on primary consumers, we know very little about the interactions between these particles and their potential consumers. One of the shortcomings in our current experimental and modeling studies of MP is the missing link of the effects of biofilms on the particle behavior in biological, chemical, and physical interactions. The fate and effects of MP mainly have been investigated in laboratory experiments, using virgin spherical particles with a uniform size distribution. However, environmental MP is characterized by heterogeneous sizes and shapes[17,18] that change with aging.[6,19] Moreover, they are mixed with natural suspended particles that may affect biofilm formation. These parameters should be included in study designs to create more realistic conditions of these mixtures and their exposure.[20] Additionally, particle properties, including those of MP (such as topography or roughness,

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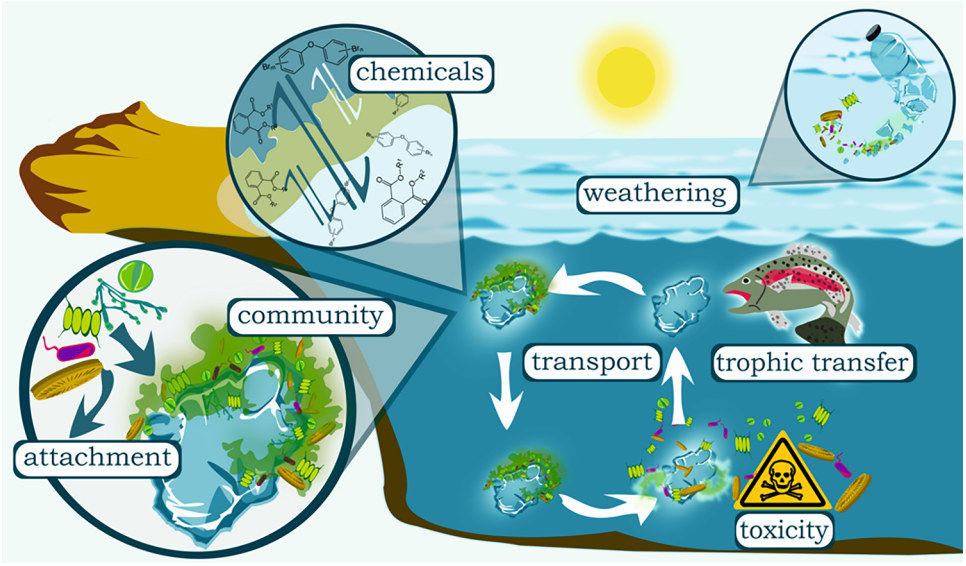

Figure 1. Key processes of the fate and potential effects of MP in the aquatic environment that are modified by biofilm formation. Biofilms on submerged surfaces are the result of selective attachment of microorganisms, facilitation, and interspecific competition in the microbial communities. Weathering processes may favor biofilm growth because of increased surface areas available for settling, which in turn may shield plastic debris from ultraviolet light. However, biofilms have the capacity to biodegrade the polymer. In addition, vertical transport and the uptake and release of plastic-associated chemicals are influenced by biofilm formation on plastic debris. Biological implications of biofilm formation include effects on trophic transfer of MP and associated contaminants, community structure of microbial assemblages, and potential toxicity to grazers.

surface charge, surface area, overall density, and many more), will inevitably change when a biofilm forms on the surface.

Upon release of a plastic item to the aquatic environment, a coating layer of inorganic and organic substances is instantaneously formed.[21] The subsequent formation of a biofilm on its surface is likely the first interaction with ambient biota, taking place within minutes to hours.[22] Biofilms are phylogenetically and functionally diverse communities of bacteria, algae, protozoans, and fungi collectively termed a microbial assemblage, biofouling community, or periphyton. These microorganisms live in spatial proximity of each other on any submerged surface mostly embedded in extracellular polymeric substance (EPS).[23] Life in a biofilm offers a variety of advantages for competition and survival strategies, including possibilities for forming stable consortia, horizontal gene exchange, accumulation of nutrients, and protection against toxic substances and desiccation.[24]

Here, we summarize the different aspects and specific future research needs of the influence of biofilm formation on plastic debris and its potential impact on the fate and effects of MP in the environment. Our specific focus is the physical effect of biofilm formation on the fate of MP and the resulting consequences for biological interactions. We specifically identify current gaps in our knowledge of the early interactions of plastic and biofilm-forming microorganisms and their reciprocal influence on weathering processes, the vertical transport of fouling MP particles, and the potential of biofilms to modify plastic-associated fluxes of chemicals. Biological aspects address trophic transfer, the community structure of the so-called "plastisphere",[25] and potential adverse effects of MP. A detailed description of the systematic literature research by means of the ISI Web of Science performed as a first step in this review is provided as Supporting Information. This query led to insufficient results (see Table S1) because of the virtual lack of studies of the investigated topic. However, several neighboring disciplines like material and medical science, nanotechnology, and food technology provide valuable insights into surface−biofilm interactions. Using the systematic literature research and cross-referencing from these disciplines, we propose some priority areas and important questions for investigating the impacts that microbial colonization may have on plastic debris in the aquatic environment.

BIOFILMS ON PLASTIC SURFACES AND THEIR PHYSICOCHEMICAL IMPLICATIONS

Attachment to New Habitable Surfaces. To elucidate the complex interactions between biofilm-forming microorganisms and surfaces available for colonization, we need to understand the attachment processes acting on macro- and microplastic (Figure 1, "attachment"). Within seconds of the first contact between ambient water and a virgin surface, a conditioning layer or film of organic and inorganic substances is formed by adsorption.[21] Microorganisms come into contact with surfaces by repulsive and attractive interactions among the surface, their cell wall, and the medium. The initial conditioning film may have the capacity to govern the colonizing community by modifying the material-specific surface properties.[26−29] The phenomenon of sorbed molecules driving the behavior of particles in fluids was just recently compared to the absorbsome[30] and the so-called eco-corona[31] of nanomaterials in a review by Galloway et al.[7] A key point of these concepts is the rapid establishment of a coating layer consisting of proteins and other biomolecules around nanoparticles in biological fluids such as serum and cytoplasm that affects the physicochemical interaction of the nanomaterials with cells and tissues.[7] Lorite et al.[32] concluded that the chemical nature of the aforementioned conditioning film appears to be more relevant for settlement of organisms than surface roughness or hydrophobicity of the initial substrate surface, which highlights the importance of this very first sorption process. The investigation of the conditioning film on MP and its close link to the concept of the eco-corona seems to be a promising

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field for future research. The universal mechanism of surfaces absorbing molecules may have far-reaching biological consequences because it is the particle's "biologically relevant entity".[33]

From a material perspective, the surface roughness,[34] topography,[35] surface free energy,[36] surface charge, electrostatic interactions,[37,38] and surface hydrophobicity[39] are generally known to be relevant parameters for the attachment process. However, Hook et al.[40] concluded that on the basis of their experiments wettability (or analogously surface hydrophobicity) and polymer topography did not affect the attachment of bacteria to synthetic polymer substrates. On the contrary, Sanni et al.[41] suggested a strong correlation of bacterial settlement and a parameter combining hydrophobicity and molecular flexibility in the specific case of poly(meth)acrylates.

Observing colonization of submerged plastic bags, Nauendorf et al.[42] suggested that surface wettability was probably of minor importance for bacterial attachment compared to surface roughness. Comparative investigations of biofilm succession on polymeric materials and other substrates suggest that the abundance of bacteria on hydrophilic stainless steel, hydrophobic polyvinyl chloride (PVC), and polyethylene (PE) was similar after colonization for 167 days.[43] Although often termed "inert", synthetic polymers exhibit important differences compared to other materials because the amount and composition of additives (chemicals that are intentionally added during manufacturing to improve the material's performance) in the polymer can also affect the species composition of organisms colonizing the surface.[44] In contrast to the work of Pedersen,[43] that of Rogers et al.[45] detected higher bacterial numbers on PE and PVC than on stainless steel during biofilm formation, which they attributed to leaching of additives as a potential nutrient source. Although of high value for our current understanding of biofilms colonizing MP, ongoing research is often observation-based[46−48] rather than mechanistically driven. However, understanding of the underlying mechanisms for eco-corona and biofilm formation and composition is crucial for predicting the behavior and fate of MP in various environmental settings. In summary, plastic materials represent a relatively recent anthropogenic substrate in aquatic ecosystems that can readily be colonized by biofilm-forming organisms. Although many studies have shown that microorganisms attach more rapidly to hydrophobic, nonpolar surfaces (such as plastics) than to hydrophilic surfaces (such as stainless steel),[23] general conclusions about the relative importance of specific mechanisms are difficult to draw, particularly for *in situ* studies. Even in controlled laboratory experiments, physicochemical properties differ between polymer types with varying monomer subunits and copolymers, differing by functional groups and additives. Plastic may also be manufactured as a composite material, further widening the range of (surface) properties. The effects of physicochemical properties driving the early attachment processes have comprehensively been reviewed by Renner and Weibel[38] and Cazzaniga et al.[49]

In response to diverse habitats and ecological requirements, microorganisms have evolved a plethora of attachment mechanisms.[50] Organism−substrate interactions have led to numerous adaptation strategies; for example, surface charge[51] and hydrophobicity of the cell walls and membranes can be adjusted[52] by forming surface structures, such as pili, curli, fimbriae,[53,54] and flagella,[55] and by regulating EPS production,[56] all of which may improve adhesion to a habitable surface. Once the coating and the first colonists of a biofilm are in place, the initial surface properties of the material are modified, which may facilitate colonization for other organisms, as demonstrated by Lobelle and Cunliffe,[57] who observed a decrease in surface hydrophobicity on submerged PE during a 3 week incubation in sea water. Additionally, environmental factors such as ionic strength, temperature, and pH may influence the attachment.[58]

Although the early formation of a biofilm on surfaces has been under scientific investigation for decades,[22,59,60] general conclusions about the underlying physicochemical processes governing early attachment of microorganisms are difficult to draw because a plethora of materials and organisms with different properties exists. As a result of the interactions between substrates and organisms mentioned above, a diverse microbial community colonizes every submerged surface.

Weathering. Among others, the fate of plastic debris in the aquatic environment is governed by weathering processes because these have significant consequences for the condition of the material and its hydrodynamic behavior[61] (Figure 1, "weathering"). Weathering describes the loss of the physical integrity of the material by abiotic and biotic influencing factors and related degradation of the material. For plastic debris, we need to consider several pathways separately, although they usually act in concert. Preceding the biological attack, photooxidation is the most common abiotic degradation pathway, at least for debris exposed to sunlight. Photooxidation may be divided into three main steps: initiation [polymer-chain scission induced by ultraviolet (UV) light and formation of free radicals], propagation (autoxidation), and termination (formation of inert products). The degradation mainly acts on the material surface that is exposed to UV light. As a result, the weathered surfaces may display a modified topography, an increase in surface roughness, and changed chemistry (e.g., becoming more polar because of the formation of carbonyl groups).[62−65] These processes may favour the adhesion of microorganisms,[23] carrying capacity of MP toward biofilm mass and, ultimately, the composition and structure of the microbial communities.[35,49] In addition, successive fragmentation into smaller particles[14,66] with a high surface-to-volume ratio is an important prerequisite for biodegradation. Over time, the surface area of plastic available for colonization by microbes increases,[67] escalating the contribution of biodegradation, changing the particle density, buoyancy, and sinking rate. However, biofilm formation may also influence abiotic aging processes, e.g., by shielding the floating plastic from UV light in the upper water layers[14] or by changing a particle's vertical position in the water column.[68,69] As a result, the exposure to light, shear stress, oxygen, and temperature will be influenced.

In addition to the effect of physical aging caused by abiotic factors, polymers are subject to biological degradation.[25,70,71] While the term biodeterioration refers to a loss of physical integrity, biodegradation encompasses the process of chemical breakdown.[72] Flemming[24] summarized the main biofilm-related processes acting on the aging of synthetic polymers, namely, (i) biofouling, (ii) degradation of plasticizers, (iii) attack on the polymer backbone, (iv) hydration, and (v) penetration of organisms into the polymer structure (e.g., fungal hyphae). Synthetic homopolymers containing C−C bonds in their polymer backbone are least susceptible to biodegradation.[72] During biodegradation, exoenzymes are released by the colonizing organisms and cause the breakdown of the polymer, finally yielding short-chain fragments, such as oligomers,

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dimers, or monomers.[73] These may then pass the cell membrane, become a carbon source, and be mineralized to CO_2, H_2O, and CH_4.[73] Comprehensive reviews of the mechanisms of biological polymer degradation are provided by Shah et al.,[73] Restrepo-Flórez et al.,[44] and Gu.[74]

Essentially unknown are the kinetics of fragmentation and the resulting emission of particles and their size distribution caused by microorganisms in the environment.[67] This is of particular relevance for the fraction of plastic that sinks to the benthos[10,14] and no longer undergoes UV-driven degradation in the euphotic zone. Thereby, high microbial activity in eutrophic waters may increase MP loads in the sediments and may promote its final removal by mineralization both in the water column and in sediments, particularly when bottoms are not hypoxic. There may be a mechanistic trade-off in the fragmentation rate due to biofilms attenuating abiotic weathering on one hand (by shielding from UV light and sinking) and causing biological breakdown on the other (biodegradation). To overcome the current lack of quantitative estimates of the importance of weathering processes for plastic debris,[6] we need to understand the changes in physical and chemical properties due to biofilm formation and thereby driving forces behind the vertical transport of MP.

Vertical Transport. From a hydrodynamic perspective, biofilm formation on plastic debris may have substantial implications. First, the fouling organisms may lead to an increase in the density of the particle and a decrease in its buoyancy.[75] The smaller the particle, the faster it can reach its critical sinking density.[76,77] Since the sinking rate is a function of particle size and density, an increase in density above that of ambient water (1.025 g/cm^3 for sea water and 1.000 g/cm^3 for fresh water) implies sedimentation[68] (Figure 1, "transport"). However, the buoyancy of particles that originally had a higher density than water may increase as a result of biofouling, rendering MP susceptible to upward transport. Furthermore, during biofilm formation, MP becomes sticky because of the EPS matrix, which promotes the formation of heteroaggregates, including MP, microbial communities, and detritus.[68] The formation of such heteroaggregates may affect sedimentation rates of algal blooms and associated microorganisms. For example, Long et al.[68] demonstrated that heavy and fast-sinking diatom aggregates displayed substantially decreased sinking rates when low-density microbeads were incorporated, whereas sinking rates of light cryptophyte cells associated with aggregates increased. Furthermore, possible preferential ingestion of MP with well-developed biofilms (see Trophic Transfer) may promote downward transport of MP particles incorporated into fecal pellets of zooplankton.[78,79] However, benthic sediments do not necessarily present an ultimate sink for plastic debris. The extent of biofouling of plastic debris may decrease because of the removal and/or digestion by benthic animals; hence, MP may regain buoyancy, leading to submerging−resurfacing cycles[80] (Figure 1, "transport").

In a recent modeling study of riverine transport of MP, Besseling et al.[81] concluded that biofilm modeled as a 0.4 μm thick monolayer of bacterial cells (1.250 g/cm^3) would introduce no changes into the overall qualitative trends and patterns in particle behavior. In this model, data on attachment efficiencies of biofilm-coated MP particles were derived from the experiments performed by Xiao and Wiesner,[82] who measured an increase in the affinity of engineered nanoparticles for porous media in the presence of biofilms. These experiments demonstrated the affected hydrodynamic behavior of the investigated nanoparticles in the presence of biofilms that may have similar consequences for transport and the ultimate fate of MP in the aquatic environment. The growth of biofilm-forming organisms largely depends on environmental factors, such as light and temperature, as well as on the trophic state of the waters.[83,84] The dynamics of the transport pathways as a function of seasonality, climate effects, and the trophic state of aquatic systems should be addressed if we are to understand and model the distribution of MP in different ecosystem compartments.

Transport of Plastic-Associated Pollutants through Biofilms. The transport of hydrophobic organic contaminants (HOCs) between plastic debris and water may be affected by biofilms because of its sorptive properties on one hand and its ability to metabolize HOCs on the other[85−88] (Figure 1, "chemicals"). In addition to the additives of (recently emitted) plastic debris mentioned above, highly persistent contaminants may be accumulated by plastic from its immediate environment and the plastic may subsequently transport and release them during residence at sea.[89−91] The chemical loads of these contaminants in MP may be enriched up to 10^6-fold compared to those of the surrounding sea water[92] and depend on the polymer/water partition ratios that can be approximated by the octanol/water partition ratios (K_{OW}).[93] Since the capacity of synthetic polymers to sorb HOCs is highly relevant for the environmental risk assessment of MP, we face the question of whether thermodynamic and kinetic processes will be influenced by biofilms, representing a superficial organic phase consisting of water, lipids, and proteins acting as both a potential sorptive phase[94] and a barrier for diffusive uptake and release of chemicals. Furthermore, EPS represents a diverse biological matrix containing polysaccharides, proteins, lipids, and other biopolymers such as humic acids,[95,96] which may contribute to the sorptive capacity of the biofilm-coated MP[97] and heteroaggregates. Humic acids are known to compete for sorptive sites and hence have the potential to attenuate the sorption of PCBs as shown for charcoal.[98] Analogous to the partitioning of HOCs into MP, synthetic polymers, such as PE, are frequently used in the field of environmental chemistry as so-called passive samplers because of their high capacity for sorbing HOCs.[99] The passive samplers are intentionally deployed in the field to sample environmental contaminants and subsequently solvent-extracted and measured in the laboratory. However, biofilm coatings can bias passive sampling rates in the field by increasing the resistance for mass transfer into and out of the polymer[100] as suggested by different sampling rates in fouled and nonfouled sampling devices.[101] In laboratory studies of the kinetics of sorption of HOCs into MP, the influence of biofilms has largely been disregarded,[90,102] despite the observed effects on kinetics in the passive samplers. Diffusion coefficients decreased by ∼4 orders of magnitude upon inclusion of a microbial biofilm during sorption of HOCs to glass beads,[103] which also emphasizes the importance of the biofilm acting as a barrier.

The release of additives may even promote microbial growth by serving as a nutrient source.[72,104] A wide range of bacteria, fungi, and algae are capable of degrading HOCs,[105] which is why they can be used, e.g., for bioremediation of surface waters *in situ* or as engineered bioreactors.[106,107] This demonstrates the high relevance of biofilms for the accumulation and/or removal via metabolization of plastic-associated chemicals,[87] which may affect their bioavailability for consumers ingesting MP. Another concern is the addition of antimicrobial agents to

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polymer materials by manufacturers to hamper microbial settlement;[108] these substances may leach and promote the spread of resistance adaptations in microbial communities.[109]

In summary, sorptive processes may lead to faster uptake and release of chemicals in MP compared to macroplastic litter due to higher surface-to-volume ratios. At the same time, however, colonization by microorganisms is facilitated because of the enlarged and weathered surfaces available for colonization that can influence the kinetics and persistence of HOCs. These two-way interactions can influence the kinetics of uptake and release of contaminants into and from the polymeric bulk phase through the active microbial interface need to be considered further to predict a more realistic scenario for risk assessment of MP being a transport and emission source of HOCs in the aquatic environment.

BIOLOGICAL EFFECTS

Community Structure. Since environmental factors and material and organismal surface properties govern the attachment of organisms, any community inhabiting a submerged surface is a result of selection processes. Microbiologists are currently investigating communities present on MP surfaces and the underlying factors that determine the community structure and succession patterns (Figure 1, "community"). Zettler et al.[25] introduced the term "plastisphere", implying that plastic-associated communities are distinct from the surrounding surface water. This assumption supports the view that plastic is a novel ecological habitat.[25,46,110,111] Studies using high-throughput sequencing showed that bacterial assemblages colonizing MP are taxonomically distinct and often less diverse than those in the water column, suspended organic matter or sediment.[110,112,113] Current studies, however, often lack a proper comparison to co-occurring natural substrates, both polymeric (e.g., cellulose, chitin, or lignin) and mineral (e.g., clay). Adequate particle controls are essential in field and experimental studies[20,114] that aim to address the specific effects of anthropogenic particles.

Although the composition of microbial communities on plastic surfaces may largely be influenced by geographical, spatial, and seasonal factors, an additional selection of a distinct community by the polymer substratum may occur.[115,116] Dang et al.[27] showed that the early microbial colonization is similar on plastic and glass surfaces during the first few days of succession. However, the lowest diatom diversity was observed on plastic, concrete, and rubber compared to that on the hydrophilic surface of iron plates and the seagrass *Posidonia oceanica*.[117] This observation indicates that plastic as a habitat may be less favorable for some species, such as diatoms, than other substrates. A recent study investigated the succession of microbial assemblages on PE in coastal sediments, suggesting a selection for specific bacterial taxa.[111] By contrast, Oberbeckmann et al.[118] concluded that the community structure on plastic surfaces is driven by conventional marine biofilm processes rather than selection of plastic-specific microbial colonizers. Interestingly, a different pattern of gene expression in microalgae grown on polypropylene and PE was demonstrated by Lagarde et al.,[75] indicating substrate-specific adaptations. The polymer-specific gene expression of sugar-synthesizing pathways may have important implications for the EPS production and subsequent formation of aggregates, which may result in a differential transport and fate of plastic particles.[75] Knowledge of the community structure and the underlying forces driving these assemblages at each succession stage will help us to elucidate the impact of plastic pollution on aquatic microbial load and diversity.[119] We need to integrate community structure and functions of the microbial communities on plastic debris because microbial activity is a crucial link between pollution as an anthropogenic pressure and the resilience of ecosystems.

Trophic Transfer. Most studies that have investigated the ingestion of MP by biota or transfer along artificial food chains used spherical, virgin MP particles and ignored the presence of biofilm under field conditions.[19,120] However, biofilm was found to facilitate trophic transfer of nanoparticles in marine systems,[121] which most likely also holds true for MP. Primary consumers may preferentially ingest particles of higher nutritional quality, such as MP carrying nutrient-rich biofilms.[122] This discrimination would be particularly pronounced in the selective feeders, such as copepods and shrimps, but also, at least to some extent, in passive feeders, such as cladocerans.[123−125] Biofilm may also increase the probability of MP adhering to the filtering apparatus in filter and suspension feeders, because neutral particles have been shown to be captured more readily than particles with a net negative charge.[126] Grazers, such as snails or copepods, may also ingest plastic fragments accidentally while feeding on the surface biofilm, as indicated by feeding marks observed on field-sampled plastic debris.[127] Zooplankton can actively explore patches of marine snow,[128] suggesting that potentially larger quantities of MP (and a broader size spectrum) incorporated into aggregates may be consumed compared to freely dispersed particles. Indeed, in suspension-feeding bivalves, enhanced uptake of 100 nm polystyrene beads embedded in marine aggregates was observed compared to that of the dispersed virgin particles.[121] Moreover, increased MP abundance may alter sedimentation rates of algal blooms, thus affecting the food supply for pelagic and benthic animals.[68] Campos et al.[129] reported nanoparticle-mediated flocculation and sedimentation of algal food resulting in a reduced rate of feeding in *Daphnia magna* under food-limiting conditions. This mechanism may potentially affect both pelagic feeders in the mixing layer and benthic communities because they may receive food of unusual quality and quantity. To conclude, biofilm formation and potential heteroaggregation may affect the uptake and susceptibility of organisms to ingesting MP by changing the physical properties and/or increasing the availability of MP particles. Biofilm coating has so far been disregarded in study designs but should be included in future studies to derive reliable uptake and ingestion rates in a more environmentally realistic scenario.

Toxicity and Adverse Effects. Because of their structural role as an interface between the overlying water and the sediments, biofilms are often used in ecotoxicology to evaluate the effect of chemicals in aquatic ecosystems.[130] In a recent study, flow cytometry was successfully applied for the detection of MP in ecological biofilms but no structural or toxicological effect was reported.[131] However, limited attention is paid to the direct adverse effects of plastic debris and the associated chemicals on the biofilms (Figure 1, "toxicity"). Potential effects may result either directly from physical and/or mechanical stress by the presence of solid particles (e.g., via adsorption of particles to the cell wall) or indirectly from plastic-associated chemicals leaching out of the polymer. Zhang et al.[132] revealed a negative effect of micrometer-sized PVC particles on the microalgae *Skeletonema costatum* only for the highest and environmentally unrealistic exposure concentration (50 mg/L).

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As they excluded shading effects by their experimental design, both physical adsorption and aggregation might have caused toxicity.[132] The available reports on suspended algae suggest effects of nanoplastic exposure on planktonic microalgae, such as inhibition of photosynthesis, promotion of reactive oxygen species,[133,134] growth inhibition, and reduced chlorophyll *a* content.[135] Until now, the exposure scenarios applied in such experiments were beyond being environmentally relevant and do not distinguish between the direct and indirect effect mechanisms. Indirect effects of plastic debris on biofilm-forming organisms may result from leaching of HOCs to the biofilm. The toxicity of plastic additives such as flame retardants[136] and plasticizers[137,138] as well as HOCs[139−141] toward microalgae was demonstrated in laboratory studies. In addition, the ingestion of plastic covered with biofilms may increase the dose of HOCs to consumers because of increased capacity to carry HOCs (with biofilms acting as an additional sorptive phase mentioned in Transport of Plastic-Associated Pollutants through Biofilms).

Another aspect of biofilm growth on MP may be its infectious capacity caused by its transport of pathogens.[142] It is known that even free EPS fragments, called "transparent exopolymer particles" (TEP), facilitate the uptake of pathogens by biota.[143] MP may present an additional vector for the dispersal of rafting communities. Plastic-associated biofilms may cause such concerns as potentially pathogenic *Vibrio* spp. were detected on floating MP.[25,144−146] However, it is unclear whether the potential for pathogen dispersal is different between MP and natural particles and whether this route can increase the rate of infection of consumers. In conclusion, knowledge of the toxicity and potential adverse effects of MP and their associated chemicals on biofilm-forming organisms and primary consumers is currently lacking.

RESEARCH PRIORITIES

As biofouling of submerged surfaces is a long-standing cause for concern in pharmacology, medical and material sciences, and food technology,[147−150] knowledge of the colonization processes from these fields can contribute to our understanding of the behavior of plastic in the environment and facilitate technical approaches to studying this behavior. The formation and succession of a biofilm on MP particles involve multilateral processes determining the respective fate of MP in the environment and the responses of biological systems to MP pollution. On the basis of the literature discussed above, we identified the following research priorities.

(1) As every submerged surface is subject to microbial colonization, we need to better understand the basic processes that are involved in the formation of a biofilm, with a particular focus on biofilm−MP interactions. Following the eco-corona concept from nanotechnology,[7,33] experiments should be designed to identify key factors that influence the physicochemical behavior of MP (e.g., particle properties and surface characteristics and/or absorbing molecules). It should be evaluated whether these factors differ for different MP materials and whether they are comparable to those of natural particles of similar size. Further, experiments should consider changes in physicochemical properties after weathering. These investigations should be performed under different weathering conditions like UV, temperature, or mechanical abrasion.

(2) Our understanding of the biofilm−plastic interactions for hydrodynamic processes, such as vertical transport, needs to be improved to parametrize predictive models of the transport and exposure of MP particles and their associated pollutants in aquatic systems. Thus, sinking and flocculation studies with environmentally representative biofilm−MP complexes are needed, on micro- and mesocosm scales.

(3) The sorption of HOCs to MP has attracted an increasing amount of attention. However, a realistic concept accounting for the effect of biofilm formation and its consequences for the kinetics of chemical partitioning is still lacking, which hinders experimental evaluations. Modeling studies in a three-phase system (water−plastic−biofilm) should be complemented by experimental studies.

(4) Virtually all experiments published to date about the effects of MP on biota lack the proper preparation of the test particles that would simulate natural biofilm coating. MP coated by biofilms (e.g., derived from preculture incubations) should be included, and the influential characteristics of different biological materials like bacteria, fungi, and different algal strains should be tested. Furthermore, particle controls need to represent natural particles similar in size, density, and biofilm colonization.

(5) The relevance of biofilms for the mode and rate of MP uptake by consumers should not be ignored when estimating feeding uptake and exposure effects under realistic conditions. Differential uptake of MP due to biofilm formation should complement the current (ecotoxicological) research on MP ingestion in artificial food chains.

(6) We need to understand the intricate interactions between microbial assemblages in water and their capacity to sustain biofilm formation on various polymer materials ("plastisphere") if we are to assess the resilience of aquatic systems to MP pollution. Therefore, the investigation and analysis of biofilms on plastic debris are encouraged so we can gain functional insight into its productivity and diversity as well as its vector role in carrying and dispersing microorganisms for reliable hazard assessment.

In conclusion, the challenge for the MP research is to account for the interactions between diverse plastic materials undergoing weathering and colonization by microorganisms in various environmental settings to provide a science-based risk assessment for the effects of plastic debris in aquatic environments.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.7b00164.

Description of the literature research by means of the ISI Web of Science and the summarized results (Table S1) (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: christoph.rummel@ufz.de. Phone: +49 341 235 1512.
*E-mail: mechthild.schmitt@ufz.de. Phone: +49 341 235 1523.

ORCID

Christoph D. Rummel: 0000-0001-5829-3908

Notes

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Manuscript I

Effects of Leachates from UV-Weathered Microplastic on the Microalgae *Scenedesmus vacuolatus*

Christoph Rummel[a], Hannah Schäfer[a], Annika Jahnke[c,d] Hans Peter Arp and Mechthild Schmitt Jansen[a]

[a] Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany

[b] Department of Environmental Science and Analytical Chemistry (ACES), Stockholm University, Svante Arrhenius väg 8, SE-114 18 Stockholm, Sweden.

[c] Department of Ecological Chemistry, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany

[d] Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, DE-52074 Aachen, Germany

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Abstract

Plastics in the environment undergo successive fragmentation and chemicals leach during residence at sea as a result of weathering processes, such as photo-oxidation, prevailing in the surface layer of water bodies. Here, we report the effects of UV-radiated microplastic leachates towards the microalgae *Scenedesmus vacuolatus*. Most leachates of the tested additive-free polymers polyethylene terephthalate (PET), polystyrene (PS) and polypropylene (PP) did not show substantial negative effects. Interestingly, polyethylene (PE) caused reduced algae biomass, cell growth and photosynthetic activity. The applied highly contaminated positive control (e-waste) induced algae toxicity. Experimental data were consistent with predicted effect concentrations based on the ionization-corrected liposome-water distribution ratios ($D_{lip/w}$) of potential polymer degradation products (mono- and dicarboxylic acids) indicating that leachates from weathering plastic were mainly baseline toxic. This study provides insight in algae toxicity elicited by leachates from UV-weathered microplastics and thereby complements the current particle- and additive-focused research.

Introduction

Interactions between plastic debris and biota have been reported for diverse taxa at different trophic levels (Anbumani & Kakkar, 2018). In most ecotoxicological studies this interaction is characterized by the ingestion or uptake of particles into the test organisms. As primary producers and due to their global abundance, microalgae has high potential to interact with microplastics (Zhang et al., 2017). Negative effects of microplastics towards microalgae may stem from the physical presence of particles or by secondary effects from leaching chemicals. The first process may lead to particle adsorption, aggregation or shading effects as discussed by Zhang et al. (2017). However, the second process comprise indirect effects by chemicals leaching from microplastic, which has gained little attention so far. Luo et al. (2019) demonstrated the release of additive compounds into water over time by microplastics and detected decreased quantum yield efficiency at photosystem II at high leachate concentrations for the microalgae *Chlorella vulgaris*. Two sensitive marine cyanobacteria *Prochlorococcus* strains were affected by plastic leachate exposures as well, with increased transcription of common stress response genes (Tetu et al., 2019). Both studies applied plastics that were most likely compounded with chemicals, as it is typically the case for plastic articles, which is why negative effects by additive leaching could have been expected.

Our previous research has demonstrated that cellular effects may be induced by plastic degradation products, *i.e.*, excluding any presumable effects of chemicals intentionally added to the plastic that may leach from the test material (Rummel et al., 2019). That study describes the induction of certain cellular stress responses such as oxidative stress by plastic leachates from four commercial mostly additive-free polymers (polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP) and polystyrene (PS)). To further investigate the ecotoxicological relevance of these plastic leachates, we tested their ecotoxicological effect potential towards an ecologically important low-trophic level representative: microalgae. Microalgae was chosen as suitable test organisms since we could ensure the test accuracy of cell-based tools and increase complexity by using a whole-organism test. Furthermore, it is a representative primary producer that provides important ecosystem functions. Following the test guideline of OECD No. 201 (OECD, 2011), we addressed potential impairment of an physiological key process, namely photosynthesis.

We hypothesized that plastic leachates generated in artificial seawater would cause impaired photosynthetic activity and cell growth. To test the hypothesis that artificially weathered microplastic leachates would cause stronger negative effects towards microalgae than the respective dark controls, we applied an artificial weathering treatment to one batch of the samples that was compared to dark controls. Our results were compared to leachates of plastic with known high content of organic contaminants (electronic waste (EW) and a computer keyboard (KB)) that

served as positive controls. To get further insights in the modes of toxic action (MoA) of UV-weathered microplastic leachates, we investigated whether polymer degradation products (carboxylic acids) previously identified as PE degradation products by Gewert et al. (2018) have the potential to explain the observed algae toxicity. The results were compared to calculated algae effect concentrations for these carboxylic acids by quantitative structure relationships between estimated effect concentrations that elicit 50 % of the maximum effect (EC_{50} values) for baseline toxicity based on Altenburger et al. (2004). Finally, the data will be compared to effect data derived from cell-based bioassays (Rummel et al., 2019).

Material and Methods

Chemicals. Diuron (as positive control, CAS: 330-54-1, Sigma-Aldrich, Steinheim, Germany), Methanol (MeOH) and Ethylacetate (EtOAc) (HPLC grade, ≥ 99.9 %, Honeywell Riedel de Haen, Fisher Scientific GmbH, Schwerte, Germany), sodium hydrogen carbonate (CAS 144-55-8, Sigma-Aldrich, Steinheim, Germany), and Grimme Boardman medium (GB) (Grimme & Boardman, 1972) were used. Mono- and dicarboxylic acids (α,ω position) of carbon chain lengths of C5, C7−C12, C14, C16, and C18 were purchased from Sigma-Aldrich (Steinheim, Germany) (detailed information in Rummel et al. (2019)).

Leachates. For each polymer type PE, PET, PS, PP, EW and KB, 200 mL of aqueous leachates in triplicates were generated as described in Rummel et al. (2019). Minor changes included the use of 40 g of each plastic material for the generation of leachates. Furthermore, after SPE enrichment, the SPE cartridges (HLB Oasis, Waters USA) were rinsed with 10 mL of LC-grade water (Optima™ Fisher Chemical, Reinach, Suisse) to eliminate salt residues in the sorbents. After drying for 2 h under vacuum in the manifold samples were stored at room temperature. Following the elution with EtOAc and MeOH and solvent evaporation, the samples were re-dissolved in 1 mL of MeOH. This corresponds to an enrichment factor (EF) of 200 based on the previous aqueous leachate and final methanolic sample volume.

Exposure. To study the effect of microplastic leachates towards microalgae, a miniaturized high-throughput algae assay based on the OECD guideline No. 201 (Freshwater Alga Growth Inhibition Test, OECD (2011)) was used. In this test setup, a synchronized culture of the unicellular green algae *Scenedesmus vacuolatus,* kept in GB medium, was exposed to the leachates in a 96-well plate. Aliquots of the leachates are blown down to dryness, re-dissolved in GB medium and serial diluted 1:2 in the well plate with 10 dilution steps of 135 µL each (highest tested relative enrichment factor (REF) = 197.8). Negative controls contained only GB medium. Reference cells were exposed to the photosystem II inhibitor Diuron (van Rensen, 2008) at 1.17 µmol/L as the highest test

concentration, eliciting 100 % effect. After verifying the absence of any interfering autofluorescence of the samples on the plate reader (Spectra Max Gemini EM, Molecular Devices, San Jose, USA), 15 µL of algae suspension were added to each well with a final algae concentration of 7.5 * 10^4 cells/mL. Plates were sealed with Parafilm and LED day light-exposed for 24 hours at 300 rpm rotation and 28 °C in a HT Multitron incubator (Infors, Bottmingen, Germany).
The biological endpoint of Chlorophyll *a* autofluorescence was measured after the addition of algae and after 24 hours of exposure on the microplate reader, the cell number was detected using a FACSCelesta (BD Biosciences, New Jersey, USA) instrument and photosynthetic capacity as maximum quantum yield (Yield I (YI)) and effective quantum yield (Yield II (YII)) after 2 h and 24 h of exposure was determined using the Imaging PAM Chlorophyll Fluorometer (M-series, Heinz Walz GmbH, Effeltrich, Germany).

Data analyses. The algae growth rate based on the parameter fluorescence was calculated according to equation (eq.) 1. For the other endpoints, cell number and photosynthetic YI and YII after 2 h and 24 h of exposure, the measured values were used for the calculation of the relative inhibition (in percent (%)) without any background subtraction (eq. 2).

$$Growth\ rate = \frac{autofluorescence_{\ 24\,h} - autofluorescence_{\ 2\,h}}{autofluorescence_{\ 24\,h}} \quad (1)$$

$$Inhibition\ [\%] = \left(1 - \frac{endpoint\ value_{\ \mathrm{sample}}}{mean\ endpoint\ value_{\ \mathrm{control}}}\right) * 100 \quad (2)$$

Endpoint $value_{sample}$ represents the measured cell number or the photosynthetic YI and YII after 2 h 24 h. The mean endpoint $value_{control}$ comprise the averaged measured values for the respective endpoints by the unexposed negative control cells. Dose-response curves and EC_{50} values were calculated for the derived endpoints applying a 4-parametric Hill model (between 0 % and 100 % with slope and EC_{50} as adjustable parameters) using the software SigmaPlot 13.0.
Concentrations are given as the product of the SPE enrichment factor (here 200) and the dilution factor from the assays resulting in the relative enrichment factor (REF). Corresponding to Rummel et al. (2019), effect units (EU) for the endpoint autofluorescence (EU_{fluo}) or cell count (EU_{cell_count}) were calculated as the inverse EC_{50} value (*i.e.*, in the unit 1/REF). If all three tested replicates of a sample type resulted in a measurable effect, the mean and the 95 % confidence interval were calculated for the triplicates for comparison between samples.

Gewert et al. (2018) confirmed the presence of carboxylic acids in aqueous leachates from UV-weathered polymers, for which we observed certain effects in previous work (Rummel et al. 2019). Hence, we tested a set of mono- and dicarboxylic acids (α,ω position) of carbon chain lengths of C5, C7−C12, C14, C16, and C18 to investigate their potential to cause algae toxicity.

We applied the QSAR by Altenburger et al. (2004) to predict baseline toxicity of the carboxylic acids towards microalgae. This QSAR is based on the hydrophobicity (K_{ow}) of the chemical of investigation. However, at the applied pH in the assay, the carboxylic acids will fully dissociate and be present in their anionic form, for which the cellular uptake is slower and smaller (Fischer et al., 2018). To account for speciation, K_{lipw} of the neutral species of the carboxylic acids was predicted by the logK_{ow}-based QSAR by Endo et al. (2011) (eq. 3) and baseline toxicity of the neutral fraction using the ionization-corrected liposome-water distribution ratios [$D_{lip/w}$ (pH 7.4)] (eq. 4) were calculated following Escher et al. (2020) (eq. 5) parameterized with the converted slope and intercept of the QSAR by Altenburger et al. (2004).

$$\log K_{lipw}\,(neutral) = 1.01\,K_{ow} - 0.12 \quad \text{(Endo et al. 2011)} \tag{3}$$

$$\log D_{lipw}\,(pH\ 7.4) = f_{neutral}\,K_{lipw} + (1 - f_{neutral})K_{lipw}/10 \quad \text{(Escher et al. 2020)} \tag{4}$$

$$\log(1/EC_{50,basline}) = 0.855\,\log D_{lipw}(pH\ 7.4) + 1.02 \tag{5}$$

(Escher et al. 2020 parameterized by QSAR of Altenburger et al. 2004)

As an effect diagnostic tool, we calculated the toxic ratios (TRs) as the ratio between predicted baseline toxicity (eq. 5) and the measured effect data to explore the MoA of microplastic degradation products. In a last step, the cytotoxicity values of the bioassays (inverse IC_{10} as TU_{bio}) were correlated to the apical endpoints for algae growth (fluorescence and cell number). To make a comparison to the cell-based EC_{10} values feasible (Rummel et al. 2019), EC_{10} values for microalgae were calculated based on eq. 5.

$$EC_x = \left(\frac{x}{100 - x}\right)^{1/h} * EC_{50} \tag{5}$$

x = x % effect (here x = 10)
h = Hill slope

Results

When testing microplastic leachates from different polymer types in microalgae, the endpoints autofluorescence and cell number were more responsive compared to photosynthesis inhibition (YI and YII) (**Figure 1 A, B, Table 1**). Therefore these two apical endpoints autofluorescence and cell number were chosen for subsequent inter-sample comparison.

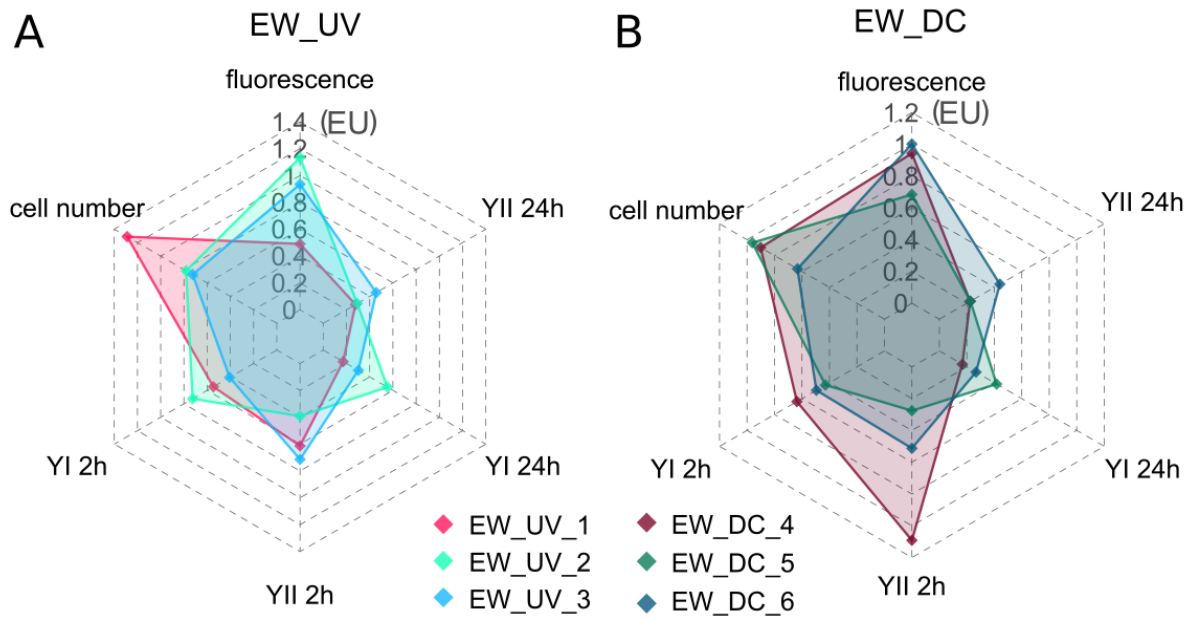

Figure 2: Radar plots of EC_{50} values of the different endpoints of leachates of EW UV-light (UV, in triplicates UV_1-3, **A**) and dark control (in triplicates DC_4-6, **B**) treatments tested in microalgae for fluorescence, cell-count and photosynthetic yield I and II after 2 h or 24 h of exposure, respectively. The units of the scale are the relative enrichment factor (REF).

Only the two positive controls EW and KB induced effects on all measured endpoints while effects of the test polymers could only be observed on algal growth (based on autofluorescence as well as cell numbers) (**Figure 1, Figure 2, Table 1**). Only one of the three dark control (DC) procedural blanks negatively affected the fluorescence of *S. vacuolatus* (**Figure 2 A**) No effects on the fluorescence could be detected for the SPE and the UV-light treated blanks. The positive control EW caused a decrease in the fluorescence compared to unexposed microalgae at mean EU values and standard deviations of EW_{DC} = 0.88 ± 0.17 and EW_{UV} = 0.85 ± 0.32. Interestingly, PE_{UV} showed higher negative effects on microalgae compared to its dark control PE_{DC}. For the remaining polymers PET, PP and PS, not every replicate (n = 3) resulted in a measurable effect on fluorescence.

One SPE blank and all replicates of the procedural blanks (DC and UV) resulted in detectable effects on the algae cell number (**Figure 2 B**). While EW showed similarly high EU mean values

for DC and UV samples of EW_{DC} = 0.83 ± 0.18 and EW_{UV} = 0.93 ± 0.3, KB_{UV} displayed a much lower effect potency than KB_{DC}. (KB_{DC} = 0.10 ± 0.02, KB_{UV} = 0.03 ± 0.01). As observed for the fluorescence endpoint, PE_{UV} induced four times higher effects towards algae cell counts than PE_{DC}. Noteworthy, most of the observed cell number effects for the tested polymers were in the range of the corresponding procedural blanks except PE_{UV}.

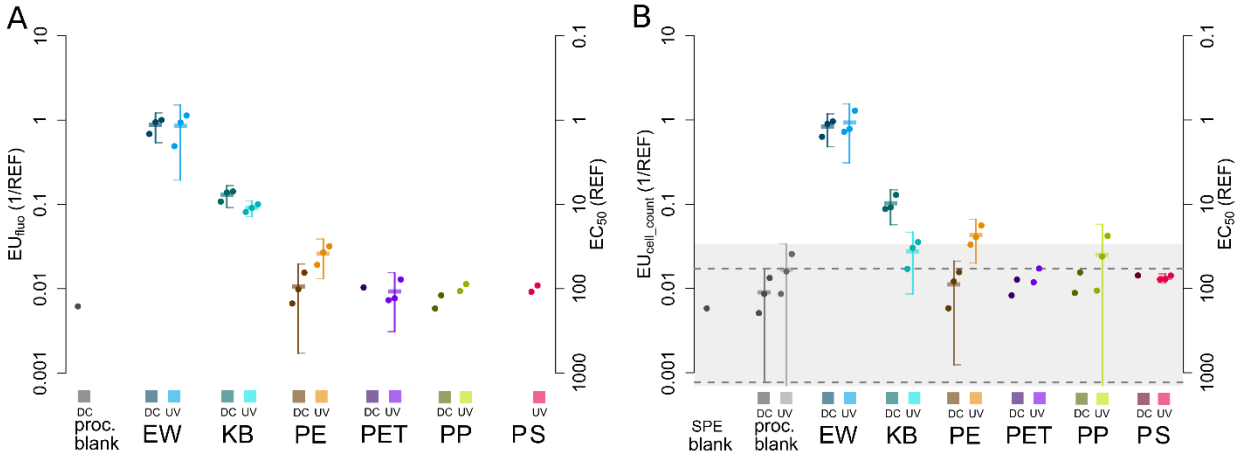

Figure 2: Effect units (EU) of autofluorescence (A) and cell number (B) of microalgae *S. vacuolatus* exposed to leachates from plastics and positive controls. EUs are given as the inverse EC_{50} (unit 1/REF) to facilitate association of high toxicity with high values; EC_{50} is additionally given as the scale to the right.. Leachate waters were generated under dark (DC) and UV-light conditions (UV) in triplicates (single data points). If all triplicates caused measurable effects the mean and 95% confidence interval were calculated and depicted as boxes and whiskers. Comparison to procedural blanks is facilitated by the dotted line and grey shaded area in B (not for since no triplicate blanks elicited effects). Missing values indicate the absence of measurable effects at the tested concentrations.

Table 1: Range of minimum and maximum EC_{50} values of autofluorescence, cell number and photosynthetic endpoints for blanks (SPE and procedural), positive controls (EW and KB) and the test polymers PE, PET, PP and PS as respective DC and UV-treatment. Concentrations are given in the unit of relative enrichment factors (REF). Single numbers are derived from a single detection where no min-max range could be reported.

Sample name	auto-fluorescence [REF]	cell number [REF]	YI 2h [REF]	YII 2h [REF]	YI 24h [REF]	YII 24h [REF]
SPE Blank	ND	172	ND	124.0	ND	ND
proc. Blank_DC	162.0	75.1 – 196.0	ND	94.0	196.0	168.0
proc. Blank_UV	ND	39.0 – 116.0	ND	ND	174.0	142.0
EW_DC	1.0 – 1.5	1.1 – 1.6	1.6 - 2.3	0.92 - 3.6	2.4-5.97	2.3 - 4.5
EW_UV	0.9 – 2.0	0.8 – 1.4	1.4 - 2.5	1.4 - 2.6	1.8 - 5.8	2.2 - 3.6
KB_DC	7.0 – 9.3	7.7 – 11.4	13.7 - 15.0	7.66 - 25.9	28.7 - 52.8	28.4 - 40.1
KB_UV	10.0 – 12.3	28.2 – 58.6	19.8 - 45.2	19.5 - 56.9	71.2 - 107.0	54.7 - 78.9
PE_DC	64.6 – 150.0	64.0 – 172	172.0	60.4 - 150	126	80.1
PE_UV	31.4 – 52.3	17.9 – 30.2	24.7 - 67.5	13.4 - 66.6	93.5	49.3 - 165
PET_DC	96.9	78.6 – 121.0	ND	ND	ND	ND
PET_UV	77.9 – 137.0	58.0 – 84.4	ND	72.7	ND	129.0
PP_DC	120.0 – 172.0	64.6 – 113.0	121	96.3	ND	ND
PP_UV	88.3 – 107.0	23.8 – 106.0	134	25.9	138	101.0 - 182.0
PS_DC	NA	70.0	ND	ND	ND	ND
PS_UV	91.5 – 109.0	70.3 – 78.0	ND	184.0	189.0	104.0

ND: not detected at REF < 198

For EW, fluorescence and cell count exhibited the lowest EC_{50} values (Fluo ($EW_{DC\ or\ UV}$) = 0.88 – 2.0; cell count ($EW_{DC\ or\ UV}$) = 0.8 – 1.6) in contrast to the photosynthetic yield (YI/II 2h-24h ($EW_{DC\ or\ UV}$) = 0.92– 5.83) (**Figure 2**). Photosynthesis was adversely affected mainly by the positive controls EW and KB as well as by PE (DC and UV) (**Table 1**). PE_{UV} showed generally stronger negative effects to YI and YII compared to PE_{DC} and was within the range of the positive control

KB. Furthermore, EC_{50} increased at 24 h compared to 2 h after dosing (**Table 1**). PET, PP and PS showed minor effects towards microalgae and, if detectable, they were mostly within the range of the blanks (**Figure 2**, **Table 1**).

Effects by Polymer Degradation Products. When testing mono- and dicarboxylic acids in the microalgae test at highest soluble concentrations mainly the monocarboxylic acids resulted in detectable effects (**Table 2**). Only dicarboxylic acids of carbon chain length C5 and C7 resulted in measurable effects while mono- and dicarboxylic acids of carbon chain length greater than eleven did generally not cause algae toxicity or impairment of the photosystem. Most TRs of the endpoints autofluorescence and cell number located in a narrow range of $1 < TRs < 10$ except for the decanoic acid with a $TR < 1$ (**Figure 3 A**).

Going one step further, we correlated the results for cytotoxicity obtained using reporter gene bioassays from Rummel et al. (2019) with microalgae results. Here, calculating the EC_{10} values for the microalgae results was necessary to facilitate a comparison to the EC_{10} and the inverse effect units (EU_{bio}) and toxic units (TU_{bio}) of the bioassays. Linear regressions of reporter gene cytotoxicity (TU_{bio}) as a function of microalgae data resulted in statistically significant correlations often with slopes close to one (**Figure 3 B, C, Table 3**).

Table 2 : QSAR and effect data of mono- and dicarboxylic acids tested in the micro algae test system

substance	CAS	MW	log P_{ow}	source	Calculated[b] EC50 [mM]	EC_{50} Fluo-rescence [mM]	EC_{50} cell count [mM]	EC_{50} YI2h [mM]	EC_{50} YII2h [mM]	EC_{50} YI24h [mM]	EC_{50} YII24h [mM]
pentanoic acid	109-52-4	102.13	1.39	SDS	8.01	14.49	9.18	9.07	8.87	16.19	14.79
pentandioic acid	110-94-1	132.12	0.256	SDS	210.83	268.59	174.54	100.90	90.85	147.07	148.19
heptanoic acid	111-14-8	130.19	2.42	SDS	1.03	1.11	0.60	1.30	1.17	5.49	2.25
heptandioic acid	111-16-0	160.17	0.61	SDS	37.72	ND	140.97	686.47	ND	456.69	ND
octanoic acid	124-07-2	144.2	3.05	SDS	0.30	0.49	0.19	1.47	0.35	11.24	1.97
octandioic acid	505-48-6	174.20	1.21	cal.[a]	11.45	ND	ND	ND	ND	ND	ND
nonanoic acid	112-05-0	158.24	3.4	SDS	0.15	1.35	0.39	2.97	2.21	32.88	7.82
nonandioic acid	123-99-9	188.22	1.57	SDS	5.60	ND	ND	ND	ND	ND	ND
decanoic acid	334-48-5	172.27	4.09	SDS	0.04	ND	1.45	1.19	4.51	ND	2.51
decandioic acid	111-20-6	202.25	2.19	cal.[a]	1.63	ND	ND	ND	ND	ND	ND
undecanoic acid	112-37-8	186.30	4.42	SDS	0.02	ND	ND	0.19	0.24	12.98	ND
undecandioic acid	1852-04-6	216.28	2.8	SDS	0.49	ND	ND	ND	ND	ND	ND
dodecanoic acid	143-07-7	200.32	4.6	SDS	0.01	ND	ND	ND	ND	ND	ND
dodecandioic acid	693-23-2	230.30	3.17	cal.[a]	0.23	ND	ND	ND	ND	ND	ND
tetradecanoic acid	544-63-8	228.38	6.1	SDS	0.001	ND	ND	ND	ND	ND	ND
tetradecandioic acid	821-38-5	258.36	4.3	cal.[a]	0.02	ND	ND	ND	ND	ND	ND
hexadecanoic acid	57-10-3	256.43	6.4	cal.[a]	3.80E-04	ND	ND	ND	ND	ND	ND
octadecanoic acid	57-11-4	284.48	8.23	SDS	1.00E-05	ND	ND	ND	ND	ND	ND

[a] Computed by XLogP3 3.0 (PubChem release 2019.06.18)
[b] calculated (cal.) by quantitative structure relation by Altenburger et al. (2004)
ND: not detected

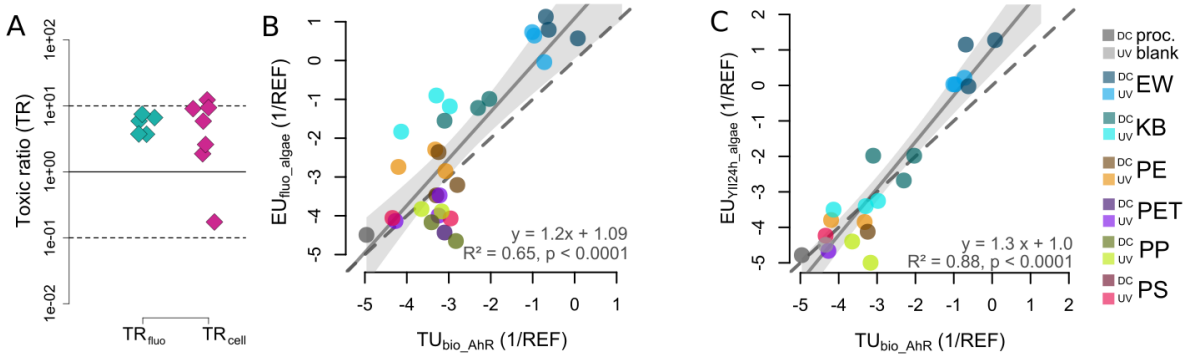

Figure 4: Toxic ratios (TRs) for reference mono- and dicarboxylic acids (C5-C18) tested in the microalgae with measured endpoints for fluorescence (fluo), cell number (cell). **B**: statistically significant correlation between cell line and microalgae data could be observed by the linear regression of the apical endpoint fluorescence for microalgae (fluo_algae) as a function of toxic units (TU_{bio}) of the AhR assay (regression parameters, coefficient of determination and p-value are included in the plot) (log-transformed data). **C**: Correlation of TU_{bio} of AhR and the photosynthetic activity at 24h after dosing for microalgae (EU_{YII24h_algae}) (log-transformed). Dashed line in C and D represents the 1:1 line. Color code for B and C shows the different polymer types with respective dark control (DC) and weathering treatment (UV)

Table 3: Parameters of the linear models ($y=ax+b$) using the microalgae endpoints (EU_{fluo}, EU_{cell}, EU_{YI24h} and EU_{YII24h}) the as y-variable as a function of the cytotoxicity values (TU_{bio}) of the reporter gene results from Rummel et al. (2019) as x-variable.

y-variable	x-variable	Slope *a*	Intercept *b*	Coefficient of determination R^2	*p* - value
EU_{fluo}	TU_{bio} AhR	1.2	1.1	0.65	<0.0001
	TU_{bio} AREc32	1.6	3.5	0.38	0.003
	TU_{bio} PPARy	-0.4	-3.3	0.0003	0.32
EU_{cell}	TU_{bio} AhR	0.8	1.2	0.08	0.05
	TU_{bio} AREc32	2.6	7.3	0.42	<0.01
	TU_{bio} PPARy	-0.29	-2.3	-0.02	0.56
EU_{YI24h}	TU_{bio} AhR	1.5	2.2	0.83	<0.0001
	TU_{bio} AREc32	2.3	5.9	0.44	<0.05
	TU_{bio} PPARy	-0.1	-1.7	-0.07	0.89
EU_{YII24h}	TU_{bio} AhR	1.3	1	0.88	<0.0001
	TU_{bio} AREc32	2.2	4.9	0.52	<0.01
	TU_{bio} PPARy	-0.3	-3.3	-0.04	0.58

Discussion

Effects of Leachates. Leachates from additive-free pre-production polymers PET, PP and PS did not show strong ecotoxicological effects on algae biomass, cell number or photosynthetic activity (**Figure 2**, **Table 1**). The measureable effects were barely differentiable from the respective blanks as seen for the endpoint cell number (grey shaded area, **Figure 2 B**). This means that no specific toxicity such as inhibition of the photosystem was induced by substances leaching from weathered PET, PP and PS microplastic in dark or UV-light treatments. One reason for the relatively low ecotoxicological potential of pre-production plastics may be the absence of additives. Capolupo et al. (2020) directly linked the high toxicity of leachates of car tire rubber and PVC on freshwater and marine microalgae to high contents of additive chemicals. Other plastic types, such as PS and PP, did not show such high ecotoxicological potential in their study (Capolupo et al., 2020). A study by Chae et al. (2020) investigated the toxicity of expanded polystyrene (EPS) towards four different microalgae species. Generally, the photosynthetic activity of all four species was enhanced by EPS leachates (Chae et al., 2020). The authors speculated about this hormesis effect that leaching DOC (such as the measured hexabromocyclododecanes, bisphenol A and UV326) might have promoted photosynthetic activity and thereby cell growth (Chae et al., 2020).

While in our study only PE showed algae toxicity to some extent, Tetu et al. (2019) detected impaired growth, photosynthetic capacity, and genome-wide transcriptional changes by LDPE and PVC leachates for an important primary producer, *Prochlorochoccus spec.* While adverse effects of plastic leachates were reduced by weathering in a study by Sarker et al. (2020), we could identify toxicity that was increased by a factor of around 2 to 3 for UV-weathered PE_{UV} compared to the dark control PE_{DC}. Interestingly, our positive control KB showed such reduced effects upon weathering which could be indicative for the photo-degradation of toxic substances leaching from the material. Similar to the results of Rummel et al. (2019), prominent effects were caused by the positive control EW with EC_{50} values of REFs around or below EC_{50} <= 1 (REF) (or reciprocal EU values >= 1 (1/REF)) meaning that no dilution or enrichment was necessary to target the observed effect. Leachates from pre-production polymers had to be enriched by factors of 18 to 190 to target the observed effects.

MoA of Degradation Products. The observed elevated EU values for PE_{UV} compared to PE_{DC} may potentially be the result of photooxidizing PE (**Figure 2, Table 1**). Gewert et al. (2018) and Rummel et al. (2019) identified potential degradation products in leachates that were generated using the identical weathering setup. The identified degradation products of PE, mainly

dicarboxylic acids of different chain lengths (C5 – C18), showed effects on all measured endpoints, however, only by acids with short carbon chain length (**Table 2**) and at high tested concentrations. Still a trend of increasing effects with increasing carbon chain length could be observed. This increase may relate to the acids' linear relationship between membrane permeability and the hexadecane/water partition coefficient (Walter & Gutknecht, 1984).

Applying the modified quantitative structure relation by Altenburger et al. (2004), we predicted baseline toxicity for the investigated carboxylic acids and calculated TRs. The observed TRs < 10 for almost all measured endpoints were in good agreement with the previously discussed non-specific disturbance of the cell membrane elicited by the acids. Moreover, the microalgae endpoints fluorescence and cell number displayed a very narrow range of TRs indicating low uncertainty between calculated and measured EC_{50} values supporting their baseline toxic mechanism of action (**Figure 2.4 B**). Based on the values of 1 < TR < 10 for the endpoints fluorescence and cell number (**Figure 2.4 B**) it can be assumed that the critical membrane concentration of 70 mmol/L_{lip} resulting in destabilization of the phospholipid bilayer was reached by the carboxylic acids (Escher et al., 2019) but there were no specific effects towards PSII. The applied QSAR is potentially not adequate for PS II inhibition since it shall serve solely to calculate baseline toxicity values.

Cellular membranes contain unsaturated fatty acids (Cid et al., 1996) that are especially prone to the attack by free radicals causing lipid peroxidation (Kellogg & Fridovich, 1975), lysis (Goldstein & Weissmann, 1977) and fatty acid deesterification (Niehaus, 1978). In this context, fluorescence is a good indicator of membrane disintegration, may highly depend on the chemicals' hydrophobicity and may therefore indicate baseline toxicity. These observations compare well to the finding that fluorescence and cell number, as apical endpoints, were the most sensitive endpoints in the leachate tests (**Figure 1**). These two endpoints, are indicative for membrane integrity, may highly depend on the chemicals' hydrophobicity and may therefore indicate baseline toxicity. Furthermore, the EU values of fluorescence correlated statistically significant to the cytotoxicity values derived from reporter gene assays with regression slopes close to one (**Figure 3**). The impairment of the photosystem is therefore an indirect effect of baseline toxicity as a similar good correlation to cytotoxicity of reporter gene assays suggests (**Figure 3**).

In a comparable way, the AREc32 cell assay, responsive to oxidative stress, was induced across all tested polymer types in Rummel et al. (2019). At high physiological concentrations, reactive oxygen species may cause cell damage and cell death (Ghosh et al., 2018) often induced by small reactive molecules (Escher et al., 2013).

Another indication for baseline toxicity as the underlaying mechanisms of toxicity was the good correlation and regression slopes close to 1 between cytotoxicity values (TU_{bio}) from the AhR assay

and EU_{bio} values of the AREc32 assay (**Figure 4, Table 3**). Accuracy of the model could potentially have been improved in the experimental setup if the same amount of plastic was used for the leaching experiment. In Rummel et al (2019), 50 g of each plastic type was leachated in ASW while in this study 40 g were applied in the weathering setup (but based on identical EF as a result of the underlying volumetric measure). Modelling the effect contribution of mono- and dicarboxylic acids using the high-resolution mass spectrometry data from Rummel et al (2019) was not feasible in this study since only dicarboxylic acids were ionizable by the applied HRMS methodology. Single compound testing, however, revealed negative effects on microalgae mainly by monocarboxylic acids.

Conclusion

Generally, enriched aqueous leachates from UV-weathered microplastic did not cause severe algae toxicity. Proof of principle was provided by the positive control EW, for which we could observe negative effects on microalgae growth and photosynthesis. Elevated toxicity by PE leachates could potentially be explained by the presence of small reactive molecules such as mono- and dicarboxylic acids that were very likely present in the leachates as a result of photo-degradation. These degradation products were mainly baseline toxic since measured data was consistent with predicted baseline toxicity for the investigated carboxylic acids. Our findings highlight that degrading pure polymers have the potential to induce negative effects on whole organisms, however, most prominent effects may stem from chemicals added to the polymers (Capolupo et al., 2020). To increase our understanding of chemical and particle toxicity of plastics, future studies should investigate algae toxicity of migrating additives and compare them to toxicity caused by the mere particles.

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Manuscript II

The conditioning film and early biofilm succession on plastic surfaces

Christoph Rummel*[a], Oliver J. Lechtenfeld[b], René Kallies[c], Annegret Benke[d], Peter Herzsprung[e], Robby Rynek[b], Stephan Wagner[bh], Annegret Potthoff[fl], Annika Jahnke[fg] and Mechthild Schmitt-Jansen[a]

*corresponding author

[a] Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany
[b] Department of Analytical Chemistry, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany
[c] Department of Environmental Microbiology, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany
[d] Department of Powder and Suspension Characterization, Fraunhofer Institute for Ceramic Technologies and Systems IKTS, Winterbergstr. 28, 01277 Dresden, Germany
[e]Department of Lake Research, Helmholtz Centre for Environmental Research – UFZ, Brückstr. 3a, 39114 Magdeburg
[f] Department Ecological Chemistry, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany
[g] Department of Exposome Analytics, Faculty of Mathematics, Computer Science and Natural Science, RWTH Aachen University, 52047 Aachen, Germany
[h]Institute for Water and Energy Management (iwe), University of Applied Science, Alfons-Goppel-Platz 1, 95028 Hof, Germany

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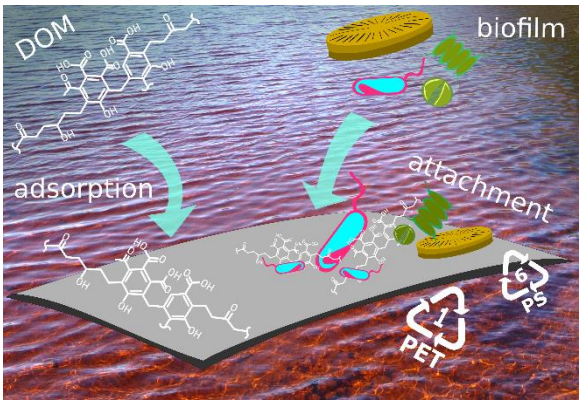

Abstract

Plastic debris in the aquatic environment has been recognized as a global pollution issue. It is still under debate if and how the 'plastisphere', a plastic-specific microbial community, can emerge and which the underlying processes are. The initial conditioning film of adsorbed dissolved organic matter (DOM) is thought to play a key role for microbial pioneer attachment and subsequent early biofilm formation. In this study, we tested the hypothesis that DOM sorbs selectively to substrates that display different surface properties. Further, we tested whether subsequent early microbial attachment is governed in a substrate-dependent manner. We investigated the adsorption behavior of stream water-derived DOM to polyethylene terephthalate (PET), polystyrene (PS), and glass as a reference material. The organic matter (OM) composition was characterized by Fourier-transform ion cyclotron mass spectrometry and compared to the DOM in the original incubation water. Only a fraction of the original stream water DOM adsorbed to the substrates. We identified major differences in the molecular OM composition between the substrates which were additionally modified by a UV-weathering treatment. The biofilm community was investigated after 24 h and 72 h of incubation by 16S and 18S rRNA gene amplicon sequencing. Early biofilm communities showed a clear time-dependency, however, we could identify a minimal but detectable substrate-specificity for biofilm attachment after 24 h. Conclusively, the adsorbed OM layer developed in dependence of the materials' surface properties and preserved the surface characteristics to some extent towards the outer OM-water interface. Subsequent material-specific colonization by microbes highlighted the importance of this first conditioning film.

Keywords

Plastic pollution, Microplastic, Dissolved Organic Matter (DOM), Conditioning film, Eco-corona, Surface properties, Microorganisms, Microbiome, FT-ICR MS

Introduction

The increasing contamination of our environment by plastic debris is cause of concern because it persists, [1] it causes negative impacts on biota[2-4] and meets two of the three criteria of a planetary boundary threat.[5] As every submerged surface is rapidly colonized by biofilms, a consortium of microorganisms, such as prokaryotes, algae, fungi and viruses, embedded in a so-called extracellular polymeric substance, researchers have begun to explore microbial communities on environmental plastic surfaces, recently. Biofilms on plastic debris are of environmental relevance since they play a key role for geochemical processes at the solid/liquid interface,[6] they may act as a vehicle or vector for microorganisms and pathogens[7,8] and may promote the spread of antibiotic resistance genes.[9] Zettler et al. (2013)[7] have coined the term 'plastisphere', which describes a microbial core community specific to plastic debris and/or specific for each type of polymer. Before such colonization can take place in natural waters, surfaces that enter the water phase almost instantaneously adsorb a layer of dissolved organic matter (DOM), the conditioning film.[10] The adsorption of DOM to solid surfaces may take place in short time frames from minutes to hours[11,12] creating a layer of organic matter (OM) of increasing thickness that eventually will reach a state of equilibrium.[13] Studies have demonstrated the influence of such conditioning on the toxicity of polystyrene (PS) microplastic particles[14] and on the sorption of hydrophobic organic compounds to TiO_2 nanoparticles.[15] Further, the adsorptive fractionation of humic and fulvic acids by mineral surfaces has been identified.[16,17] Whether different polymer surfaces display distinct sorption behavior towards OM remains so far unexplored. However, for a comprehensive interpretation and extrapolation of the afore-mentioned study results this knowledge is an indispensable prerequisite. Within hours to days, microorganisms attach to the surface either actively (by cell appendages) or passively by attractive and repulsive forces which marks the beginning of biofilm succession.[18,19] This first conditioning film is believed to have major impacts on biofilm formation[20-23] and may alter the fate, stability and ecotoxicological potential of particles in a medium.[24-26] Taylor et al. (1997)[20] concluded that all investigated types of surfaces (metals, minerals and polymers) were not modified in the same way even when exposed to the same surface-active solutes. Furthermore, the study reveals that bacterial accumulation could be best described by thermodynamic parameters such as the surface free energy γ (SFE). [20] By the application of high-throughput DNA sequencing, scientists identified plastic-specific communities that differed from other solid or natural substrates.[27-29] Contrarily to these findings, other studies have not indicated such difference and report that microbial community structures on plastic surfaces may be mainly driven by geographical, spatial and seasonal factors that select for a distinct community.[30,31] The characteristics and consequences of the adsorption of DOM on microplastic (MP) particles that

attribute to a so-called "eco-corona" has recently gained scientific interest.[32] A recent study raised the question about the role of conditioning films for bacterial adhesion on plastic substrates.[33] To test this hypothesis, we investigated the substrate-bound OM using three-dimensional excitation emission matrix spectroscopy (3D EEM). Our results of a pilot study suggested differential adsorption of fluorescent DOM to different polymeric surfaces even under a UV-weathering pre-treatment of the surfaces (**Pilot study SI 1**, **Figure S 1**, supporting information (SI)).

In this study, we conducted refined analyses under the hypotheses that I) polymer surfaces selectively adsorb DOM even under the influence of altered surface properties due to weathering, II) if our observation of material-specific DOM sorption holds true (i.e. not masking the surface by uniform surface-OM) we expect the microbial community to be governed via initial adsorption of DOM leading to material-specific communities. To test these hypotheses, we characterized the DOM composition of stream water (SW) and the fraction that adsorbed to submerged glass, Polyethylene terephthalate (PET) and PS sheets by Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR MS). The potential influence of changing surface properties under the impact of weathering (i.e. UV-induced photooxidation) was investigated by including a batch of artificially weathered substrates. To investigate the quality of the conditioning film on different surfaces and its potential influence on early biofilm formation we used the identical SW in parallel setups and explored subsequent early bacterial and eukaryotic community formation by 16S and 18S rRNA gene amplicon sequencing.

Material and Methods

Material and Chemicals. The test polymers were selected based on their importance for commercial use and the availability of pure, mostly additive free polymer sheets. Polyethylene terephthalate (PET) and Polystyrene were purchased from Goodfellow GmbH (Hamburg, Germany) with a thickness of 1 mm and 1.2 mm. Microscopy slides were purchased from Labsolute, Th. Geyer GmbH & Co. KG (Renningen, Germany). Chemicals used were Methanol (MeOH) of LC-grade (Honeywell, Riedl de Haën, Seelze, Germany), 2 % Wofasteril (Kesla Pharma Wolfen GmBH, Bitterfeld- Wolfen, Germany) and monoethylene glycol (MEG) (Carl Roth GmbH + Co. KG, Germany). We used 50 mg styrene-divinyl-polymer sorbent (Bond Elut PPL, Agilent Technologies, Santa Clara, CA, United States) in our DOM experiments. Filters used for the DOM and the biofilm experiment were 3 µm Isopore TSTP 04700 (Millipore, Merk KGaA, Frankfurt, Germany) and 0.2 µm Isopore GTTP04700 membrane filters (Millipore, Merk KGaA, Frankfurt, Germany).

Artificial weathering. Detailed description of the artificial weathering is provided in the SI (**Exposure scenario SI 2**, SI). Briefly, 6.5 x 2 cm soda lime microscopy slides (reference substrate) as well as PET and PS slides were used. All slides were thoroughly cleaned with wofasteril and MilliQ. Weathering was simulated for 288 h using the solar simulator XE-1 Xenon test chamber (Q-lab Deutschland GmbH, Saarbrücken, Germany) at 0.68 W/m² at 340 nm on a batch of microscopy glass slides (hereafter glass_UV), PET (PET_UV) and PS (PS_UV). A batch of glass, PET and PS (hereafter glass_DC, PET_DC, PS_DC) slides serving as negative dark controls were put concurrently in the test chamber wrapped in aluminum foil to guarantee same processing and temperature conditions (**Figure S 2 A**, SI).

Material properties. There were two separated sampling events for SW sampling. One for the incubation of substrates for subsequent analyses of surface properties and another sampling of SW for the main study of DOM sorption analyses via FT-ICR MS and concurrent microbial community analyses. For all materials, we determined the contact angles for two test liquids water and MEG, the hereof calculated SFE before and after DOM incubation and measured FTIR spectra to calculate the carbonyl index of PET and PS. Further details are provided in the SI (**Material Properties SI 3**, SI).

DOM sorption experiment. A detailed description of the DOM sorption experiment, the FT-ICR MS measurements and instrument settings can be found in the SI (**DOM sorption experiment SI 4, Table S1**). Briefly, we incubated 13 slides each of glass, PET and PS (DC and UV) at room temperature for 1 h in 0.2 μm filtered SW. After retrieval, the slides were rinsed with MilliQ water and MeOH and enriched via solid-phase extraction. FT-ICR mass spectra were recorded using a solariX XR (Bruker Daltonics Inc., Billerica, MA, USA) in ESI negative ionization mode in the mass range of 150 – 1000 m/z. Raw spectra were processed with Compass DataAnalysis 5.0 (Bruker Daltonics Inc., Billerica, MA, USA). To account for background signals stemming from the material itself (leaching of plastic-associated substances) we incubated a separate batch of slides in MilliQ water, rinsed them thoroughly with MeOH and measured with FT-ICR MS (see also **DOM sorption experiment SI 4**).

DOM data analyses. Molecular formulas were assigned to signals in the range 150-1000 m/z allowing for elemental compositions $C_{1\text{-}80}$ $H_{1\text{-}198}$ $N_{0\text{-}4}$ $O_{0\text{-}40}$ $S_{0\text{-}1}$ with an error range of ± 0.35 ppm according to Lechtenfeld et al. (2014)[34]. Briefly, the following rules were applied: $0.3 \leq H/C \leq 2.5$, $0 \leq O/C \leq 1$, $0 \leq N/C \leq 1.25$, $0 \leq DBE \leq 25$ (double bond equivalent, $DBE = 1 + 1/2\,(2C - H + N)$,[35] $-10 \leq DBE\text{-}O \leq 10$,[36] and element probability rules proposed by Kind and Fiehn (2007)[37].

Isotopologue formulas (13C, 34S) were used for quality control but removed from the final data set as they represent duplicate chemical information. Molecular formulas detected in the procedural blanks were removed for each corresponding sample type. Molecular formulas are referred to as "molecules" in this text although each formula may represent different isomers.

Statistical analyses of DOM data. Statistical evaluation was performed in R studio Version 1.2.5019.[38] Van Krevelen plots were generated to display the relative degree of saturation and oxygenation (H/C over O/C ratio) of molecules. For Figures 1 and 2, the molecular formulas of each substrate (glass, PET or PS (DC or UV)) were rigorously reduced to those co-occurring in the SW. Following this conservative approach, we do not consider those signals that may derive from a certain substrate and any bias by peak signals stemming from the plastic was unlikely since the signals under consideration were derived from the incubation SW. Mass peak signal intensities of molecular formulas were normalized by the sum of intensities in each sample and normalized intensity ratios (ΔRI) of DC and UV samples were calculated according to equation 1 (Eq.1). The ΔRI values represent the enrichment on the substrate dark control (1 > ΔRI. > 0.6), similar sorption (0.6 > ΔRI > 0.4) or enrichment on the UV treated substrate (0.4 > ΔRI > 0). Molecular formulas were grouped according to their degree of saturation (H/C), oxygenation (O/C) and modified aromaticity index (1 + C – 0.5 * O - S - 0.5 * (H + N) / (C – 0.5 * O - S - N)[39] as described in the SI (**DOM data analysis SI 5**). The intensity-weighted population density D_k in percent (%) was calculated based on the summarized relative intensities in each compound class following Perminova (2019)[40] (Eq. 2). Furthermore, molecular formulas were grouped by the four prevailing elemental compositions of CHO, CHN, CHNOS, CHOS. Hierarchical cluster analysis (HCA) applying the agglomeration method "complete" on Bray-Curtis dissimilarities was performed on the blank-corrected data using the R package *vegan* [41]. The valid number of clusters was estimated using the partitioning technique "silhouette"[42] in the package *factoextra* [43] (also used for the amplicon data).

$$\Delta\, relative\ intensity = \frac{rel.\ Intensity\ (sample\_DC)}{rel.\ Intensity\ (sample\_DC) + rel.\ Intensity\ (sample\_UV)} \qquad \text{Eq.1}$$

$$D_k = \frac{\sum_{k=1}^{N_i} I_i}{\sum_{j=1}^{N} I_j}\ ,\ k = 1,2,\dots n, \qquad \text{Eq.2}$$

where D_k is the contribution of compound class of total intensity in percent (%), ΣI_i is the sum of relative intensities in each compound class, ΣI_j the total relative intensity of all molecular signals.[44]

Early biofilm succession. A separate batch of 24 substrates each of glass, PET and PS (DC and UV) were inserted randomly in mountings to place 12 slides each vertically in two test aquaria

(**Figure S 2 B and C,** SI). Upon the start of the experiment ca. 14 L of non-filtered SW (identical SW as used for **DOM sorption experiment**) was filled in two aquaria and the mountings (with the same set of substrates) were submerged for biofilm succession. After 24 h and 72 h three slides of each material were retrieved from each of the two aquaria (total number of six slides) and put into 0.2 μm filtered SW to wash off loosely attached colonizers and to avoid cross-contamination with pelagic organisms randomly getting trapped on the surface biofilm. A pooled sample was taken by scraping off the surfaces with a scalpel and snap frozen in liquid nitrogen for amplicon sequencing. Inoculation SW was sampled at the start of the experiment (0h) using fractionated filtering onto 3 μm and 0.2 μm membrane filters to account for the particle-associated (3 μm) and the pelagic (0.2 μm) inoculating communities.[31] Since microbial community studies with low quantities of biomass have raised awareness to consider procedural blanks to account for reagent and laboratory contamination[45,46] we included one sample of 0.2 μm filtered SW and one 0.2 μm filter and 3 μm filter serving as procedural blanks to account for all processing after sampling.

Amplicon sequencing. Biofilm DNA was extracted using the Nucleo Spin™ Soil kit (Macherey-Nagel™, Düren, Germany) followed by Illumina MiSeq sequencing according to the Illumina library preparation workflow described in the "16S Metagenomic Sequencing Library Preparation" protocol[47] (see details in **Amplicon sequencing SI 6**, SI). Applying the open-source bioinformatical pipeline Qiime2[48] the demultiplexed raw reads were denoised with "dada2"[49] and truncated at a Quality Score of 25 to gain enough overlap between the forward and the reverse reads. After chimera removal the taxonomic identification was performed using the SILVA SSURef release 132[50] and clustered at 97 % similarity threshold for amplicon sequence variant (ASV) assignment.[51] Rarefaction curves showed that the number of observed ASVs remained constant with increasing sequencing depth (**Figure S 4**, SI). Sequencing data are available via NBCI SRA bioProject accession number PRJNA646354.

Statistical Analyses of sequencing data. Further processing and statistical analyses were performed in R studio using the packages *phyloseq*,[52] *microbiome*[53] and *ampvis2*.[54] ASVs occurring in the blank samples were subtracted from the data set. Beta-diversities based on "Bray Curtis" dissimilarities were calculated using the *vegan* package.[41] A valid number of clusters was estimated as described above. Data reduction and visualization of community dispersion was performed using a multidimensional scaling by Principal coordinate analyses (PCoA).[55] This was followed by permutation tests to identify ordination-driving taxa that showed a relative abundance of > 1 % at a significance level of $p < 0.001$ (in case of prokaryotic ASVs) and $p < 0.01$ (for eukaryotic ASVs) with corresponding coefficients of determination of $R^2 > 0.8$ (pro- and eukaryotic ASVs). Different thresholds were chosen to reduce the number of significant taxa and for graphical reasons in the

ordination plot. The assignment of different taxonomic ranks in the eukaryotic community were due to the respective degree of classification depth in the reference database. Statistical differences between pelagic (pooled 0.2 μm and 3 μm fraction) and biofilm communities (pooled attached communities), between different substrates (glass, PET and PS), different treatments (DC and UV) and time points (24 h, 72 h) were tested using permutational multivariate analyses of variance (PERMANOVA)[56] with 1000 random permutations to test for group mean differences. Pairwise permutational MANOVA followed by Benjamini and Hochberg *p*-value adjustment[57] was used as a post hoc test to identify potential differences between groups.

Results

Surface properties. The pristine materials (glass_DC, PET_DC and PS_DC) differed in their physico-chemical properties. Artificial weathering caused a decrease in surface hydrophobicity for the materials PET_UV and PS_UV and an increase in the polar share of the surface energy (**Table 1, Figure S 5,** SI). For the reference material glass, the measured parameters did not change during weathering and remained within the range of the standard deviation. Visual inspection of the polymeric substrates revealed deformations of the PET_UV sheets whereas strong yellowing could be observed for PS_UV (**Figure S 2,** SI). OM adsorption caused an increase in the materials' contact angle for PET (DC and UV) and PS_UV (increase between 2-6°) while the increase for glass_DC was within the uncertainty range of the standard deviation (**Table 1**). The FTIR spectra showed differences between weathered and non-weathered polymer substrates (**Figure S 6**, SI). PET_UV generally showed smaller and wider peaks than PET_DC. A strong increase in the carbonyl peak could be detected for PS (DC to UV) resulting in a correspondingly increasing carbonyl index (**Table 1**). PET showed a decrease in the carbonyl index upon artificial weathering.

Table 1: Surface properties such as surface free energy γ (SFE), contact angles for water and monoethylene glycol (MEG) (n = 4 - 6) and the FTIR based carbonyl index of the dark control (DC) and weathered (UV) substrates glass, PET and PS before and after DOM incubation.

	sample	Surface free energy γ [mN/m]	Dispersive share (SFE) [mN/m]	Polar share (SFE) [mN/m]	Contact angle θ for H_2O ± stdv [°]	Contact angle θ for MEG ± stdv [°]	Carbonyl Index
pre DOM incubation	glass_DC	35.3	13.5	21.8	67.2 ± 5.9	48.8 ± 4.5	NA
	glass_UV	37.8	8.7	29.1	64.1 ± 0.7	52.0 ± 3.2	NA
	PET_DC	31.3	15.8	15.5	73.6 ± 1.5	52.9 ±8.0	9.47
	PET_UV	42.7	13.8	29.0	57.9 ±0.8	37.8 ±2.6	7.42
	PS_DC	25.9	11.9	14.0	79.9 ± 2.6	63.5 ± 5.9	1.09
	PS_UV	33.7	18.1	15.6	71.3 ± 0.9	47.9 ± 1.6	1.69

post DOM incubation						
glass_DC	32.9	15.4	17.5	71.1 ± 5.5	50.7 ± 7.8	NA
glass_UV	37.7	12.1	25.6	64.0 ± 3.5	47.2 ± 3.7	NA
PET_DC	28.4	13.9	14.4	77.1 ± 2.3	58.5 ± 4.4	NA
PET_UV	38.9	18.8	20.1	64.1 ± 1.6	38.6 ± 1.1	NA
PS_DC	27.0	11.4	15.6	78.0 ± 1.9	62.3 ± 5.1	NA
PS_UV	31.7	18.4	15.6	74.7 ± 0.9	51.3 ± 1.3	NA

NA = not analyzed

DOM sorption. Between 1141 (glass_UV) and 7968 (SW) molecular formulas were assigned to 11756 – 19516 signals in the FT-ICR mass spectra (**Table S 2,** SI). Highest relative peak intensities centered around H/C = 1 and O/C = 0.6 (**Figure S 7**, SI) as the only maximum region for the SW. Pronounced occurrence of sulfur-containing molecular formulas indicated the large influence of anthropogenic inputs to this urban stream (**Figure 1B**).[58] SW-DOM selectively adsorbed to different substrates which could be identified by substrate-specific OM molecular composition (**Figure 1 and 2**, **Figure S 7**, **S 8**, **S 9**, SI). This adsorption of DOM to the different substrates was generally characterized by a small fraction of "unsaturated O-poor" molecules of 4 % in SW compared to corresponding largest fractions of "unsaturated O-poor" molecules with 17 % - 43 % in all substrate-bound OM samples (**Figure 1A**). While the SW displayed a weight-averaged molecular mass of 446 Da, all substrate-bound OM showed larger masses in the range of 495-600 Da (**Figure S 10, Table S 2,** SI).

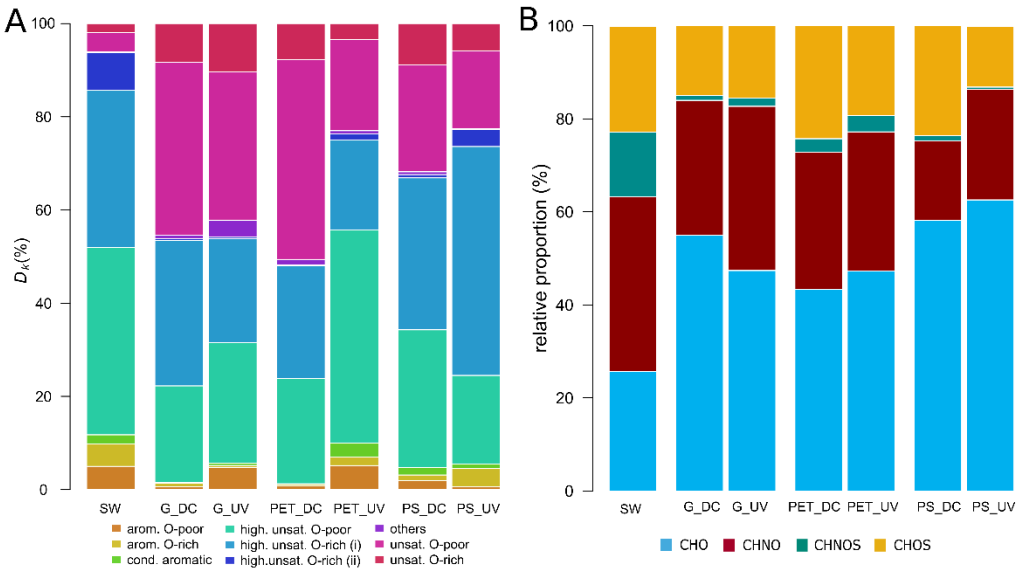

Figure 1: Quality of SW-DOM and surface-bound OM fractions based on **A**) the summarized relative intensity of each compound class defined by Kamjunke et al. (2019)[59] and **B**) relative proportion (%) of the prevailing (> 1 % occurrence) molecular formula classes CHO, CHNO, CHNOS and CHOS.

Preferentially adsorbed compound classes on glass were "unsaturated O-poor" (DC and UV 37 % and 32 %), "highly unsaturated O-rich (i)" (DC and UV 31 % and 22 %) and "highly unsaturated O-poor" (21 % and 26 % for DC and UV respectively) formulas (**Figure 1A, Table S 4**). We detected 296 molecular formulas for glass-bound OM (DC and UV) that were in common with the SW (**Figure 2A, Table S 3**, SI). Almost half of them (49.3 %) displayed similar signal intensities for both glass_DC and glass_UV (**Table S 3**, SI). Overall, both glass_DC and glass_UV showed similar sorption patterns towards DOM which was reflected in a homogenous distribution without conspicuous maximum or minimum region of molecular formulas with a distinct degree of saturation and oxygenation (**Figure 2A, Figure S 7**).

The greatest difference in the DOM adsorption pattern between DC and UV samples could be identified for PET and PS (**Figure 1 and 2, Figure S 7, S 8, S 9**). For molecular formulas co-occurring on PET_DC, PET_UV and SW, more than 55 % of the molecular formulas were enriched on PET_DC basically with more saturated molecular formulas of H/C > 1.2 and O/C < 0.6 (**Figure 2A**). Only a minor fraction of 5 % was enriched on PET_UV that was less saturated (H/C < 1.4). The higher amount of unsaturated molecules on PET_UV was reflected in the prominent relative increase of aromatic O-rich and O-poor and condensed aromatic molecules of up to more than one order of magnitude between PET_DC and PET_UV (**Figure 1A, Table S 4, Figure S 7**). This separation by the degree of saturation was especially detectable for signals unique to PET_DC and PET_UV (**Figure 2B**). PET_DC adsorbed two-fold more "unsaturated O-poor" molecules (43 %) than PET_UV (20 %). The HCA reflects the prominent differential sorption behavior of the pristine PET_DC and weathered PET_UV that separated them on different branches (**Figure 3**).

PS_DC tended to adsorb a substantially lower amount of "highly unsaturated O-rich (i)" molecules (33 %) compared to PS_UV (49 %) (**Figure 1A**). Molecular formulas with 1 <H/C 1.6 and 0.3 < O/C < 0.5 were enriched on PS_DC while molecules with H/C < 1.5 and O/C > 0.5 were enriched on PS_UV (**Figure 2C, S9E, F**). While the difference between PET_DC and PET_UV was mainly driven by the degree of saturation, PS_UV showed a clear trend towards higher oxygenated substances compared to PS_DC (**Figure 2C**). The unique features of the OM signature of PS were in accordance with the results of the HCA in which PS_DC and PS_UV were neighbor groups (**Figure 3**). Further, the relative proportions of formula classes CHO, CHNO, CHNOS

and CHOS of substrate-associated OM differed between the substrate types (glass, PET and PS) and were distinct from original SW DOM (**Figure 1B**).

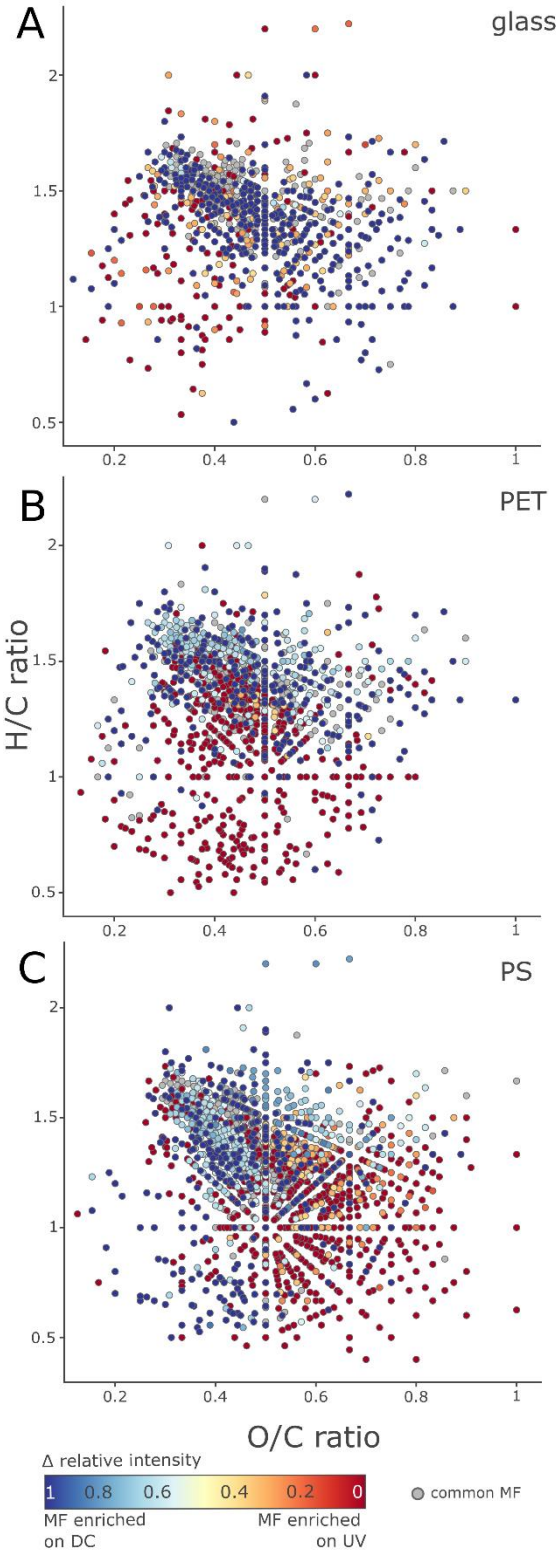

Figure 2: Van Krevelen diagrams (H/C over O/C ratios) representing the differences in the relative signal intensities of adsorbed OM in comparison with dark control (DC) and weathered (UV) substrates glass (A), PET (B) and PS (C) based on equation 1 (Δ relative intensity). Molecular formulas (MF) of each substrate

were reduced to those that co-occurred in the incubation SW. Solid blue and red molecular formulas represent those that were unique to the DC or UV substrate, respectively (without unique MFs see **Figure S 9**).

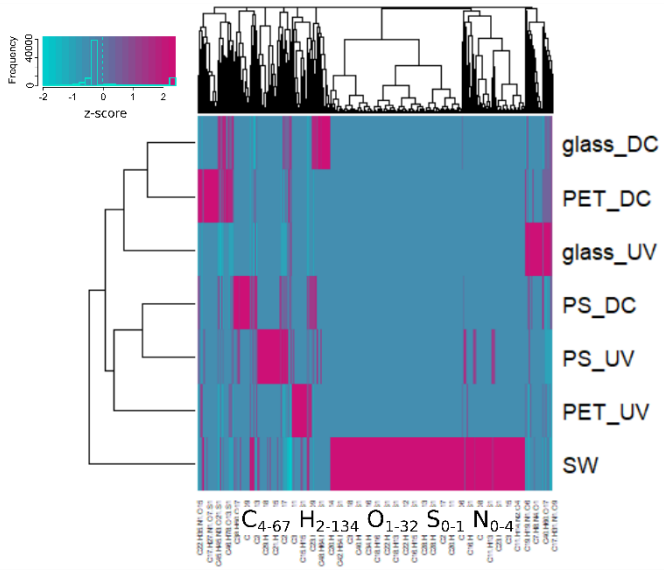

Figure 3: Hierarchical Cluster Analyses of Bray Curtis dissimilarities based on the blank-corrected scaled intensity data of all molecular formulas for the samples (SW), glass, PET and PS with respective dark control (DC) and weathering treatment (UV).

Microbial community analyses. We identified differences in relative abundances of the twelve most dominant prokaryotic ASVs between the inoculating community and the first microbial colonizers on the substrates (**Figure 4A**). *Falvobacterium* was the most abundant in both, the pelagic (0.2 µm) and the particle-associated (3 µm) SW fraction which is a typical genus for urban waters.[60] Genera such as *Prosthecobacter*, *Rheinheimera*, *Luteolibacter* and *Fluviicola* were present in either pelagic or particle-associated fractions. While *Methylotenera* (Methylophilaceae) was only present in low relative abundance in the inoculum (1 – 2 %), it was the most abundant first colonizing taxon after 24 h of incubation on glass (9 – 12 %) and with even higher abundances on the polymeric substrates (14 – 19 %) with indication of a treatment effect (DC vs UV). *Methylotenera* remained the predominant genus after 72 h of incubation with constantly high relative abundances especially on the two weathered polymeric materials PET_UV and PS_UV with both more than 18 %. After 72 h of incubation, the abundance of *Prostecobacter* (Verrucomicrobiaceae) increased by a factor of up to seven. Noteworthy, PET_UV and PS_UV showed the highest Chao1 Richness and Shannon Diversity values for the prokaryotic community with concurrently lowest Pielous Evenness values after 72 h which indicates selective processes leading to a community structure predominated by only a few taxa (**Figure S 11**, SI).

The inoculating eukaryotic community displayed high relative abundance of the ciliate family Peniculia (**Figure 4B**). A yet to be classified eukaryote predominated the 0.2 µm fraction while other prevailing pelagic taxa were ciliates (Ciliophora) and photosynthetic heterokonts (Ochrophytes). The radial centric diatoms (Melosirids) were amongst of the most relative abundant eukaryotic pioneer colonizers with preference to polymeric surfaces after 24 h. In contrast, they displayed higher relative abundances on glass after 72 h. Generally, the investigated early biofilm formation over 72 h of growth was highly dynamic with respect to abundances between the substrates and over time.

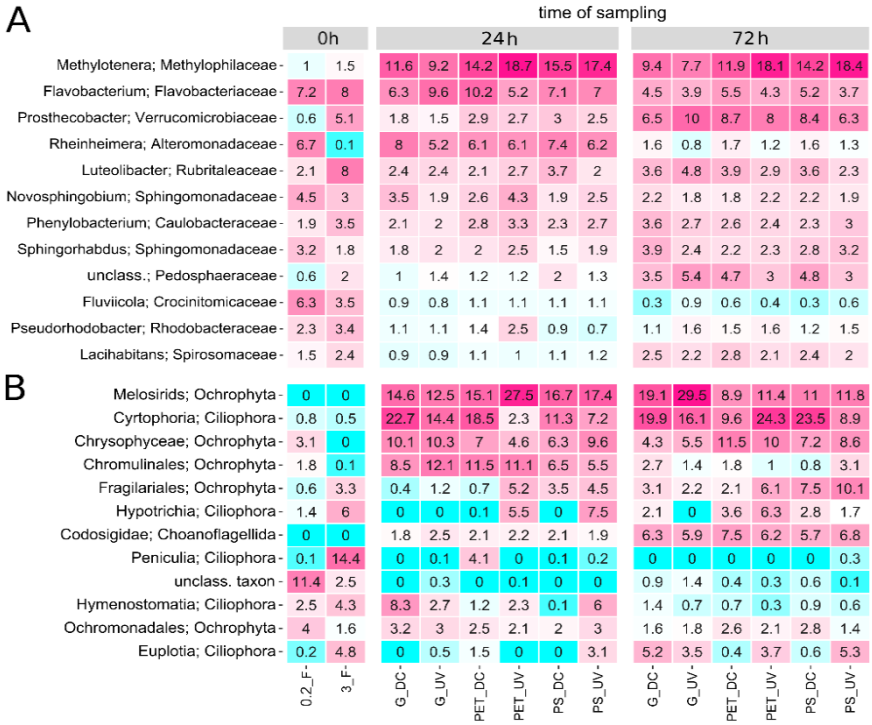

Figure 4: Relative abundances of the twelve most abundant taxa (where available with added genus) of prokaryotic and eukaryotic communities based on 16S (**A**) and 18S (**B**) gene amplicon sequencing after zero (0.2 µm (0.2_F) and 3 µm (3_F) filter fraction), 24 h and 72 h of incubation. Biofilms grew on glass (G), PET and PS with respective dark control (DC) and weathered (UV) substrates. The assignment of different taxonomic levels in the eukaryotic community resulted from the respective degree of taxonomic classification depth.

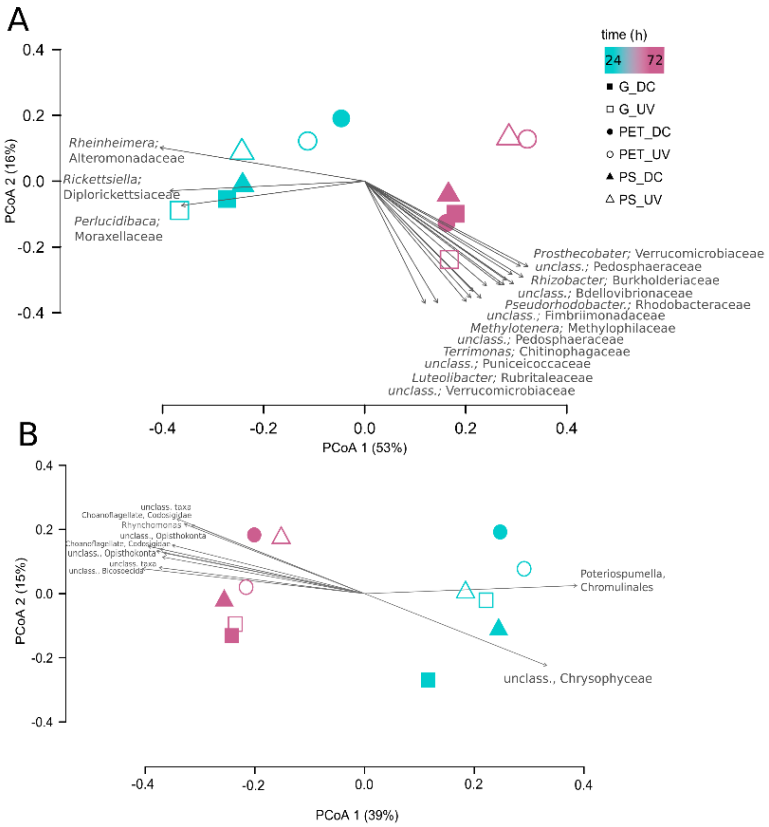

Figure 5: Principal coordination of prokaryotic (**A**) and eukaryotic (**B**) communities based on Bray-Curtis dissimilarities. Color codes represent the consecutive sampling events, different symbols and fills represent the substrate types glass (G), PET, PS with corresponding dark control (DC; filled) or weathered (UV; empty) treatment. The species scores represent statistically significant species driving the ordination which were identified by permutation tests. The assignment of different taxonomic levels in the eukaryotic community resulted from the respective degree of taxonomic classification depth.

For the prokaryotic community, the first PCO (53 % explained total variance) separated the samples along the time course in two distinct clusters (four when including the inoculating pelagic community (**Figure S 12A**, **S 13A**, SI)) (**Figure 5A**). A separation along the second PCO (16 % explained variance) was mainly driven by the different substrates. The pioneer colonizing communities on day one tended to group material-specifically by the substrates glass, PET and PS indicated by their close spatial proximity for corresponding dark controls and weathered samples (DC and UV) (**Figure 5A**). This clustering could be confirmed by a cluster analysis (**Figure S 13A**, SI). For the 24 h samples, the resulting dendrogram separated the substrates on different branches and grouped the corresponding DC and weathered UV-samples on the same branches (**Figure S 13A**, SI). In contrast, there was no material-specific separation after 72 h of incubation, the communities converged and grouping of the two weathered polymers was detected (**Figure 5A**, **S 13A**, SI). There was a statistically significant difference between the inoculating pelagic and the

attached biofilm-forming community ($F = 11.49$, $p < 0.001$, $R^2 = 0.42$, permutations=1000 (**Figure S 12A**, SI).

We could identify the substrate type (filters, glass, PET or PS), treatment (DC or UV) and the time course (24 h or 72 h) as statistically significant descriptors applying PERMANOVA (substrate: $F=8.47$, $p < 0.001$, $R^2 = 0.49$; treatment: $F = 04.75$, $p < 0.01$, $R^2= 0.18$; time: $F=6.73$, $p < 0.01$, $R^2 = 0.13$, permutations $n = 1000$) without significant interaction between these factors ($p > 0.05$). However, PERMANOVA only revealed statistically significant differences between each biofilm community (glass, PET and PS) and the pelagic SW community ($p < 0.05$) but not between biofilm samples from different substrates ($p > 0.05$). Taxa that were significantly associated with the separation of samples along the PCOs were also among those with high abundances as discussed earlier in Section 3.3 (**Figure 4A, Figure 5A**). Highly correlated with the first two PCO axes and predominant in their relative abundance were the genera *Methylotenera* mainly driving pioneer colonization, *Prosthecobacter* (Verrucomicrobiaceae) and *Rheinheimera* (Alteromonadaceae).

Similar to the prokaryotic data set, the eukaryotic communities were more dispersed after 24 h than after 72 h of incubation (**Figure 5B**). The first two PCOs explained up to 54% of variation which was driven by a separation along the time course of the experiment. A weak differentiation according to the substrate could also be detected for the eukaryotic community (i.e. for PET). Taxa that were significantly correlated with the PCOs were mainly flagellates (Poteriospumella, Chromulinales), unclassified Choanoflagellates, unclassified Chrysophyceae or Discicristata and Bicosoecida. There was a statistically significant difference between the inoculating SW and the biofilm communities ($F = 11.49$, $p < 0.001$, $R^2 = 0.42$) (**Figure S 12B**, SI). Furthermore, we could identify a significant influence of the substrate ($F = 8.47$, $p < 0.001$, $R^2 = 0.49$), the treatment ($F = 4.75$, $p < 0.001$, $R^2 = 0.18$) and time ($F = 6.73$, $p < 0.001$, $R^2 = 0.13$) without statistically significant interaction between those factors. A pairwise permutation revealed a significant difference between each respective substrate and the pelagic community ($p < 0.05$) but not between the biofilm samples themselves ($p > 0.05$).

Discussion

Surface properties. Whereas our surrogate material glass remained unaffected by the simulated weathering, we identified a decreased surface hydrophobicity for the two polymeric substrates after weathering. Following recently proposed surface classification thresholds for the water contact angel $\theta < 65°$,[61,62] we can conclude that glass_DC and, with respect to its large standard deviation,

glass_UV as well as PET_UV were weakly hydrophilic. The polymer samples PET_DC and PS_DC and PS_UV can be classified as weakly hydrophobic. The absence of changes in the FTIR spectra between glass_DC and glass_UV was due the inertness of glass (**Figure S 6A,** SI). Interestingly, the investigated polymeric materials (except PS_DC) became more hydrophobic upon DOM adsorption indicated by increased contact angles. As a result of surface conditioning by OM the materials remained distinct in their surface properties which is of relevance for further interpretation of the microbial community data. The change in material properties after conditioning certainly depended on the chemistry of the sorbates since Talluri et al. (2020)[23] observed increased hydrophilicity of polymeric materials after conditioning with BG-11 medium.[23] Modified surface characteristics after conditioning or eco-corona formation were also detected by Saavedra et al. (2019)[63] Whether the detected changes in surface hydrophobicity were caused by natural OM adsorbed from the SW or might be a result of an OM layer mixture of adsorbed OM and leaching substances from the plastic cannot be differentiated with the method applied. Further interpretations of the FTIR spectra can be found in the SI (**Surface Properties SI 7**, SI).

DOM sorption. Potential bias in the OM signatures may be caused by the applied experimental design by rinsing the substrates and enrichment via SPE. Still, similar OM signatures on glass surfaces with similar properties (DC and UV) demonstrated the robustness and reproducibility of the method applied using one pooled sample. Special attention was paid to the potential source of uncertainty by leaching substances and a discussion on our study limitations can be found in the SI (**Limitations of DOM experiment SI 10**, SI**)**. It should be noted that the summarized relative intensity shares of compound classes, the relative proportion of formula classes (**Figure 1A & B**) and the comparison of relative peak intensities (**Figure 2**) were based on molecular formulas stemming from the SW. However, the total entity of surface-bound DOM may be a mixture of adsorbed OM and OM that has leached from the polymer itself. The number-based contribution of MFs detected in the blanks that were in common with the respective MFs found in the adsorbed OM varied between 2 % (for PS_DC) and 23 % (for PET_UV).

The fractionation of SW-DOM to substrate-associated OM is most likely the result of selective adsorption driven by the physico-chemical properties of the surfaces of the different substrates (**Figure 1**). The absence of major changes in glass surface properties during weathering may have caused comparable sorption affinity to DOM for both glass materials which was reflected in highly similar OM signatures on glass slides irrespective of their weathering treatment (**Figure 2**). Differences in the peak intensities may be a result of low concentrations of OM sorbed to the glass (**Figure 2**). The observed low adsorption affinity of OM to glass surfaces compared well to low or no mass detection of OM (Suwannee River Humic Acid and Fulvic Acid) on silica surfaces (SiO_2)

made by Li et al. (2018)[11] Consequentially, molecules were measured around their detection limits which may explain the observed variation.

The most pronounced changes in the contact angle, the hereof-calculated SFE and carbonyl index for PET were in good agreement with the identified differences in the relative intensities of adsorbed OM. The generation of carboxylic acid end groups and moieties during weathering [64] may facilitate hydrogen bond formation for interactions with DOM. Similar to our results, Aflori and Drobota (2015)[65] attributed the altered sorption and adhesion behavior of PET towards collagen to surface modifications as a result of UV weathering. Taking into account that weathering induced a decrease in hydrophobicity and changes in the SFE of PET and PS, we can deduce that these changes in material surface properties might have caused substantial differences in the sorption behavior between PET_DC and PET_UV and PS_DC and PS_UV. Furthermore, the OM fingerprint of PS differed substantially from that of PET and glass which may also be attributed to its highly hydrophobic characteristics even for the weathered surface of PS_UV.

Li et al. (2018)[11] identified hydrophobic interactions to have a major impact on the deposition masses of OM onto hydrophobic PS surfaces. High ionic strength (in the presence of e.g. divalent cations) favored OM adsorption onto PS surfaces already at Mg^{2+} concentrations of 0.5 mM.[11] Since typically measured Mg^{2+} concentrations in the investigated SW ranged from 0.5 – 0.8 mM we can assume a substantial contribution of this surface adsorption mechanism to our observed high OM adsorption behavior of PS. This mechanistic explanation is supported by the observation of increased OM adsorption affinity to PS with increasing ionic strength.[66] Finally, our observations of preferential adsorption of higher molecular weight molecules to all investigated surfaces were in good agreement with studies by Davis and Gloor (1981)[67] on Al_2O_3, whereas Chi and Amy (2004)[68] observed the lower molecular weight of DOM to be adsorbed more favorably onto mineral surfaces. There were many CHOS and CHNOS formulas in the SW that might have influenced the substrates' surface properties due to their likely amphiphilic character. No equalizing masking effect that converged the surface properties could be observed. Moreover, surface properties (e.g. contact angle) post DOM incubation were shifted by a similar magnitude to more hydrophobic properties for most investigated substrates. Surfaces conditioned by an OM film seemed to reflect the underlying surface characteristics to a certain extent by passing the surface properties on to the OM-water interface.

We could confirm our first hypothesis that DOM adsorbs selectively to different (polymeric) substrates. Our second important observation is that the same polymeric material displayed altered OM sorption behavior when surface properties were pre-modified by artificial weathering. Upon

contact of DOM with the investigated substrates, it competes for binding sites. The adsorbed OM is consequentially the result of this selective mechanism. Whether the observed material-specific OM fractionation on different macro-sized polymer substrates applies to micro- and nanoplastic particles as well needs to be addressed in future research since this may have consequences not only for colonization but also for their toxicity. A recent study demonstrated reduced acute toxicity of nanoplastic particles due to a corona formation by humic substances.[14] Scanning electron microscopy demonstrated this corona formation on the surface of MP which reduced the sorption affinity to microalgae and minimized their ecotoxicity.[14] The importance of OM coatings of nanoparticles was demonstrated by a DOM signature-dependent sorption of phenanthrene.[15] Additionally, natural DOM signatures underlie seasonal and geographical fluctuations;[69] they may therefore vary with study site and time, and thus the investigated processes should also be considered under varying conditions. Noteworthy, plastics may leach a certain fraction of OM[70,71] that is available for adsorption to natural minerals and colloids.[72,73] Whether surfaces that have different conditioning OM fingerprints might also differ in early community structure will be discussed in the following.

Microbial communities. The observed high prokaryotic richness with co-occurring low evenness of the weathered PET_UV and PS_UV in contrast to the other samples may stem from species selection since these measures indicate the dominance of a minor number of taxa. Our results confirm what is commonly observed: substrate-associated microbial communities, including assemblages on MP substrates, differ from the inoculating community they originated from.[7,74-77] This difference resulted from adaptation to a certain life strategy or life cycle stage to an either sessile life form or as pelagic free living organisms. In our study, *Methylotenera* was a dominant primary colonizing taxon that was significantly correlated with the PCOs. However, it did not further increase in relative abundance until 72 h after the initial attachment. If this taxon depends on the first conditioning film it may stagnate in growth as a result of the primary adherents' utilization of the conditioning film as indicated in the initial reduction of total organic carbon levels by Siboni et al. (2007)[78]. Furthermore, this taxon seem to have a preference towards the polymeric substrates PET and PS especially to the weathered substrates PET_UV and PS_UV. *Methylotenera* is an obligate methyl utilizer[79] and was identified to play a key role in oil-contaminated soil and grouped together with bacteria that were mainly associated with organic contaminant degradation.[80] This taxon was recently detected to be present in high relative abundances in PE and PS-associated communities as well.[75]

The gammaproteobacterium *Ideonella* spec. was detected on all polymeric samples on both sampling days, however, in low abundances (data not shown). *Ideonella* was recently identified to be capable

of degrading PET.[81] These low abundant but distinct members of the community give rise to a material-specific selection to some extent. The material-specific clustering of the very first colonizing bacteria (after 24 h, **Figure S 13A**) may be driven by the material's unique surface characteristics which still, as discussed above, remain after a layer of DOM has been adsorbed. This observation highlights a common pattern between material surface properties, DOM signature and the early microbial colonization on different substrates. Noteworthy, the high relative abundances of many taxa on the two weathered materials PET_UV and PS_UV after 72 h support the hypothesis of being a more favorable substrate for settling. This might be due to potentially easier access to the polymer as a carbon source[82] or due to weathering-induced changes of the surface properties that support adhesion and that still remain even after sorption of an OM layer. Eyheraguibel et al. (2017)[70] demonstrated that water-soluble oligomers from polyethylene films could be biodegraded by *Rhodococcus rhodocchrous* after more than half a year indicating the potential use of plastic-derived leachates, despite this investigation had another time frame.

The material-specific attachment could also be detected for the most abundant eukaryotic pioneer colonizer which underlines our hypothesis. The material specificity disappeared after 72 h, however, the weathering effect became more prominent which may be a sign of accessibility of degraded polymers as a carbon source. Hypothetically, this observation of material specificity may continue under certain conditions as seen for marine studies where a material selectivity of mature biofilms on plastic and glass substrates could be observed.[27,77] Oberbeckmann et al. (2017)[75] hypothesized material specificity under certain nutrient-limited conditions that may also be reflected in the conditioning film. In our study, any nutrient limitation was unlikely since the incubation water showed high levels of DOC (8.5 mg/L) and because the stream is impacted by rural and municipal sources with generally high nutrient loads (NO_3 = 4-13 mg/L, PO_4 = 0.1-0.3 mg/L based on long-term records). The material-specific communities on day one (although not significant but detectable for the most dominant pioneer colonizers) may have been controlled indirectly by the surface's physico-chemistry that passes on its distinct features to the OM-water interface forming a distinct conditioning film of adsorbed OM. The presented data supports this hypothesis since the material properties were still not similar after OM adsorption (no equalizing masking effect) and OM signatures appeared to be material-dependent. The herein proposed process of early biofilm formation on polymer surfaces is supported by studies with similar results but on non-polymeric materials.[21,83,84]

The analysis of microbial communities attaching to the above discussed conditioned substrates provided minimal support for our second hypothesis of a community structure with tendency of material-specific primary colonization. However, this tendency diminished until sampling after 72

h of incubation and the weathering effect became more prominent. Generally, the microbial communities converged with time which was already observed by Harrison et al. (2014)[85] for plastic-associated communities and which is in accordance with the hypothesis proposed in the framework of the species sorting theory: It states that the power of species sorting during microbial community assembly is dictated by habitat conditions, duration and the structure of the source community.[86] The question remains if nutrient-limitation may further promote a material-specific 'plastisphere' after the herein demonstrated material specificity of early microbial attachment processes have faded. Whether such plastic-associated communities show similar overall ecological functional capacities (such as nutrient turnover and/or primary production) as communities on natural substrates should be investigated in future studies.

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Appendix A. Supplementary Information

Supporting Information to this article can be found free of charge at http://dx.doiXXXXXXXXXX

Additional Information includes Pilot Study (SI 1), details on weathering exposure scenario (SI 2), material properties (SI 3), DOM sorption experiment and respective data analyses (SI 4 and 5), details on amplicon sequencing (SI 6), details on surface properties (SI 7), results on the DOM and microbial community study (SI 8 and 9). Furthermore, it includes the tables for DOC concentrations (table S1), metadata for FT-ICR MS measurements (Table S2), number of molecular formulas enrichend on differnet substrates (Table S3) and a summary of assigned compound

classes and formula classes (Table S4 and S5). Supplementary figures provided are 3D EEM preliminary results (Figure S1), experimental setups (Figure S2), Quality Control for FT-ICR MS measurements (Figure S3), Rarefaction curves (Figure S4), surface properties (Figure S5), FTIR data (Figure S6), van Krevelen plots (Figure S7, S8, S9), histograms of molecular masses (Figure S10), Alpha Diversity values (Figure S 11) and the PCoA and hierarchical cluster analyses (Figure S12 and S13).

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Manuscript III

Biofilm succession on plastic surfaces (in preparation)

Christoph Rummel[a], Thomas Neu[b], René Kallies[c], Mechthild Schmitt-Jansen[a]

[a] Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany

[b]Department of River Ecology, Helmholtz Centre for Environmental Research – UFZ, Brückstr. 3a, 39114 Magdeburg

[c] Department of Environmental Microbiology, Helmholtz Centre for Environmental Research – UFZ, Permoserstr. 15, 04318 Leipzig, Germany

This manuscript is currently in preparation

Introduction

Microbial communities can greatly differ from the surrounding pelagic water community (Oberbeckmann et al. (2017), Kesy et al. (2019)). In Rummel et al. (2021) (in preparation), we detected high taxonomic variation between early microbial communities grown on different polymer substrate types. This phenomenon may eventually be ecologically relevant if a material-specific community structure and function holds true for mature biofilms since biomass-wise they will represent the major fraction of aquatic biofilms (in contrast to small amounts of new available habitable surfaces where rapid biofilm succession takes place). Researchers have detected plastic-specific core-communities, often termed "Plastisphere" (Zettler et al., 2013). However, epiplastic communities would then play an ecological role if they are present at relevant abundances (that certainly depends on plastic loads and inputs) to cause any downstream response by *e.g.* providing beneficial key processes to ecosystem functioning or modulating or restricting certain functions. In order to draw some general conclusions on biofilm succession on different polymers with respect to their ecological functioning, we conducted a four week incubation study using stream water (SW) in microcosms. We were specifically interested whether there may be differences in biofilm growth between different polymer substrates and between those that were artificially pre-weathered. In Rummel et al. (2021) (in preparation), we demonstrated changes in material surface properties under artificial weathering conditions that might have affected the early succession. Therefore, we wanted to test the hypothesis if these differences remain for mature biofilms and if this may have implications for their biomass or photosynthetic capacities. During the course of the experiment of over 4 weeks, we took samples and measured biofilm biomasses of different components, the photosynthetic capacity and investigated the prokaryotic community. Biofilm biomass was calculated based on voxel counts derived from confocal Laser Scanning Microscopy (cLSM) and subsequent imaging analyses. The biofilms' photosynthetic activity was measured using Puls Amplitude Fluorometry (PAM) while the community structure was analyzed by next generation sequencing techniques (NGS).

Material and Methods

Material and Chemicals. 37 % Formaldehyde solution (Sigma Aldrich) was used as a fixative for the cLSM samples. Mostly additive free polymer sheets of Polyethylene terephthalate (PET) and Polystyrene were purchased from Goodfellow GmbH (Hamburg, Germany) with a thickness of 1 mm and 1.2 mm. Glass microscopy slides were purchased from Labsolute, Th. Geyer GmbH & Co. KG Renningen, Germany).

Artificial weathering. Detailed description of the artificial weathering is provided in the SI of *Manuscript II* (Exposure scenario SI 2, SI). In short, each sample type was artificially weathered using a QLab QSun solar simulation for 288h of exposure. Hereafter, these pre-weathered samples were named glass_UV, PET_UV and PS_UV while the controls kept in darkness were named glass_DC, PET_DC and PS_DC

Biofilm microcosms. Detailed description of the microcosms and culturing of biofilms is given in *Manuscript II*. In short, biofilms were grown on polymer and glass slides for 1, 3, 7, 12, 20 and 32 days in various microcosms filled with 14 L stream water. Stream water got renewed every week during the course of the experiment to circumvent any nutrient depletion (absence of nutrient limitation was surveyed via chemical analyses – data not shown). On all sampling days, a subset of 5 slides from randomly chosen aquaria were retrieved from the microcosms and carefully rinsed in 0.2 μm filtered stream water to remove loosely attached biofilm fractions. On sampling days 7 – 32, two slides of each material were then measured at the Imaging PAM (Walz) at six randomly chosen measurement points. A subset of the slides were scraped off and snap frozen in liquid nitrogen for NGS (day 1-32). The slides with the remaining biofilm were then fixed in formaldehyde for subsequent CLSM analyses (day 1- 32).

Confocal Laser Scanning Microscopy. Biofilm samples were stained using lectin-specific fluorochrome Alexa 568 to investigate the glycoconjugates of the EPS matrix (Neu & Kuhlicke, 2017), SYBR Green as a nucleid acid-specific stain widely used to detect prokaryotes and autotrophic organisms were detected by the autofluorescence signal of Chlorophyll (Luef et al., 2009). On each slide 10 measurement points were acquired using the TCS SP1 and a TCS SP5X (Leica, Wetzlar, Germany) and the LAS AF software in the respective emission signal ranges.

Data evaluation was done using an in-house KNIME workflow to analyze and count voxels of each channel (Bacteria, Algae and EPS) (great acknowledgments for supervision go to Elisabeth Teixido). As can be seen in Figure .1 and the time series of Figures .2 – .4, autofluorescence of the (mostly weathered) polymers was especially critical in the green channel for bacteria determination. Black and white thresholds for voxel determination were difficult to set and either over or under

estimated the amount of voxels to count. Hence, the KNIME workflow was extended by a Gaussian filter to subtract the blurry background from each z-stack and to increase measurement accuracy (see **Figure .1**).

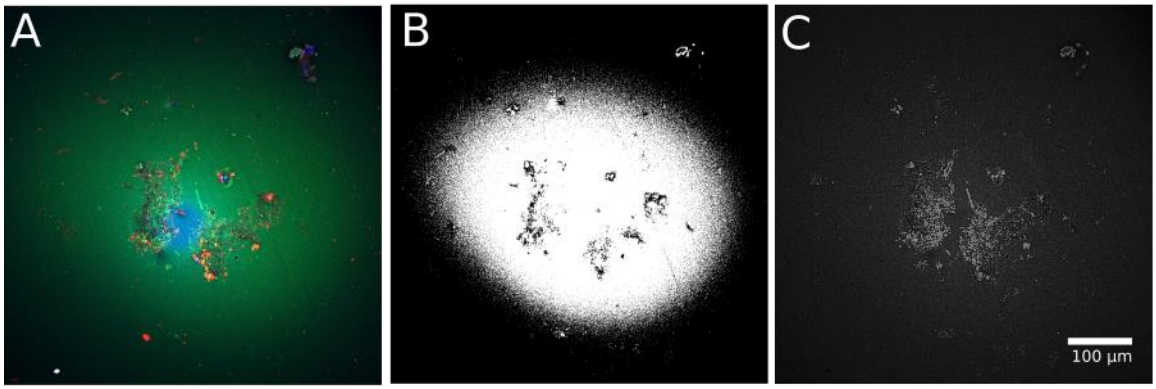

Figure 1: Application of a Gaussian filter in the green channel (Bacteria) of the z-stack cLSM images to subtract blurry background signals stemming from interfering autofluorescence of the substrate. **A:** Maximum projection of a z-stack of PET after 7 days of incubation. **B:** Black and white image without correction in the green channel. **C:** Gaussian filter corrected image of the SYBR green channel for accurate biovolume calculation.

Next Generation Sequencing. A detailed description of the DNA extraction and NGS protocol can be found in the Material and Method section of *Manscript II* and in "Illumina 16S protocol (Illumina)".

Statistical analyses. cLSM data (Bacteria, Algae and EPS biovolumes) was log transformed and cLSM and PAM data was fitted by a specific generalized additive mixed model (GAMM). For best-fit model selection the cLSM and PAM responses were fitted as a function of time and weathering treatment using the R package "mgcv" to test for differences between the substrates and weathering treatment. Gamma distribution was chosen since cLSM data is count data. To increase model accuracy we included the different microcosms as a random factor and to account for any potential bias by biofilms growing in different microcosms. Based on varying number of knots for time (k=2-6) and inclusion of the factors substrate and weathering the best fit model was chosen using the Akaike Information Criterion (AIC) and predicted mean and confidence interval of the mean were plotted over time in R studio.

Microbial community data based on amplicon sequence variants (ASVs) was bioinformatically generated using Qiime 2.0 as described in *Manuscript II*. Non-metric multidimensional scaling plot was calculated based on Bray Curtis dissimilarity matrix using the *vegan* package in R Studio.

Results

Biofilms in the microcosms showed increasing growth rates until roughly day 7 and growth seemed to stagnate after day 7 and 12. There was no detectable nutrient depletion that could have explained this observation (data not shown). Biovolumes of bacteria, algae and EPS matrix over 32 days of incubation did not reveal any clear differences between the substrates glass, PET and PS (confidence bands overlap), however, generalized additive model including substrate as a factor resulted in the smallest AIC and therefore in best model fit (**Figure .5 A-C**). Only on sampling day 7, there is a slight indication of different algae biomass on the different substrate types based on the fluorescence values (F0) measured by PAM (**Figure .5 D**). CLSM confirmed higher corresponding algae biomass for PET than for PS and glass (**Figure .5 B**). No difference in photosynthetic activity was observed between the biofilms on different substrates. Growth rates differed statistically significant between PET_DC and PET_UV indicated by a lower AIC when including the factor age in the fitted GAMM (**Figure .6 D**) since PET_DC displayed higher abundance of Prokaryotes. This difference remained until the end of 32 days of incubation. Interestingly, more EPS at early time points for PET_DC could be detected (**Figure .6 F**). Also for PS_DC higher bacterial abundance was detected for early biofilm colonizers (day 1 & 3) however these differences disappeared with ongoing incubation time. PS_DC and UV showed varying bacterial growth rates until day 12 which then leveled off to a constant growth until day 32. Algae growth was very similar across all substrates and treatments. For glass as a control resembling a natural substrate, all investigated biofilm structures (bacteria, algae and EPS) showed similar growth pattern irrespective of the weathering treatment.

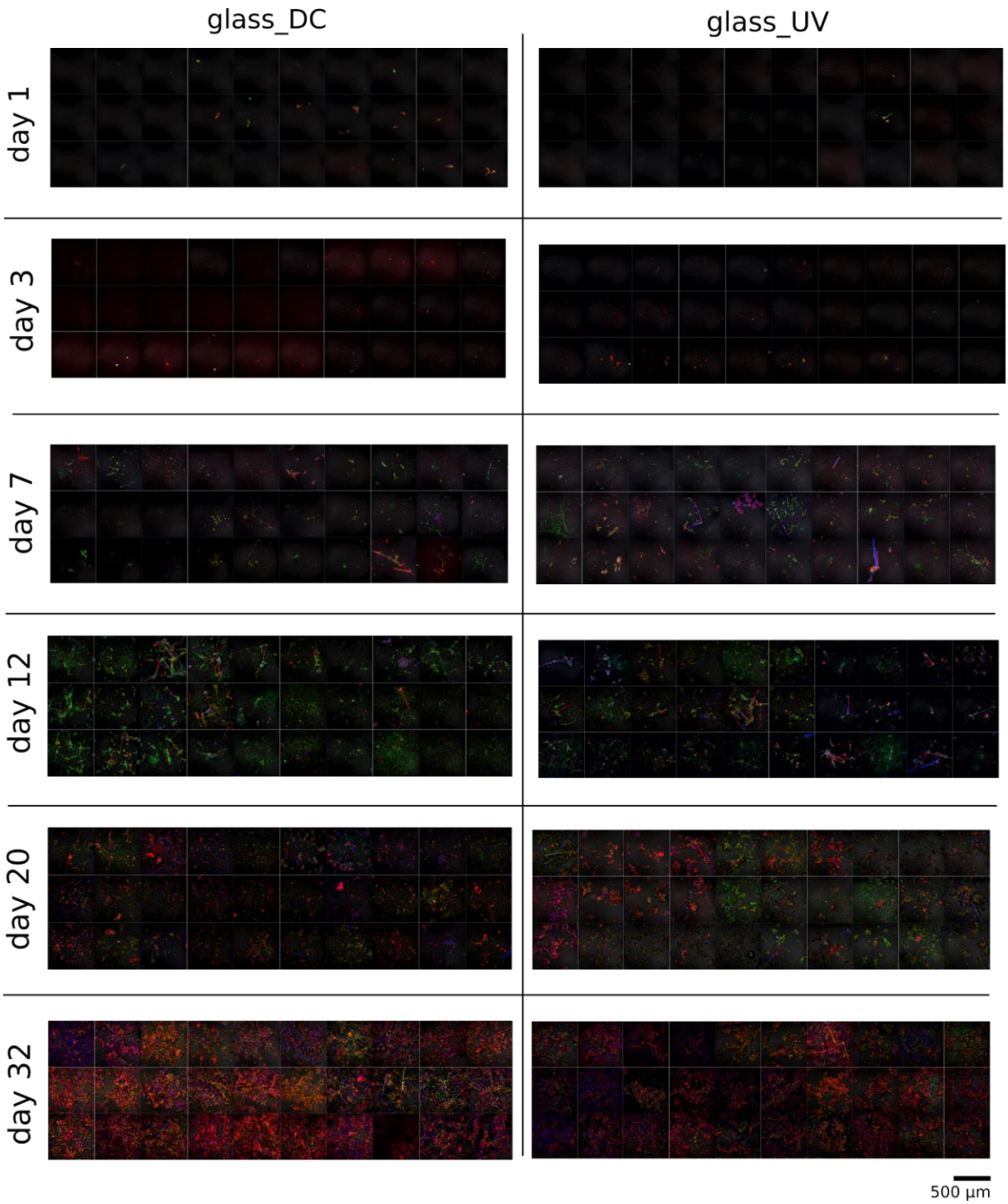

Figure 2: Time series of biofilm cLSM images of glass DC (left) and UV (right) of 32 days of incubation. Stainings correspond to green=Prokaryotes, blue=algae/Chlorophyll, red=EPS matrix.

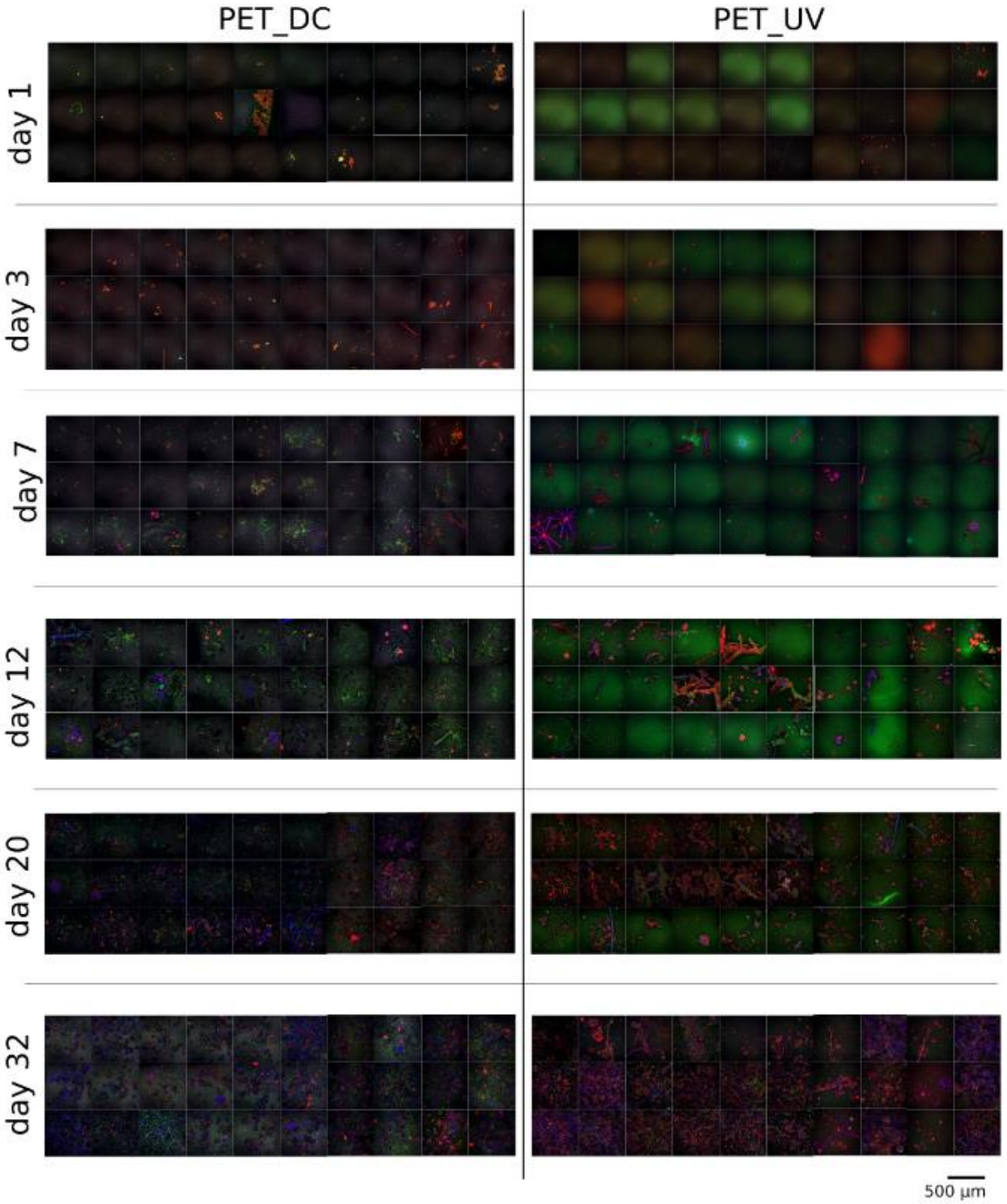

Figure 3: Example for the time series of biofilm cLSM images of PET DC (left) and UV (right) over 32 days of incubation. Stainings correspond to green=Prokaryotes, blue=algae/Chlorophyll, red=EPS matrix.

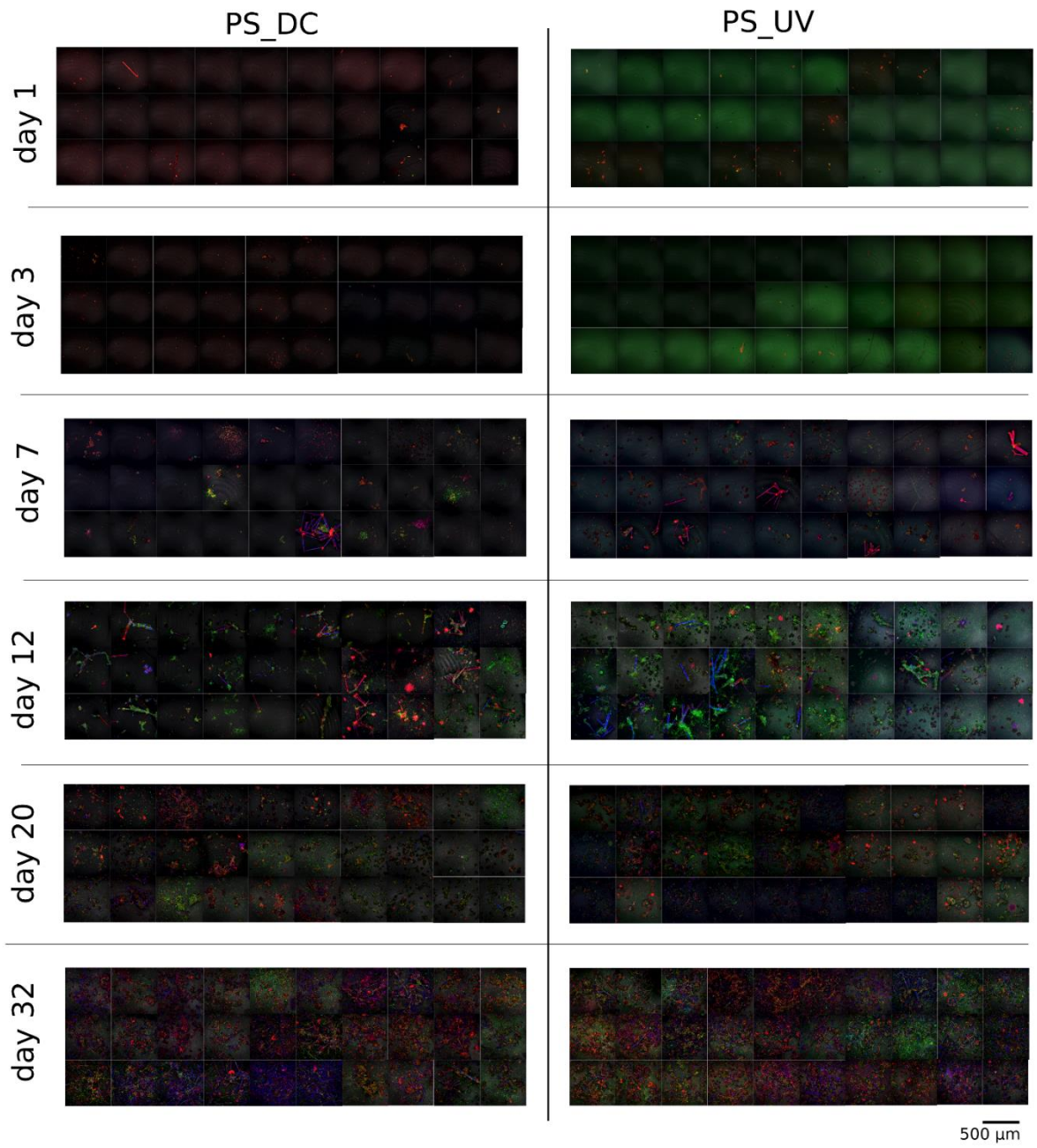

Figure 4: Time series of biofilm cLSM images of PS DC (left) and UV (right) of 32 days of incubation. Stainings correspond to green=Prokaryotes, blue=algae/Chlorophyll, red=EPS matrix.

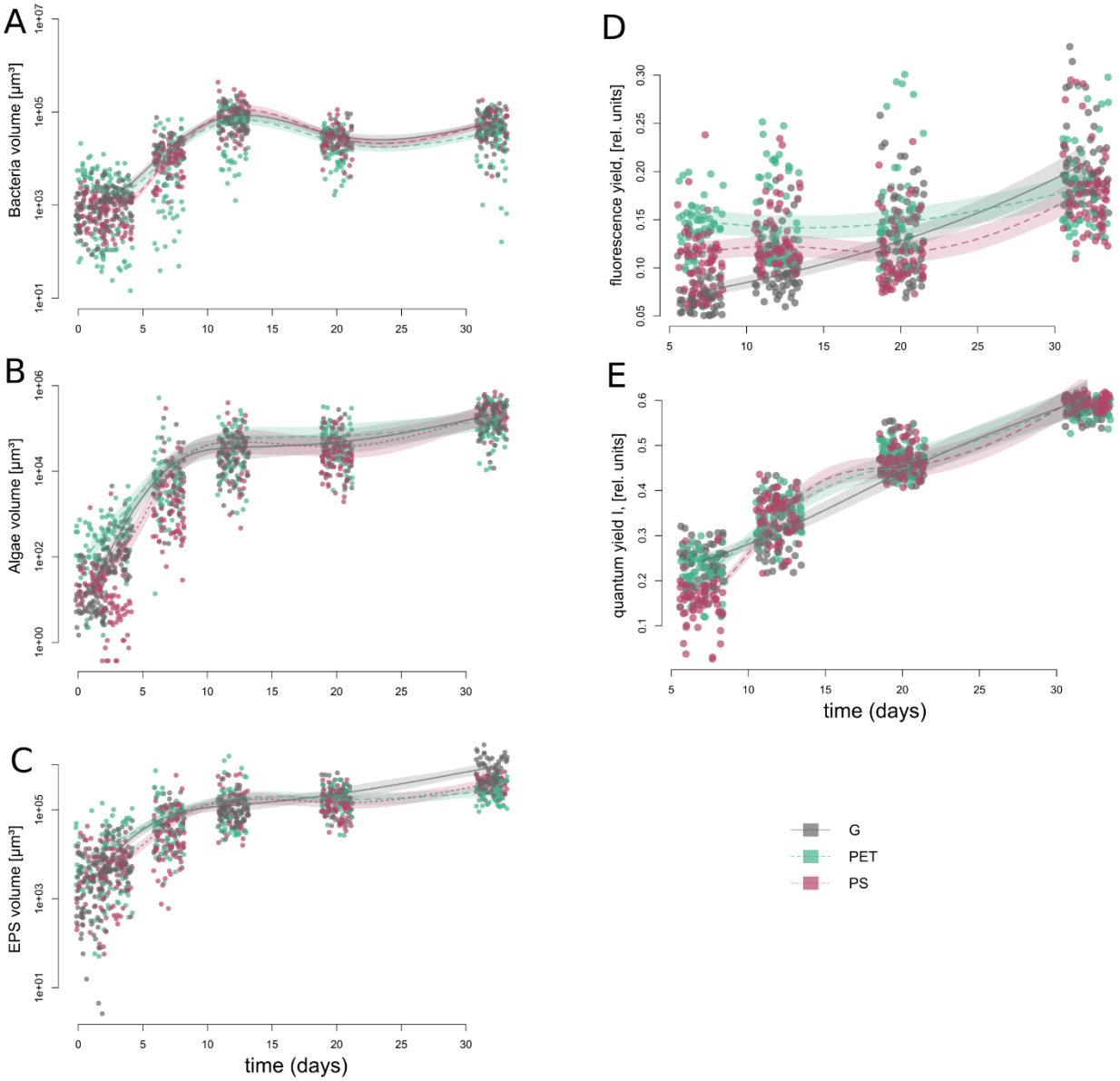

Figure 5: CLSM and PAM data fitted with a generalized additive mixed models. The biovolumes of bacteria, algae and EPS matrix over 32 days of incubation do not reveal any significant difference between the substrates glass, PET and PS (**A-C**). PAM data (**D:** F0, fluorescence yield; **E:** quantum yield) on sampling days 7-32. Y-axis is log-scaled

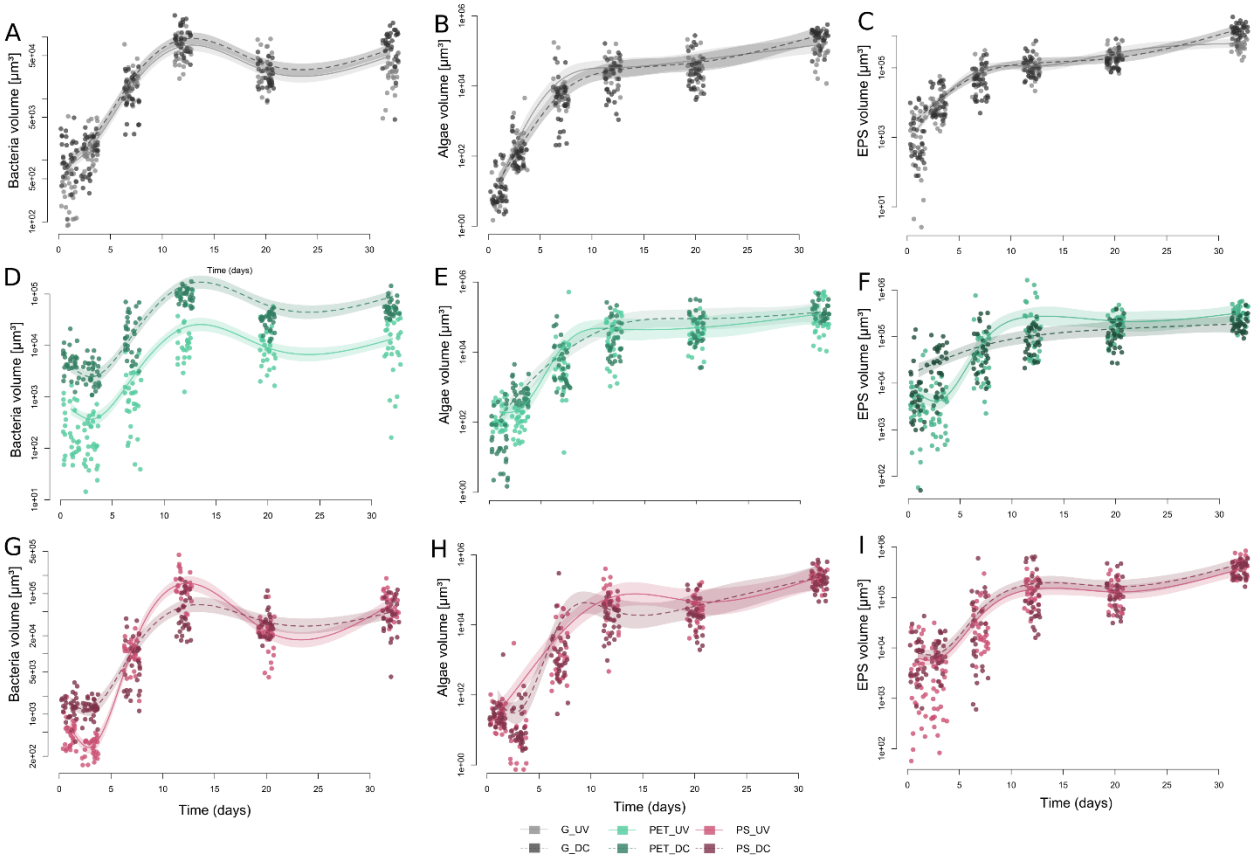

Figure 6 CLSM data fitted with a generalized additive mixed models on the biovolumes [µm³] of bacteria, algae and EPS matrix comparing weathered (UV) and pristine (DC) substrates over 32 days of incubation. **A-C:** comparison of glass_DC and UV in the respective biovolumes. **D-F:** PET_DC shows higher abundance of Prokaryontes than PET_UV, while there is no clear difference in algae biomass. At sampling time 1 and 3 PET_DC showed also higher EPS shares. **G-I:** Biovolumes of PS_DC and PS_UV. Solid and dashed lines are the predicted means with confidence bands as shaded areas. Y-axis is log-scaled

The biofilm community structure follows clearly the same trend as already described in *Manuscript II*. At early time points, biofilm community differed greatly between substrates and treatments which more and more equalized to a similar community structure with increasing incubation time (**Figure .7**). Noteworthy is the great dissimilarity between PET_DC and PET_UV at the very first sampling day 1 which was reflected in the NMDS by locating those samples most distant. Glass_DC and UV lay close together even on sampling day 1. The inoculating community did not change much during the course of the experiment and, accordingly all 0.2 µm and 3 µm filter samples grouped together (**Figure 7**).

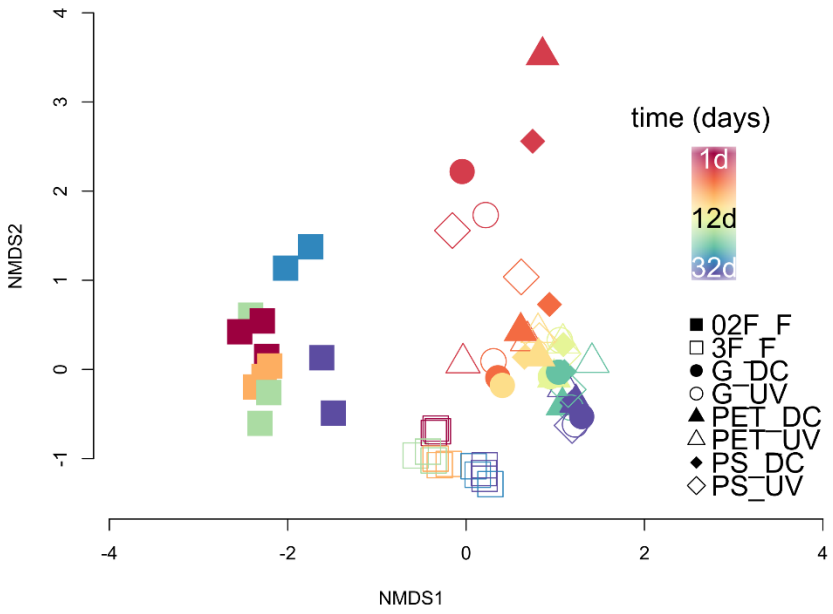

Figure 7: Non metric multidimensional scaling plot on the Bray Curtis dissimilarity matrix of ASVs. Color code represent the different sampling days 1-32, symbols indicate the different substrates with solid and empty symbol for dark and UV treated samples.

Discussion

The most essential conclusion from the presented data is, that after more than four weeks of incubation almost all measured and evaluated endpoints (biomass, photosynthesis, community structure) did not reveal any differences between biofilms grown on either glass, PET or PS substrates. Nor we did observe differences between the UV and DC treated substrates. The period of greater variance and detectable differences were the early time points between day 1-12. Interestingly, only the polymeric materials displayed variation in growth at the early phase of colonization which may reflect the influence of the materials surface properties. Similar to the DOM quality on glass (*Manuscript II*), no differences in bacteria, algae or EPS could be detected on glass_DC and UV which may be attributed to the absence of any changes with weathering due to the inertness of the material. Differential growth could only observed for the substrates where the material properties changed with weathering (PET and PS) from more hydrophobic to hydrophilic substrates (see section "material properties" in *Manuscript II*). This variation was especially pronounced for PET which was then reflected even in the highly dynamic community structure at early colonization. Further, differences in bacterial growth between PET_DC and UV may be the result of different amount of EPS at early time points potentially enabling the increased cell adhesion and attachment.

Other authors detected significantly different biofilm compositions on Polymethylmethacrylate (PMMA) compared to polycarbonate and glass (Vosshage et al., 2018). In this study this altered

biofilm quality had even indirect effects on higher trophic levels via grazing. These structural findings contrast other molecular community studies which compare well to our NGS results of converging communities. Harrison et al. (2014), Li et al. (2017) and Pinto et al. (2019) reported highly comparable data of community succession with strong convergence within a few days, or weeks or months.

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