Gulizia, Alexandra M. (2023) ***The preparation and characterisation of microplastics containing contaminants.*** **PhD Thesis, James Cook University.**

The preparation and characterisation of microplastics containing contaminants

This thesis was submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Natural and Physical Sciences)

by

Alexandra M. Gulizia (BSc. Hons.)

2023

College of Science and Engineering

James Cook University

Statement of sources

DECLARATION

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. The information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

This thesis is dedicated to my late Nonnas, Michelina Caligiuri and Giuseppina Gulizia,

La tua fiducia è stata preminata, sono davvero una dottoressa di scienza!

Acknowledgments

Firstly, I would like to extend my utmost gratitude to my supervisors, A/Prof. George Vamvounis, Dr. Cherie Motti and Dr. Lynne Van Herwerden for their unwavering guidance and mentorship throughout my Ph.D. Thank you for taking a chance on me almost 5 years ago, from the very beginning you were pillars of knowledge and support, with an endless drive and focus I always strived to match! I would not have had as many successes during my time here if it weren't for you all. I never imagined a line of work that could so perfectly combine my academic interests, and it remains a "pinch-me" moment that my Ph.D., experience has been one of constant learning with exciting new avenues to collaborate and grow as a researcher. Your passion for research and willingness to explore the field of microplastics with me alongside has allowed me to pursue a career I always dreamed of.

Throughout my Ph.D., I always felt like I existed in a limbo between the chemistry, marine science and engineering disciplines, however, it was this web of collaborators and inter-disciplinary thinking from peoples across JCU Engineering, Chemistry and AIMS that shaped the direction and outcomes of my thesis. I extend a special thanks to Dr. Bronson Philippa and Dr. Marina Santana (my unofficial supervisors) who helped me navigate the mathematical and marine science challenges, providing words of advice, boundless knowledge and ideas. To Kishan Patel, Eve Brodie, Renee Daunmuller, Sarah Bloom and Jess Zacharuk, your work in the lab facilitated this thesis, and gave me a stepping stone to build upon for my research. To Prof. David Bourne, Dr. Peter Thomas-Hall, Dr. Yui Sato, Dr. Yun-Kit Yeoh, Dr. Richard Edmunds, Dr. Sara Bell and Dr. Felicity Keuk, you all were instrumental in the completion of my final chapter; comprehending the bioinformatics and microbiology sections would not have been possible without your swift answers to my seemingly endless list questions. Of course, my thesis would not have been so joyously completed without my Ph.D-brother by my side, Dr. Angus Shephard, completing this milestone alongside you has been a privilege and I am grateful that we got to share this experience together.

To my family; especially my parents, brother, Aunty Joan, Uncle Tony and many cousins, when I left Melbourne in 2018 I could not have predicted to still be here in Townsville almost 6 years later. You were always there to share my excitement and show empathy when I struggled, your belief in me allowed me to believe in myself. To my Mum – my biggest supporter – you are always in my corner and always available to listen to me (no matter the time of day or night), and I truly cannot thank you enough for the fierce and limitless care you show me. To my Dad, you pioneered the direction of my life, nurturing and encouraging my passion for learning and research science from a young age, I feel honoured to be shadowing you and somewhat following in your footsteps. To my brother and sister in-law, you are both insanely driven and successful, and a constant reassurance that anything is achievable with hard work and people by your side. Lastly, to my best friends and soul sisters, Brodie Visintin,

Emma Peixoto and Alex Chenney; you guys are truly the lights of my life and a reminder that lifelong friendships transcend time and distance. Alex, meeting you was a chance encounter and without you, Townsville would not have felt like a home.

Finally, I would like to acknowledge the Traditional Owners and Custodians of the Land and Sea Country upon which I have conducted this body of work, and to which I have called home: the Wulgurukaba, Bindal, Djabugay, Yirrganydji, and Gimuy Yidinji Peoples. I extend my respects to their Elders past, present and emerging, and thank them for the contribution as our Country's first scientists. This Country always was and always will be Aboriginal land.

Publications associated with this thesis

Gulizia, A. M.; Brodie, E.; Daumuller, R.; Bloom, S. B.; Corbett, T.; Santana, M. M. F.; Motti, C. A.; Vamvounis, G. Evaluating the Effect of Chemical Digestion Treatments on Polystyrene Microplastics: Recommended Updates to Chemical Digestion Protocols. *Macromol. Chem. Phys.* **2022**, *223* (13), 2100485. https://doi.org/10.1002/macp.202100485.

Gulizia, A. M.; Patel, K.; Philippa, B.; Motti, C. A.; van Herwerden, L.; Vamvounis, G. Understanding Plasticiser Leaching from Polystyrene Microplastics. *Sci. Total Environ.* **2023**, *857*, 159099. https://doi.org/10.1016/j.scitotenv.2022.159099.

Gulizia, A. M.; Philippa, B.; Zachurak, J.; Motti, C. A.; Vamvounis, G. Plasticiser leaching from polyvinyl chloride microplastics and the implication for environmental risk assessment. Mar. Poll. Bul. 2023, 195, 115392. https://doi.org/10.1016/j.marpolbul.2023.115392

McCormick, M. I.; Chivers, D. P.; Ferrari, M. C. O.; Blandford, M. I.; Nanninga, G. B.; Richardson, C.; Fakan, E. P.; Vamvounis, G.; **Gulizia, A. M**.; Allan, B. J. M. Microplastic Exposure Interacts with Habitat Degradation to Affect Behaviour and Survival of Juvenile Fish in the Field. *Proc. R. Soc. B Biol. Sci.* **2020**, *287* (1937), 20201947. https://doi.org/10.1098/rspb.2020.1947.

Conference Presentations

Gulizia, A. M., Brodie, E., Santana M. MF., Bloom, S. B., Daunmuller, R., Corbett, T., Motti, C. A., Vamvounis, G., **2020**. Chemical digestion methods: what are the real impacts on microplastics? RACI Queensland Annual Chemistry Symposium [*Online*]

Gulizia, A. M., Brodie, E., Santana M. MF., Bloom, S. B., Daunmuller, R., Corbett, T., Motti, C. A., Vamvounis, G., **2020**. Chemical digestion methods can degrade microplastics during extraction. MICRO2020 [*Online*]

Gulizia, A. M., Brodie, E., Santana M. MF., Bloom, S. B., Daunmuller, R., Corbett, T., Motti, C. A., Vamvounis, G., **2021**. Evaluating the effect of common chemical digestion treatments on microplastics. RACI Queensland Student Chemistry Symposium [*Townsville, Australia*]

Gulizia, A. M., Patel, K., Motti, C. A., Vamvounis, G., **2021**. Characterising the leaching behaviours of microplastics. AIMS Student Seminar Day [*Townsville, Australia*]

Gulizia, A. M., Patel, K., Motti, C. A., Vamvounis, G., **2021**. The leaching of plasticisers from microplastics. Society of Environmental Toxicology and Chemistry Australasia Conference [*Online*]

Gulizia, A. M., Patel, K., Philippa, B., Motti, C. A., Van Herwerden, L., Vamvounis, G., **2022**. Understanding the leaching behaviours of plasticisers from polystyrene microplastics. RACI National Congress [*Brisbane, Australia*]

Gulizia, A. M., Patel, K., Philippa, B., Motti, C. A., Van Herwerden, L., Vamvounis, G., **2022**. Understanding the leaching behaviours of plasticisers from polystyrene microplastics. JCU HDR Engineering Conference Day [*Townsville, Australia*]

Gulizia, A. M., Patel, K., Philippa, B., Motti, C. A., Van Herwerden, L., Vamvounis, G., **2022**. Understanding the leaching behaviours of plasticisers from microplastic polymers. Australian Marine Science Association 2022 [*Cairns, Australia*]

Gulizia, A. M., Patel, K., Philippa, B., Motti, C. A., Van Herwerden, L., Vamvounis, G., **2022**. Understanding plasticiser leaching from microplastic polymers. AIMS Student Seminar Day [*Townsville, Australia*]

Gulizia, A. M, Bell, S., Keuk, F., Santana, M. MF., Yeoh, YK., Sato, Y., Edmunds, R., Van Herwerden, L., Motti, C. A., Bourne, D., Vamvounis, G. **2023**. Biofilm formation on plasticised polystyrene and polyvinyl chloride microplastics in the marine environment. Society of Environmental Toxicology and Chemistry [*Townsville, Australia*]

Statement of contribution of others

Supervisors

A/Prof. George Vamvounis, James Cook University

Dr. Cherie Motti, Australian Institute of Marine Science

Dr. Lynne Van Herwerden, James Cook University

Volunteer and student contributions

Eve Brodie

Renee Daunmuller

Sarah Bloom

Jess Zachurak

Taylor Corbett

Kishan Patel

Additional support

Microplastics team

Bronson Philippa, Marina Santana and Emily Washington

JCU Engineering and Chemistry

Sandile Gwebu, Troy Poole, Josef Kunta, Ruilan Liu, Yang Liu, Mark Robertson, Angus Shephard and Zhifang Guo

JCU AAC

AIMS team

David Bourne, Michaela Miller, Peter Thomas-Hall, Yun-Kit Yeoh, Yui Sato, Sara Bell, Richard Edmunds, Felicity Keuk, Gretel Waugh, Katarina Damjanovic, Lee Bastin, Tom Barker, Andrea Severati and Pirjo Haikola

Funding bodies

JCU and the Australian Government Research Training Program and Stipend

JCU Competitive Research Training Grant

AIMS@JCU Pilot Research Award

AIMS@JCU Science Communication Award

RACI

Reef Restoration and Adaptation Program (RRAP)

Chapter Number	Publication details	Extent of contribution of co-authors
2	**Gulizia, A. M.**; Brodie, E.; Daumuller, R.; Bloom, S. B.; Corbett, T.; Santana, M. M. F.; Motti, C. A.; Vamvounis, G. Evaluating the Effect of Chemical Digestion Treatments on Polystyrene Microplastics: Recommended Updates to Chemical Digestion Protocols. *Macromol. Chem. Phys.* **2022**, *223* (13), 2100485. https://doi.org/10.1002/macp.202100485.	AG, GV, CM, MS conceptualised the study. AG, TC, EB and RD conducted the digestions and analysis. SB conducted photoluminescence analysis. All experimental work was conducted under close supervision of AG. All co-authors reviewed and edited the final manuscript.
3	**Gulizia, A. M.**; Patel, K.; Philippa, B.; Motti, C. A.; van Herwerden, L.; Vamvounis, G. Understanding Plasticiser Leaching from Polystyrene Microplastics. *Sci. Total Environ.* **2023**, *857*, 159099. https://doi.org/10.1016/j.scitotenv.2022.159099.	GV, LVH, CM and AG conceptualised the study. KP prepared the microplastics. AG conducted all experimental and analysis. BP conducted mathematical modelling. All co-authors reviewed and edited the final manuscript.
5	**Gulizia, A. M.**; Philippa, B.; Zachurak, J.; Motti, C. A.; Vamvounis, G. Plasticiser leaching from polyvinyl chloride microplastics and the implication for environmental risk assessment. *Mar. Poll. Bul.* **2023**, 195, 115392. https://doi.org/10.1016/j.marpolbul.2023.115392	GV, CM, AG and BP conceptualised the study. AG prepared the microplastics and conducted all experimental and analysis. JZ obtained the microscope images. BP conducted mathematical modelling. All co-authors reviewed and edited the final manuscript.

Abstract

Plastic pollution, especially microplastics (<5 mm in diameter), are ubiquitous contaminants in aquatic environments, and their impact on ecological functions is an area of growing concern. Microplastics are heterogeneous in nature, and comprise of a wide variety of polymers (polyproplyene, polystyrene, polyvinyl chloride, etc.,) with complex morphologies (i.e., size, shape, density, and colour) and chemical properties (i.e., incorporation of chemical additives such as plasticisers). It is these highly variable and complex physiochemical properties that make measuring and predicting the environmental distribution, behaviour and ecological consequences of microplastics challenging. This thesis employed a controlled approach to deconstruct the complexity and reactivity of microplastic contamination in aquatic environmental matrices, with the aim to improve technical accuracy for future experiments and environmental risk assessments (ERAs). To that end, controlled samples were used to 1) develop non-destructive chemical digestion protocols for isolating microplastics, 2) understand the leaching dynamics of plasticised plastics and 3) elucidate the relationship between microplastics, plasticisers and the microbiome. These experiments will help to develop effective mitigation strategies by harmonising the microplastic isolation methodology, determining the factors that contribute to plasticiser leaching in the environment, and revealing their relationship with the marine microbiome.

List of abbreviations

ABS	Acrylonitrile butadiene styrene
ACN	Acetonitrile
ANOVA	Analysis of variance
Ar-NO$_2$	Aromatic nitration
ATR	Attenuated total reflectance
BFR	Brominated flame retardant
BPA	Bisphenol A
BPF	Bisphenol F
BPS	Bisphenol S
CaCO$_3$	Calcium carbonate
CDCl$_3$	Deuterated chloroform
CMFSW	Calcium/magnesium free-seawater
D	Diffusion coefficient
DBP	Dibutyl phthalate
DCM	Dichloromethane
DEHP	Diethylhexyl phthalate
DEHT	Dioctyl terephthalate
DOPO	Dihydrooxaphosphaphenanthrene
DSC	Differential scanning calorimetry
E_A	Activation energy
EDC	Endocrine disruptive chemical
EDTA	Ethylenediaminetetraacetic acid
EPA	Environmental Protection Agency
ERA	Environmental risk assessment
EtOH	Ethanol
FT-IR	Fourier Transform-infrared spectroscopy
gDNA	Genomic DNA
GIT	Gastrointestinal tract
GPC	Gel permeation chromatography
GPGP	Great Pacific Garbage Patch
H$_2$O$_2$	Hydrogen peroxide
HBCD	Hexabromocyclododecane
HCl	Hydrochloric acid
HClO$_4$	Perchloric acid
HDPE	High density polyethylene
HNO$_3$	Nitric acid
HPLC	High-performance liquid chromatography
IAA	Phenol:chloroform:isoamyl alcohol
K$_2$S$_2$O$_8$	Potassium peroxodisulfate
KCl	Potassium chloride
KOH	Potassium hydroxide
K_{OW}	Octanol-water partition coefficient
LC$_{50}$	Lethal concentration at 50% mortality
LDPE	Low density polyethylene
MeOH	Methanol
Mw	Weight-average molecular weight
Na$_2$P$_2$O$_7$	Tetrasodium pyrophosphate

Na_2SO_4	Sodium sulfate
NaCl	Sodium chloride
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
OD	Optical density
OTU	Operational taxonomic unit
PAE	Phthalate acid ester
PC	Polycarbonate
PDI	Polydispersity index
PE	Polyethylene
PES	Polyester
PET	Polyethylene terephthalate
PEVA	Poly(ethylene-vinyl acetate)
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
PLA	Polylactic acid
POP	Persistent organic pollutant
PP	Polypropylene
PS	Polystyrene
PUR	Polyurethane
PVC	Polyvinyl chloride
PVP	Polyvinyl pyrrolidone
rRNA	Ribosomal RNA
SDS	Sodium lauryl sulfate
SEM	Standard electron microscopy
Tg	Glass transition temperature (°C)
TGA	Thermal gravimetric analysis
THF	Tetrahydrofuran
T_m	Melting point temperature (°C)
UV	Ultraviolet
WHO	World Health Organisation
Wt. %	Percentage by weight
WWTP	Wastewater treatment plant
$\delta*$	Boundary layer coefficient

Table of contents

Introduction: our world wrapped in plastic

During the dawn of the plastics revolution in 1941, Yarsley & Couzens published a book entitled *Plastics,* which anticipated the many ways that plastics could influence everyday lives in the future[1]. They described, *"an [unbreakable] world of colour and bright shining surfaces"* endowed with plastic. Where babies were fed from plastic bottles, children played with plastic toys, *"cleaned their teeth and brushed their hair with plastic brushes, clothed themselves in plastic clothes, [and] wrote their first lesson with a plastic pen ... in a book bound with plastic".* The text continued through an exhaustive list of potential plastics in homes, workplaces and industries, and finally, ominously concluded the lifecycle of human plastic consumption *"in a hygienically enclosed plastic coffin".* Nearing a century since its publication, these once abstract and inconceivable applications of plastics have become a modern reality and has cemented the development of synthetic polymer materials as one of the most influential innovations of the 20th century[2,3]. Major commodity polymers such as polyethylene (PE; 116 million t/year), polypropylene (PP; 68 million t/year), polyvinyl chloride (PVC; 38 million t/year), polyethylene terephthalate (PET; 33 million t/year), polyamide (27 million t/year) and polystyrene (PS; 25 million t/year) have provided industries with exciting and unmatched manufacturing efficiency (prompting the replacement of traditional and natural materials), physiochemical diversity (pertaining to their high mechanical strength, thermal stability and degradative resistance) and optimisation potential (through the incorporation of functional chemical additives)[4]. Together these factors have facilitated the worldwide monopoly of plastics and their application in almost every market economy, including packaging (146 million t/year), building and construction (65 million t/year), textiles (59 million t/year) and consumer (42 million t/year) industries. With no foreseeable end in sight, the demand for plastics continues to rise exponentially[5]. However, the consequences of society's complete reliance, massive overconsumption and mismanagement of plastics are looming, and at the current rate of plastic waste generation (exceeding 275 million t/year in 2015), natural environments are suffering under the strain of immense plastic pollution[6–8]. Perhaps, Yarsley and Couzens' description of man enclosed in a "plastic coffin" may have instead fallen to the fate of the natural world.

Plastic pollution in aquatic environments

Aquatic environments including oceanic and freshwater matrices are considered the biggest accumulation zone of plastic debris worldwide. Marine systems such as the North Pacific (e.g., the Great Pacific Garbage Patch (GPGP)[9]), Indian and Atlantic Oceans contain more than 79% of the world's floating plastic waste, which is estimated to contain a combined mass exceeding 270, 000 million metric tonnes[10]. Plastic debris has been detected even in enclosed (e.g., the Mediterranean Sea[10]) and landlocked seas (e.g., the Dead Sea[11]), as well as in remote marine ecosystems such as the Polar regions and the deep sea[12–14]. Much of this debris is emitted directly into the ocean from land-based sources (e.g., littering, landfill run-off[6]), however, freshwater rivers (e.g., Chang Jiang and Ganges River), wetlands, ponds and lakes can also be highly polluted with plastics[15–18], and thus can contribute significantly to the influx of plastics into the ocean[18]. Still, challenges in employing standardised and universal methodologies[19], research biases favouring oceanic environments[20] and the sinking and sedimentation of bio-fouled and/or high-density polymers (e.g., PVC and PET)[21,22] means that current global estimates of plastic pollution in aqueous environments is still considered extremely conservative[23,24]. These inconsistencies have highlighted the critical need to consider the physiochemical complexity of plastics during environmental contamination studies, as well as to recognise the multifaceted relationship between plastics, the natural environment and wildlife, which can impact their distribution and fate in different aquatic compartments.

Plastics and wildlife

Organisms at all trophic levels can interact with plastic debris in their environment in some way. In few cases, such as with microorganisms, the formation of a biofilm on the surface of plastics can promote polymer biodegradation and aid in the complete removal of these materials from the environment[25]. As well, growing evidence suggests that reef-building corals can act as long-term sinks for certain plastics (e.g., microplastics), creating semi-/permanent deposition zones, which reduce pollution levels in the surrounding waters[26]. For the most part however, aquatic plastic debris is a significant hazard to wildlife. Reviews published by Azevedo-Santos *et al.,* (2019) and Kuhn & Franeker (2020) compiled records of aquatic animal interactions with plastic debris, showing that 53% of seabirds, 70% of marine mammals, 100% of turtles and 99% of fishes surveyed have encountered plastics in their lifetime[27,28]. Even more alarming, is that up to 83% of these encounters could result in debilitating harm (e.g., lacerations, loss of limbs, gut obstructions) and/or death[29]. Many of these physical injuries occur as a result of entanglement, inhalation, accidental and mistaken ingestion of discarded plastic items, such as abandoned fishing lines, six-pack drink rings and other single-use consumer items[30]. However, plastics debris can also mobilise and transport organic contaminants present in the water table (e.g., persistent organic pollutants; POPs) as well as introduce synthetic chemicals into novel ecosystems (e.g., plastic-associated additives)[31], which can lead to a magnitude of ecotoxicological effects[32,33].

Plastics containing synthetic additives

Plastics contain a cocktail of synthetic chemical additives used to manipulate the aesthetic, thermal and mechanical properties of the polymer material (Table 1.1). Plasticising agents and flame retardants such as those containing phthalate acid esters (PAEs such as diethylhexyl phthalate; DEHP), diphenols (e.g., Bisphenol A; BPA) and halogens (brominated flame retardants BFRs; e.g., hexabromocyclododecane; HBCD) are the most common and highly manufactured plastic additives, and are vital for engineering safe, long-lasting and highly selective plastic materials[34]. They add malleability and flexibility to otherwise hard and rigid polymers (e.g., PVC), increase thermal stability, reduce flame spread and supress smoke formation. However, they are also known endocrine disruptive chemicals (EDCs), and due to their extended and concentrated use in plastic products are routinely detected in gaseous[35], terrestrial[36] and aquatic matrices[37], as well as in animal and human tissues[38–40]. Exposure to these additives can interfere with hormone production (e.g., estrogenic and anti-androgenic potency), has been linked to severe developmental and reproductive disorders, and possesses carcinogenicity, immune-, cyto- and genotoxicity potential[39,41,42].

Concerns of plasticiser and flame retardant exposure was first raised in humans, specifically because of their relationship with plastics[43]. Current toxicological data supports toxic exposure of plasticisers and flame retardants to humans through dietary and dermal contact with contaminated materials[39,44–46], inhalation of indoor dust[47,48], as well as leaching from medical devices and feeding bottles[49]. Consequently, special attention has been given to developing *non-toxic* chemicals such as dioctyl terephthalate (DEHT), dibutyl phthalate (DBP), bisphenol S (BPS) and dihydrooxaphosphaphenanthrene (DOPO) to replace traditional additives (e.g., DEHP, BPA and HBCD, respectively) in high-concern consumer items, as well as synthetising non-toxic polymer alternatives such as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), polylactic acid (PLA or, starch), polycarbonate (PC) and acrylonitrile butadiene styrene (ABS)[50,51]. However, in many cases, the toxicity potential of these alternatives mimics that of the original additive and/or polymer (e.g., BPS, DEHT and ABS)[42,52–54]. Already plastics constitute a significant exposure source of these additives to humans, however, recent studies also suggest that plastic pollution in aquatic ecosystems can transfer and release additives into waterways and to biota[55]. Whereby these processes are significantly accelerated as plastics fragment and degrade into smaller particles (i.e., microplastics; < 5 mm in diameter)[56].

Table 1.1: Descending list of the most common plastic additives, market value, associated function(s) and toxicity potential[37,52,55,57–60].

Additive	**Market value in 2020 (billion USD/year)**	**Common substances**	**Function**	**Range in plastic materials (wt. %)**	**Toxicity**
Plasticisers	15	Phthalate acid esters (PAEs), terephthalates, diphenols	Increases flexibility and softness	10 – 70	High: acute and chronic
Fillers	10	Calcium carbonate, silica, talcum	Cost reduction	< 50	Low
Flame retardants	8	Phosphorus-, boron- and nitrogen-based acids and hydrates	Reduces flammability	3 – 25	High: acute and chronic, bioaccumlative potential
Stabilisers and antioxidants	2	Organophosphite, phenolic antioxidants	Minimises oxidative degradation	0.05 – 3	Moderate: toxicity still under investigation
Colourants	15	Azo and anthraquinone dyes	Colouring aid	0.001 – 10	Low
Lubricants	6	Mineral oils, silicone, synthetic hydrocarbons	Reduces friction between molecular chains	0.1 – 3	Low

Microplastics; a small but mighty problem

Plastics are commonly categorised based on their size distribution into mega- (>50 cm), macro- (5 – 50 cm), meso- (0.5 – 5 cm) and microplastics (<5 mm). Culmination of recent plastic contamination data indicates that more than 92% of all debris collected from the ocean were microplastic particles less than 4.75 mm in diameter, which amounted to an excess of 4.86 trillion pieces globally (Figure 1.1)[16,61]. Furthermore, 75% of these microplastics were derived from plastics manufactured prior to the 1990s, suggesting that microplastic contamination in the environment has yet to reach its peak[61].

Microplastics have vastly different and complex manufacturing origins and degradative histories, and thus can also be distinguished by their source origin, as either primary or secondary microplastics[62]. Primary microplastics (15 – 31% of all oceanic microplastic contamination[63]) are purposefully manufactured at microscopic size, such as microbeads used in personal care products, glitters and pellets. These plastics are small enough to pass unimpeded through household and municipal drains, bypass wastewater treatment plants (WWTPs), and thus can be introduced into the environment by discharge after use and/or in accidental spills during transportation[64,65]. Secondary microplastics such as fibres from weathered car tires, fishing lines and clothing as well as coatings from maritime vessels are the dominant origin of microplastics in the ocean, and are formed as larger debris items degrade through photo oxidation (i.e., UV exposure), bacterial assimilation (i.e., biofilm formation) or mechanical weathering (i.e., wave action) and fragment into smaller particles[62]. These properties can greatly impact the morphological and chemical characteristics of the microplastic, determining their accumulation zone (e.g., buoyancy) and toxicity potential (e.g., leachability), as well as their interaction likelihood (i.e., ingestion/depuration rates) by wildlife[66].

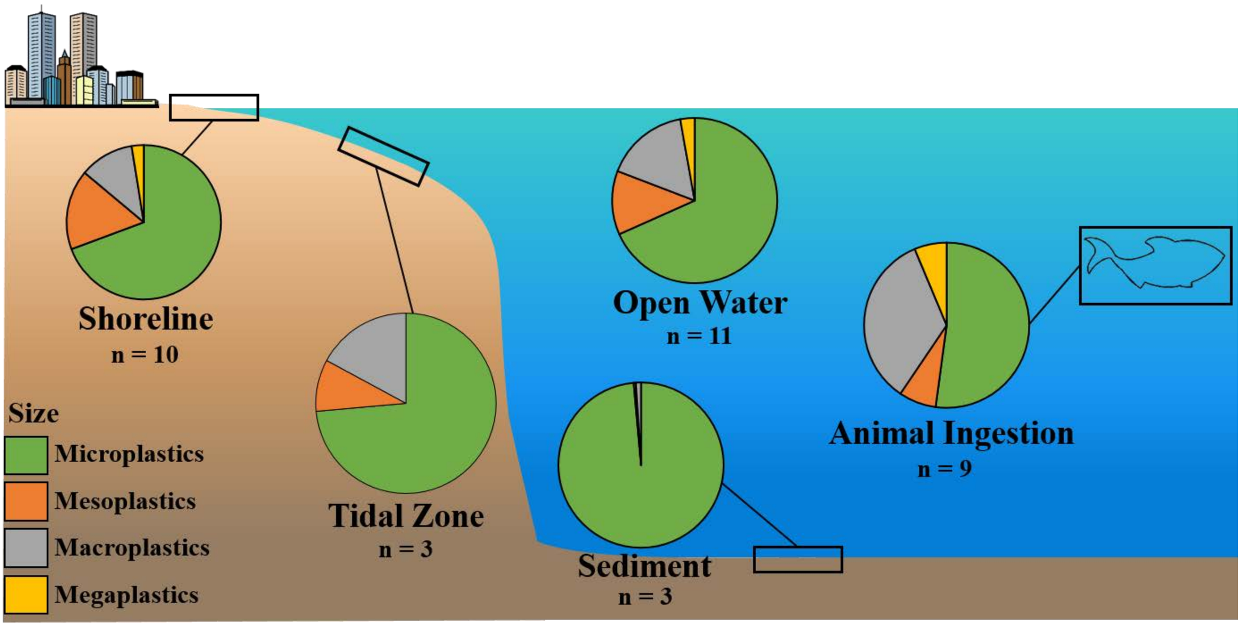

Figure 1.1. Size distribution of plastic in different compartments of the aquatic environment. Pie charts represent the average percentage of plastic debris per number of items (categorised according to typical nomenclature: megaplastics > 50 cm; macroplastics 5 – 50 cm), mesoplastics (0.5 – 5 cm) and microplastics (<5 mm)). Only studies published after 2013, which quantified contamination levels of three or more plastic size classes were included. Number of studies in each area is indicated (n). Exact numerical values and references are presented in Table S1.1.

Chemical properties of microplastics

Identifying the polymer origin of microplastics present in aquatic ecosystems can be determinate of their major accumulation zones and interactions with biota occupying different compartments of the water table (i.e., pelagic or benthic)[5]. Unsurprisingly, major commodity polymers such as PP, PE, PVC, PS and PET are the most prevalent microplastics detected in aquatic matrices worldwide[61,67–69], however, differences in the chemical structure (i.e., density) of these polymers influence their accumulation zones. Low density polymers such as PP (0.90 g/cm^3), PE (0.91 – 0.97 g/cm^3) and PS (0.96 g/cm^3) tend to occupy surface waters and pelagic habitats, as they are positively buoyant in seawater (~1 g/cm^3)[61,68,70–72]. Whereas, high-density and/or neutrally/negative buoyant polymers such as PET (1.38 g/cm^3) and PVC (1.42 – 1.48 g/cm^3) comprise the large majority of microplastics detected in sub-surface, benthic and sedimentary matrices[22]. Other factors such as biofilm formation[73] and chemical adsorption[74] can also alter these densities, causing buoyant polymers to sink and/or aggregate with sediment particles. Thus understanding how these processes impact the long-term fate and behaviours of microplastics in the environment can elucidate their bioavailability, ingestion and interaction rates with different benthic and pelagic organsisms[66]. However, other morphological characteristics of microplastics can also influence and further complicate these interactions.

Morphology of microplastics

Recent studies investigating the ingestion preferences and depuration rates of microplastics by biota have identified a species-specific relationship between the shape, colour and size of microplastics[75–79]. It was found that microplastics closely resembling an organism's diet will be preferentially ingested, indicating that ingestion of certain microplastics is highly dependent on their morphological characteristics. As microplastics decrease in size, they become significantly more bioavailable to direct and indirect ingestion by low-trophic level organisms (i.e., bivalves[80] and planktonic organisms[76]) and filter feeders (i.e., corals[81] and whales[82]), and thus can biomagnify and bioaccumulate in carnivorous and apex predators (i.e., fishes and mammals[66,83]). Once ingested by biota, differences in microplastic shape (e.g., fibres (48.5%), fragments (31%), beads (6.5%), films (5.5%) and foam (3.5%)) can impact depuration rates[84]. Both *in-situ* and *in-natura* studies have observed long-term retention of microplastic fibres and fragments by organisms, as these shapes are easily tangled and lodged in tissues and gut contents[84]. Accumulation of microplastics within biota leaves them vulnerable to long-term exposure consequences and may accelerate and/or enhance toxicity (e.g., additive leaching[85]). However, quantification and characterisation of microplastics ingested by organisms can often be a difficult and erroneous task, thus significant research attention has been directed at developing and strengthening their clarification and extraction methods from environmental sample matrices[86–88].

Sampling microplastics in environmental matrices

Accurate and non-destructive methods to separate, extract and characterise microplastics from environmental sample matrices (e.g., aqueous, sediment, biological) is a critical aspect of microplastics research[86–88]. However, owing to their small size, the standardised methods developed for collecting larger plastic debris (e.g., manual sorting) can rarely be applied with ease and accuracy to microplastics[86,89]. In many cases, completely novel detection (e.g., fluorescent staining[90–93]), separation (e.g., floatation, elutriation, chemical and enzymatic digestion[86,88]), and characterisation (e.g., spectral profiling[94,95]) techniques have been developed, many of which still require extensive troubleshooting to ensure precise and accurate data output for environmental risk assessments (ERAs)[94].

Detection

As plastics fragment into micro- and nanoplastics, their detection and identification in complex sample matrices becomes increasingly difficult. A technique commonly employed to remedy these challenges is fluorescent staining and photoluminescence microscopy[90–92]. These techniques are most common in studies investigating the interaction parameters (e.g., ingestion, toxicity) of microplastics with wildlife[96–98], however, have also been used for distinguishing plastics from sand, sediment and other natural particles[90,92]. Shim *et al.,* (2016) reported on the applicability of Nile Red staining for the analysis of PE, PP, PS, PVC, PC, polyester, polyamide and poly(ethylene-vinyl acetate) (PEVA) microplastics (100 – 300 µm in diameter)[90]. The Nile Red staining method was successfully applied to PE, PP, PS, PC and PEVA microplastics, and using fluorescence microscopy achieved an average of 98% detection rate. This detection efficacy rivals, and in many cases exceeds what can be achieved using other commonly used methods, such as visual identification, thermal desorption-gas chromatography-mass spectroscopy (89 – 99%), infrared red (IR) and Raman microscopy (89 – 102%)[94,95]. However, the chemical properties of the polymer, microplastic shape as well as the staining methodology (e.g., temperature and solvent) can influence fluorescence intensity[91,99], and many studies achieved low detection and recover rates (0 – 42%) due to insufficient staining of certain polymers[91]. Furthermore, in many cases, microplastics are also subject to chemical and enzymatic separation and extraction procedures, which can degrade the dye, reduce fluorescence and limit recovery[100]. Separation and extraction

Several techniques have been developed to separate and extract microplastics from complex sample matrices, these include, floatation separation, chemical and enzymatic digestion. Floatation techniques can be easily implemented for sedimentary and biological matrices, either standalone, with elutriation, combined with a surfactant or in a hypersaline solution (density floatation). While these floatation techniques are cheap, easily implemented and non-destructive, recovery rates peak at an average of 84% (10 – 97%[101]), and declines when separating high-density polymers (e.g., PET, PVC[102]) in complex biological matrices (e.g., whole organisms)[103]. Chemical and enzymatic digestion procedures were developed as a more robust means to separate and extract microplastics. Acidic (e.g., nitric acid; HNO_3, hydrochloric acid; HCl, perchloric acid; $HClO_4$), alkaline (e.g., sodium hydroxide; NaOH, potassium hydroxide; KOH), oxidative (e.g., peroxide; H_2O_2) and enzymatic (e.g., Proteinase-K, Trypsin) conditions can be implemented alongside elevated temperatures and extended treatment times, as well as in combination with density floatation to increase recovery efficiency[86]. Santana *et al.,* (2022) compared the efficacy of HNO_3 (70%; 3 – 12 h; 22°C) and KOH (10% and 1M; 24 – 48h; 22°C) for the extraction of microplastics from marine biota; coral, sponge, ascidian and sea cucumber[88]. Under all tested KOH conditions, efficient removal of coral (16 – 20%), sponge (17 – 21%) and sea cucumber (21 – 23%) tissue could not be achieved, however, acidic conditions were successful at clarifying all tested biological material after only 3 hours (95 – 100%). Similar outcomes in studies using strong oxidising agents have also been achieved by Dehaut *et al.,* (2016) using potassium peroxodisulfate (0.27M $K_2S_2O_8$; 24h; 65°C[104]), and Avio *et al.,* (2015) using a combination of density floatation (1.2 g/cm^3 sodium chloride (NaCl)) and H_2O_2 (15%; overnight; 50°C[105]). However, the caustic and degradative nature of these treatments can often result in modification of the plastic particle, reducing recovery efficiency and impacting accurate characterisation[104–109].

Characterisation of microplastics

Microscopy and FT-IR are both qualitative techniques used to characterise important chemical and physical properties of recovered microplastics. While these methods can be used to assess biofouling, organic matter adsorption and polymer degradation[88,100,102,110,111], they are most commonly used to identify unknown polymers collected from the environment[112]. To identify unknown polymers, the IR profile of the collected plastics can be compared for similarities and searched against commercially available polymer library databases (e.g., Polymers and Additives; NICODOM Ltd.). Microscopy (i.e., stereomicroscopy and scanning electron microscopy (SEM)) is also often used in unison with FT-IR to help verify the polymer source and origin, as well as to assess the size, shape, colour and surface morphology of the particle[100]. However, given the mostly qualitative nature of these techniques, any unbeknownst changes to the chemical profile and/or the plastic morphology during sample processing can skew polymer identification and physical assessment[112], and potentially alter the conclusions of the study, e.g., unrealistic estimates of contamination levels and ingestion rates[86]. Therefore, accurate data output relies on robust, standardised and non-destructive separation, extraction and detection techniques. Yet, a comprehensive chemical evaluation of the impacts of common digestion protocols on microplastics is not available[86]. This is a critical gap in the microplastics literature, as results from environmental sampling research are used to develop ERAs.

Plastic leachates

Ecotoxicology studies, both in the laboratory and the environment are vital to understanding the exposure consequences of microplastics on wildlife. Research to develop ERAs of microplastics has shifted beyond investigating the physical impacts of ingesting microplastics (e.g., blockages, tissue damage etc.,[29]), and instead is beginning to focus on the toxicity of plastic leachates[113–115]. Plastic leachates can include residual monomers (e.g., styrene and vinyl chloride), sorbed organic pollutants (e.g., POPs) and additives (e.g., plasticiser and flame retardants), and can be released into biological, gaseous, terrestrial and aquatic matrices via leaching and evaporation from plastic materials[34,57,116–119]. Plasticisers are some of the most highly concentrated chemicals detected in aquatic ecosystems worldwide and as such are recognised internationally by the Environmental Protection Agency (EPA) and the World Health Organisation (WHO) as hazardous and toxic environmental pollutants.

Plasticisers in aquatic environments

PAE and diphenol plasticisers such as DEHT, DEHP, BPA and BPS are among the most significant plasticiser pollutants detected in aquatic environments. Figure 1.2 is a concerning overview of their accumulative concentrations in aquatic ecosystems worldwide: exceeding 11.60 µg/L in industrial harbours, 2.18 µg/L in river systems, 7.00 µg/L in inland lakes and seas, 0.24 µg/L in coastal and surface waters and 0.99 µg/L in Arctic regions. The highest concentrations of these additives are generally associated with effluent from industrial discharge points (e.g., Auckland Harbour ~11.60 µg/L) and run-off from WWTPs and landfill environments[42], however, plasticisers can also be released into the environment as leachates from plastic materials[120]. Therefore ecosystems prone to excessive plastic and microplastic loading can accumulate especially high concentrations of aqueous plasticisers[12,26,69,121–124]. However, the exact mechanism of additive release as well as the factors impacting the leachability and leachable dynamics of microplastics remains to be clarified.

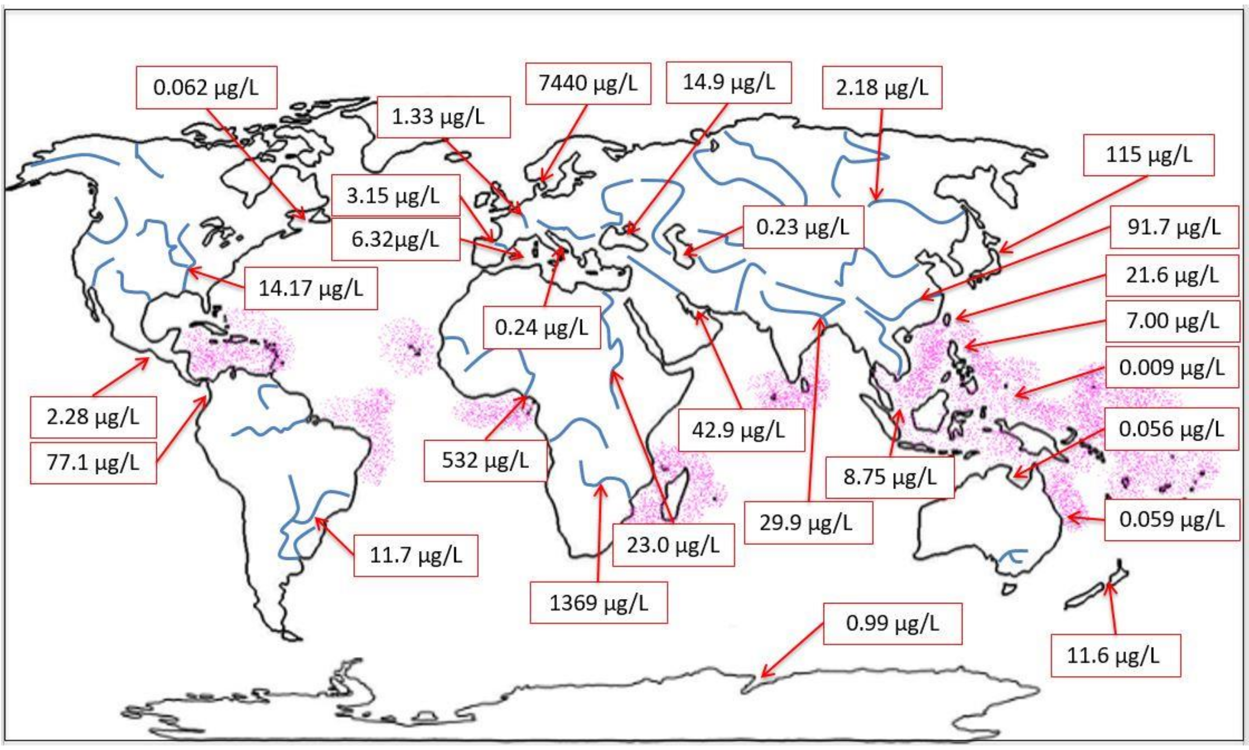

Figure 1.2. Accumulative concentrations (µg/L) of dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPS) and bisphenol S (BPS) detected in freshwater and marine environments worldwide using all available peer-reviewed data up to 2022. Major coral reef ecosystems harbouring the majority of the ocean's biodiversity are indicated by pink shading. A complete methodology breakdown that includes plasticiser type(s), concentration range (lower – upper limit) and references is presented in the supplementary information as Table S1.2.

Plastic leaching studies

The physiochemical properties of microplastic polymers and additives, as well as their chemical interactions and the surrounding abiotic conditions can be deterministic of the leaching behaviours of microplastics in aqueous environmental matrices. Studies by Garde *et al.,* (2001), Sun *et al.,* (2019), Suhrhoff *et al.,* (2016) and Henkel *et al.,* (2022) were pivotal to characterise these behaviours, and together suggested that microplastic leaching is controlled by molecular diffusion and boundary layer properties[118,125–127]. Garde *et al.,* (2001) demonstrated the size-dependent leaching of antioxidants from PP microfilms of different sizes and thickness, and suggested that the release kinetics of additives will change with diffusion length[125]. Sun *et al.,* (2019) built upon this theory by investigating the diffusion-limited leaching kinetics of BFRs from ABS and HDPE microplastics, and found that smaller microplastics with a lower glass transition temperature (*Tg*; indicative of polymeric free-volume[128] and correlated to plasticisation efficiency[34]) will have the highest additive leaching fluxes (i.e., higher diffusion coefficients)[118]. From these studies, key physiochemical properties of the polymer such as free-volume as well as the bonding interactions between additive and the polymer backbone were identified as deterministic of additive leaching, specifically because of their influence on molecular diffusivity[45,118,119,125,129]. However, this theory alone cannot be used to explain the different leaching behaviours of additives under fluctuating environmental conditions, as was observed by Suhrhoff *et al.,* (2016) and Henkel *et al.,* (2022)[126,127]. Suhrhoff *et al.,* (2016) investigated PS microplastic leaching into aqueous matrices with fluctuating water agitation, salinity and UV exposure, and found that water agitation strongly increased the leaching of additives[126]. They attributed these accelerated leaching behaviours to the hydrophobicity of the additives, hypothesising that agitation enhanced additive solubility in the surrounding solution and thus facilitated leaching. This idea was further explored by Henkel *et al.,* (2022) who investigated phthalate leaching from PVC microplastics, and extended the leaching model which included molecular diffusion to also consider the partitioning of additives across the microplastic-surface water boundary[127]. For microplastics leaching in aqueous systems, the boundary layer refers to a thin layer in the immediate vicinity surrounding the particle, whereby partitioning across this layer can be influenced by flowing water (i.e., agitation)[130–132]. While the combined diffusion and boundary layer partitioning theory is a promising approach to explain, characterise and enable the prediction of additive leaching from microplastics, it still requires extensive further investigation before it can be confidently used and applied into ERAs.

The research which pioneered the development of the diffusion and boundary layer theory was achieved through a culmination of a variety of studies investigating different high-concern polymer-additive compositions with varying origins, morphologies and degradative histories as well as under a range of abiotic conditions. However, without characterising and/or controlling all the physiochemical and abiotic parameters that may impact leaching, it is difficult to isolate the exact properties determining these behaviours. For example, many leaching studies used plastics collected from the environment and/or purchased from a proprietary consumer source, and neglected to characterise the polymer-additive chemical profiles prior to experimentation[120,133]. These plastics can have vastly different physiochemical properties (e.g., molecular weights data, free-volume and surface morphology[62]), additive composition (e.g., type(s) and concentration[34]) and reactivity when exposed to different conditions (e.g., accelerated aging[134–136]), which may skew leaching behaviours. The same can be said about leaching studies that are undertaken *in-natura* or alongside laboratory animal exposure/ecotoxicology studies implementing fluctuating/uncontrolled abiotic conditions (e.g., temperature, agitation and pH)[85,137,138]. Before a predictive leaching model can be extended to include the complexity of microplastics in the environment, the theory must first be validated under controlled conditions and using plastics with characterised and homogenous chemical profiles[127]. With this knowledge, researchers can go on to predict additive leaching and exposure concentrations in laboratory, environmental and biological matrices contaminated with microplastics[33,36,85,139].

Plastic leachates and the microbiome

Microplastics research has been as fairly recent inclusion in the field of microbiology, however, by utilising microbial assays and DNA isolation and sequencing techniques, there is much to learn about how microplastics and plastic leachates impact marine[140], soil[36,141] and biological microbiomes[78,142]. While these microbial communities are dynamic and can contain an abundance of different species, the leaching behaviours of microplastics present in these matrices has been shown to change the natural community structure[78,141,143]. For example, a recent study comparing the impacts of virgin and DBP containing PVC microplastics in soil, saw an increase in nitrogen fixing bacteria only in the presence of the plasticiser[141]. This indicated that DBP leaching from PVC prompted these community changes. Similarly, plasticised microplastics ingested by fish can induce gut dysbiosis (i.e., a negative imbalance of microbiota), as leached plasticisers can preferentially bind to microbial hormone receptors and interfere with the hosts' hormonal signalling[78,142]. These changes are seldom observed in the presence of virgin microplastics, and highlights how influential leaching is to the health of microbial communities, wildlife and the environment[78,143].

Marine microplastic-associated biofilms

Microorganisms play foundational roles in marine biogeochemistry by providing sources of food for primary consumers, assimilating biological and organic compounds and supporting ecosystems through their involvement in nutrient cycling[143]. Many microorganisms are also capable of activity in extreme environmental conditions, such as in super saline waters (e.g., coastal lagoons), across a variable temperature gradient (e.g., Polar Regions, coral reef ecosystems) and under high pressure and low sunlight availability (e.g., deep sea). Aforementioned, many of these environments are also contaminated with microplastics[12,69,144]. Microplastics can be colonised by microbial communities comprising of fungi, diatoms and bacteria, which form biofilms on the surface of the plastics often referred to as the "plastisphere"[73,145–147]. Given the extreme mobility of microplastics in the environment as well as their resistance to degradative stressors, biofilm formation on microplastics offers colonising microorganisms enhanced dispersal opportunities, superior protection from physical weathering (e.g., wave action) and in some cases, easy access to nutrients (e.g., through adsorption of biogenic particles[148])[149]. However, the factors impacting colonisation (e.g., abiotic surroundings, microplastic physiochemistry), microbiome dynamics (e.g., core community structure, diversity, and abundance), as well as the implications for the fate of microplastics in marine systems (e.g., ingestion likelihood, biodegradation) is not well understood[150].

Research classifying microbial communities forming the plastisphere is still quite novel, however, using DNA extraction and sequencing techniques (e.g., 16S rRNA amplification) dominant taxa can be identified[151]. Microplastics have been shown to form unique and dynamic biofilm communities that are taxonomically distinct from the surrounding waters, however, the colonisation and long-term adhesion of these communities is not well understood[151,152]. Compilation of recent studies has revealed that bacteria from the phyla Proteobacteria, Bacteroidetes, Cyanobacteria and Firmicutes are the most commonly reported and overrepresented taxa identified on microplastics dispersed in marine water worldwide[25,153]. Members of these phyla are the most abundant (10 – 30% of the total marine bacteria counts) and widely distributed bacteria in the ocean (including in coastal, offshore, sediment, deep sea and hydrothermal ecosystems)[154], and thus it is likely many are generalist and/or opportunistic colonisers with the ability to freely attach to any submerged substrate[155]. Still, no taxa are known to preferentially or predominately colonise plastics, and thus the degree to which community composition, especially amongst dominant taxa, differs among microplastic substrates occupying different ecological niches (i.e., benthic or pelagic) remains unclear[25] . Identifying similarities in microplastic-biofilm community composition is important to understand bacteria commonly and closely associated with microplastics, and will allow data pertaining to pathogen transmission and biodegradation to be fed into microplastic ERAs. Moreover, it will allow researchers to more easily identify factors impacting microbial colonisation and community structure, such as geography and seasonality (e.g., changes to abiotic conditions), as well as the physiochemical properties of the plastic[73,156,157].

Biogeographic and microplastic physiochemical properties impacts the plastisphere

A plethora of microorganisms have been identified in the plastisphere, such as opportunistic bacteria and zoonotic parasites carrying pathogenic risk to larger organisms, bacteria capable of degrading aliphatic and polycyclic aromatic compounds[158,159], as well as fungi[160], marine insect eggs[161] and photosynthetic diatoms[156,161]. However, biofilm community composition (i.e., species diversity), presence and abundance of these taxa is influenced heavily by seasonal and spatial variations, as well as the physical and chemical properties of the microplastic. In many cases, the biofilm community can be representative and characteristic of a specific ecological compartment[147]; with site specific bacterial communities identified on microplastics collected from different waterways worldwide[149,161–163], fluctuating in community composition and taxa presence/abundance with spatial (i.e., depth in the water table)[153] and seasonal variations[153,156]. Moreover, recent research has also highlighted the importance of polymer physiochemistry (e.g., morphology, polymer type and additive presence) on a developing and maturing biofilm[157,164,165]. Several studies, including those published by Zettler *et al.,* (2013), Agostini *et al.,* (2021) and Webb *et al.,* (2009), identified taxonomically distinct and polymer-specific biofilms forming on PE, PP, PET, PS and PVC microplastics (< 300 µm in diameter ~ 1.2 cm^3)[147,149,166]. These differences were attributed to changes in surface morphology (e.g., roughness, porosity and surface[167]) as well as the reactive properties of the polymer and any incorporated additives (e.g., chemical structure of the polymer backbone, leaching behaviours and surface functionalisation[168–170]). These parameters are hypothesised to impact attachment and adhesion opportunities, while additive leaching behaviours may act to either promote (e.g., by acting as nutrient sources[169]) or mitigate (e.g., antimicrobial/toxic properties[142,170]) microbial growth. However, the relationship between microplastics and the microbiome has yet to be fully elucidated, and it can only be speculated how biogeography and different polymer-additive compositions may impact specific microbial growth behaviours and overall biofilm community composition.

Implications for microplastics contamination

Biofilm formation on microplastics could have both negative (e.g., transfer and introduction of exotic and pathogenic microbes into new environments and the food wed) and positive (e.g., enhanced biodegradation pathways) implications for the environment. Regarding the latter, as research classifying the plastisphere grows, so too does the phylogenetic database of plastic-degrading microorganisms[171]. While these taxa are still quite rare in nature and can require optimised abiotic conditions to assimilate plastics, harnessing their metabolic activity is a promising route for bioremediation of plastics in the environment[172–174]. Many studies have utilised enrichment cultures to promote the growth of known plastic degraders to understand biodegradation pathways of microplastics[172,175]. Degradative changes to microplastics colonised by these bacterial genera include pits, cracks and grooves conforming to the bacterial shape[146,176], changes in crystallinity, compressive strength, hydrophobicity[153,177], and infrared profiles[111,178]. While biodegradation pathways of several commodity polymers have been successfully described[179], there is limited reliable information available regarding microbial degradation of PP, PS and PVC[180]. Bacteria such as *Pseudomonas*, *Xanthobacter*, *Rhodococcus*, *Corynebacterium* capable of assimilating PP, PS and PVC polymers exist in marine environments[171], however, their isolation and behaviour in microplastic-biofilms remains controversial[180]. While there is still much to understand about the complexity of marine microplastic-associated biofilms, accelerated and enhanced biodegradation pathways offers exciting potential to naturally remove these polymers from marine environments with minimal energy expenditure[73].

Current study

Mitigating the effects of microplastics in aquatic environments ultimately hinges on developing effective management strategies, which can only be achieved through the development of holistic ERAs[62]. The work outlined in this thesis presents three distinct but interrelated topics vital to microplastics research, whereby each topic addresses critical aspects of ERA improvement and development[181], by 1) harmonising chemical extraction methodology (Chapter 2), 2) characterising and predictive modelling the leaching dynamics of plasticisers from different microplastic polymers under fluctuating aqueous conditions (Chapter 3 – 5) and 3) elucidating the relationship between plasticised-microplastics and the marine microbiome (Chapter 6). Ultimately, the data obtained throughout the five data chapters of this thesis will positively influence the experimental design and outcomes of future research, by allowing researchers to implemented non-destructive and standardised methodologies, and ensuring that leaching behaviours and biofilm formation is considered and included in experiments undertaken both *in-situ* and *in-natura*. The following sections are a brief overview of the experimental aims and outcomes of this thesis.

Chapter 2

Chapter 2 applied a molecular approach to deconstruct the effects of chemical extraction protocols commonly applied in environmental quantification studies of microplastics collected from aqueous, biological and sedimentary matrices. Using PS as a model polymer, virgin PS was incorporated with a fluorescent dye (Nile Red), processed into irregularly shaped microplastic fragments (200 µm – 5 mm) and exposed to common digestion reagents (alkaline, oxidative and acidic) under varying temperatures (30 – 90°C) and exposure times (1 – 48 hours). Utilising chromatographic (gel permeation chromatography (GPC)), spectroscopic (FT-IR, nuclear magnetic resonance (NMR) and photoluminescence spectroscopy) and microscope (optical and SEM) techniques, the chemical and morphological effects of trialled digestion protocols were evaluated. A trend in enhanced microplastic reactivity (i.e., polymer degradation/morphological changes) was associated with higher temperatures, longer exposure times and with the caustic nature of the reagents, as well as with the decreasing size of the microplastic. With this information, recommendations were made for updates to future protocols and application of chemical digestion techniques.

Chapter 3

Chapter 3 aimed to characterise, understand and model the leachable properties of PS microplastics ($\lessapprox$ 200 µm in diameter) incorporated with plasticisers (DEHT, DEHP, BPA or BPS). Prepared microplastics were exposed to controlled, environmentally relevant aqueous media (i.e., changes to temperature, salinity, pH and wave action) and the leaching behaviours quantified using GPC and high-performance liquid chromatography (HPLC). Using thermal characterisation techniques (DSC and thermal gravimetric analysis (TGA)), the polymer-plasticiser interactions impacting diffusivity were characterised, and the dominant forces driving plasticiser leaching were determined. It was found that factors impacting diffusivity (e.g., water temperature, microplastic size and polymer-plasticiser interactions) and boundary layer properties (e.g., plasticiser hydrophobicity and wave action) had a significant impact on leaching rate and concentration. Following this, the experimental data was fitted to a numerical diffusion and boundary layer model and the leaching kinetics determined and quantified. The experimental methodology and model developed in this study was then applied to the following two data chapters in order to further validate the developed model for aged microplastics and different polymer types.

Chapter 4

Chapter 4 utilises the leaching modelled developed in Chapter 3 to assess leaching from degraded PS microplastics. PS microplastics with different weight-average molecular weight values (*Mw*; 35k – 350k g/mol) were incorporated with plasticisers (DEHT, DEHP, BPA or BPS), processed into irregularly shaped microplastics ($\lessapprox$ 200 µm in diameter) and exposed to varying seawater temperatures up to 60°C. Utilising controlled conditions and quantifiable changes to molecular weight, the relationship between polymer degradation (specifically molecular weight) and accelerated leaching can be deconstructed. Data obtained in this chapter indicated that molecular weight is not a determining factor in plasticiser leaching, and suggested that other degradative properties (e.g., size) are most influential.

Chapter 5

The rate-limiting factors of PAE and diphenol leaching from microplastics were outlined in Chapter 3 and 4 using PS as a model polymer, and thus in Chapter 5, these model findings were applied to DEHP and BPA plasticised PVC microplastics ($\lessapprox$ 200 µm in diameter). Using methods previously developed, plasticised PVC microplastics were prepared and exposed to agitated seawater up to 60°C to understand how different polymer-plasticiser chemical interactions impact microplastic leachability. Leaching kinetics from PVC followed similar trends as PS, impacted by temperature (diffusion-limited; BPA) and controlled by surface rinsing (boundary layer-limited; DEHP), respectively. However, leaching concentration from these microplastics was significantly lower, indicating that enhanced plasticisation efficiency may result in a higher proportion of polymer-associated-plasticiser and thus less concentrated leaching. These data further validate the numerical model developed in Chapter 3 of this thesis, and highlighted the potential differences in ecotoxicological consequences of plasticised polymers in aquatic matrices.

Chapter 6

Chapter 6 was an investigation of biofilm formation on microplastics in marine environments. Using the plasticised PS and PVC microplastics prepared and characterised in Chapters 3 and 5, microplastics were exposed *in-situ* to seawater representative of coastal, central Great Barrier Reef World Heritage Area, Australia for 0, 7 and 21 days, and biofilm formation analysed as function of polymer and plasticiser type, and exposure time. Microbial colonisation and biofilm composition was analysed using high-throughput 16S rDNA gene sequencing using the bioinformatics software QIIME2 and differences in community composition and taxon relative abundance compared statistically using a general linear model in RStudio. Results indicated that the biofilm forming on PS and PVC microplastics were significantly different to the surrounding seawater, indicating that microplastics can create specific niches for microorganisms, which are impacted by polymer type, plasticiser presence/absence and exposure time. Key polymer biodegradation parameters were also evaluated (i.e., *Mw*, spectral profiles and surface morphology), and highlighted how polymer-plasticiser composition promote the colonisation of bacteria with putative degradative potential and could accelerate the metabolic activity.

Chapter 7

As evidenced in this thesis, the behaviours of microplastics in different laboratory (Chapter 1) and aquatic (Chapter 2 – 5) matrices, as well as with the marine microbiome (Chapter 6) can have severe implications for research output and development of comprehensive ERAs. However, many of the current methodologies applied in environmental and biota toxicological studies are not underpinned by comprehensive chemical assessment, and thus can result in inconsistent and unrealistic findings. The goal of this Ph.D., thesis was to provide the necessary knowledge to inform more accurate experimental designs by deconstructing the behaviours of microplastics in environmental matrices. This information is vital knowledge underpinning microplastic behaviours, and is necessary to develop more accurate and comprehensive ERAs of microplastics in aquatic environments.

Chapter 2: Evaluating the effects of chemical digestion treatments on polystyrene microplastics

Gulizia, A. M.; Brodie, E.; Daumuller, R.; Bloom, S. B.; Corbett, T.; Santana, M. M. F.; Motti, C. A.; Vamvounis, G. Evaluating the Effect of Chemical Digestion Treatments on Polystyrene Microplastics: Recommended Updates to Chemical Digestion Protocols. Macromol. Chem. Phys. **2022**, 223 (13), 2100485. https://doi.org/10.1002/macp.202100485

Introduction

The cost effectiveness and easy manufacturing of synthetic polymers has encouraged the application of plastic products in almost every market economy worldwide. However, global mismanagement and inadequate waste disposal strategies has resulted in the ubiquitous discharge of plastic materials into aquatic environments[13,182]. Microplastics (<5 mm in diameter) are an especially hazardous by-product of the plastic's industry, and represent a prevalent size category of plastics with unique environmental fates and behaviours[183]. Owing to their small size, microplastics can be easily transported worldwide via wind and water currents and interact with biota across all trophic levels; from planktonic and benthic organisms, to carnivorous and apex predators[80,82,83,184]. These occurrences have sparked a plethora of studies investigating the exposure pathways and long-term toxicity consequences of microplastics with marine life. A critical aspect of these studies relates is their retrieval from complex sample matrices (e.g., aquatic, sedimentary and biological), which is often achieved by implementing isolation and separation techniques during sample processing[185]. Accurate quantification and characterisation of microplastic loading in target organisms hinges on employing non-destructive and standardised separation protocols. Strengthening these protocols can only be achieved through comprehensive and rigorous chemical assessment of their suitability in different sample matrices[88] and with different microplastic compositions (e.g., chemical and physical properties)[86].

Existing methods for isolating microplastics from environmental sample matrices include manual visual sorting, density floatation, elutriation and chemical digestion[102,186]. Although manual visual sorting and density floatation techniques are widely applicable for most major commodity polymers (e.g., polypropylene (PP), polyvinyl chloride (PVC) and polystyrene (PS)) and environmental sample types (e.g., seawater[185] or sediment[102]), they do not offer the same recovery efficiency for complex matrices (e.g., fatty fish tissue[88]) as chemical digestions treatments. For this reason, chemical digestion treatments in combination with high temperatures and prolonged exposure times are commonly employed to achieve more robust and efficient microplastic recovery[104,106,108,109,187–193]. Common digestion treatments involve immersion of the contaminated matrix in alkali (e.g., alkali hydroxides including potassium hydroxide (KOH) and sodium hydroxide (NaOH)), oxidative (e.g., hydrogen peroxide (H_2O_2)) and/or acidic (e.g., nitric acid (HNO_3)) media under ambient temperatures. However, temperatures approaching 100°C and exposure times exceeding 1 month are not uncommon for digestion of complex tissues such as whole juvenile fish, invertebrates and parts of the gastrointestinal tract (GIT)[108,187,193]. Implementation of these especially harsh conditions (e.g., HNO_3 at elevated temperatures[187]), while effective at removing biological material[86,88], can react with, deform and/or completely deteriorate the microplastic[104–107]. Furthermore, the choice of digestion methodology (i.e., reagent type, concentration, exposure temperature and time) is often at the discretion of the experimenter, and thus digestion suitability and efficacy can significantly vary between studies[86,88].

Implementing protocols without proper assessment of their suitability (i.e., protocol standardisation) can lead to inconsistent comparisons between studies and an inaccurate determination of the level (i.e., number of ingested particles) and type (i.e., microplastic polymer type, size, shape and/or colour) of contamination within a sample. Despite this, standardised digestion methods that prescribes a suitable and non-destructive chemical treatment remains elusive and is an area that requires critical evaluation to prevent inconsistent reporting.

To identify the isolated microplastics as well as to establish the extent of microplastic degradation during chemical digestion, Fourier Transform-Infrared Spectroscopy (FT-IR) and microscopy techniques such as standard electron microscopy (SEM) are commonly employed post-digestion[90–92,111]. While mostly qualitative techniques, examining the infrared profiles (i.e., using a library database) and microscope images can be used with a high degree of confidence to identify unknown microplastic polymers and characterise important morphological features (e.g., size, shape and colour)[88]. However, for assessing protocol suitability and polymer degradative susceptibility during digestion, a combined chemical approach using FT-IR and microscopy, as well as quantitative spectroscopic techniques such as gel permeation chromatography (GPC) and photoluminescence spectroscopy offers more accuracy and allows for evaluation of the polymer's reactive pathways. These methods can be used to identify changes in the functional groups along the polymer chain (e.g., oxidation[111]), measure fluorescence quenching of an incorporated dye (e.g., Nile Red[90–93,96,98]) and analyse average molecular weights distribution (i.e., chain scission). Therefore, when developing and recommending non-destructive chemical digestion methods for microplastic recovery, a suite of chemical and physical techniques should be used in combination to assess microplastic reactivity.

This chapter will characterise the effects and evaluate the suitability of routinely applied alkaline (KOH and NaOH), oxidative (H_2O_2) and acidic (HNO_3) chemical digestion protocols on PS microplastics incorporated with a fluorescent dye (Nile Red). PS polymer reactivity was also assessed as a function of temperature (30 – 90^{P}C), exposure time (1 – 48 hours) and microplastic size (200 µm – 5 mm in diameter) to encompass the different digestion conditions[86], as well as the range of microplastics present in environmental samples[61,194]. While some studies have already reported on the degradative impacts on these conditions (especially acids and oxidants[109,187]), application of these caustic protocols is often at times necessary for the robust clarification of complex organic matrices (e.g., biological material containing high concentrations of calcium carbonate[88]). Therefore, using a range chemical (i.e., FT-IR, proton-nuclear magnetic resonance (^{1}H-NMR), photoluminescence spectroscopy and GPC) and physical (i.e., optical and SEM) characterisation techniques, a comprehensive understanding of the reactivity and degradative susceptibility of PS microplastics can be attained. Guiding recommendations for the non-destructive isolation of polymers using chemical digestion and allowing researchers to predict with greater accuracy the most suitable protocol for their sample and/or polymer type.

Experimental Section

Materials

PS (Sigma Aldrich, average molecular weight = 192K g/mol, batch number MKCL2807), Nile Red (Sigma Aldrich), tetrahydrofuran (THF) (Unichrom, HPLC grade), deuterated chloroform ($CDCl_3$) (Sigma Aldrich, 99.99 atom%), KOH (AnalaR; 85% grade), NaOH (Univar; 97% grade), HNO_3 (RCI Labscan Limited; 32% grade), and H_2O_2 (Univar; 32% grade) were used as received. Dichloromethane (DCM) (Univar, ACS grade) was distilled prior to use.

Microplastic preparation

Additive free and virgin PS was chosen as it is well-defined and commercially available control standard, highly susceptible to reactivity and easily soluble in common laboratory solvents. Thus allowing for quantification using a range of chemical characterisation techniques (e.g., GPC), easy reproduction, chemical manipulation and assessment of its reactive pathways. Additionally, while previous reports have indicated that the degree of polymer reactivity during chemical digestion is not molecular weight-dependent[102,104,192], factors such as additive presence, size and surface porosity may enhance the degradative impacts[88,90]. Therefore, PS with an average molecular weight and across a range of dominant microplastic size classes was chosen to represent the diversity of recalcitrant polymers present in environmental matrices[61,67,194].

Virgin PS was dissolved in DCM at ambient conditions with constant stirring until homogenous. Once dissolved, a 4:1 solution of THF and Nile Red (0.5 μg/mL) was added and allowed to stir until homogenous in colour[90,195]. This fluorescent PS solution was cast on a watch glass, where the solvents were evaporated to form a plastic membrane. The dried membrane was then processed using a Magic Bullet – Nutribullet® 900 Series blender and sieved over a stainless-steel screen sieve (Glenammer Sieves) to afford irregularly shaped microplastics 300 μm – 1 mm in diameter (median size = 1 mm; Figure 1). A Thermo Scientific Nicolet iS5 FT-IR spectrometer equipped with an attenuated total reflectance crystal head (ATR) diamond head attachment was used to confirm the incorporation of Nile Red into these microplastics (Figure S2.1)[91,111]. Spectra were baseline corrected using default PerkinElmer parameters using the PerkinElmer Spectrum software. For the size-dependent study, additive free and virgin PS was processed into small (200 – 300 μm in diameter), medium (400 μm to 1 mm in diameter) and large (5 mm in diameter) particles using methods described above (Figure S2.2).

Digestion conditions

Microplastics (0.5 g, 5 – 200 fragments depending on size) were exposed to up to nine different digestion conditions using KOH (1.8 M; 15 mL), NaOH (10 M; 15 mL), H_2O_2 (9.8 M 15 mL) and HNO_3 (15.8 M; 5 mL), which were chosen because of their prevalence in literature[86,88] (Table 2.1). Due to HNO_3 being a potent oxidiser and unstable at high temperatures (boiling point of 83°C)[196], HNO_3 digestions at 90°C were not conducted. Microplastics were immersed in digestive solution (5 – 15 mL depending on reagent molarity) and maintained under constant conditions using a temperature-controlled water bath (Grant JB Instruments). Following chemical digestion, microplastics were washed with deionised water to remove excess reagent and air dried overnight under ambient conditions.

Table 2.1 Digestion conditions used across these studies (reagent concentration, digestion time and temperature)

Reagent	**Digestion Time (hours)**	**Temperature (°C)**
KOH (1.8 M)	12, 24*, 48	30, 60, 90
NaOH (10 M)	12, 24*, 48	30, 60, 90
H_2O_2 (9.8 M)	12, 24*, 48	30, 60, 90
HNO_3 (15.8 M)	1, 2, 12, 24*	30, 60

* 24 hours digestion conditions were only used in the size-dependent study

Sample analyses

Spectral analyses

The spectroscopic signatures of PS were obtained using FT-IR, ^{1}H-NMR, photoluminescence spectropscopy and GPC. The infrared spectra of treated microplastics were obtained using FT-IR as described above. To compare spectral similarity (%) post-digestion, the infrared profile of all treated microplastics were applied to the PerkinElmer COMPARE mathematical algorithm with the following filters: resolution, intensity, noise and H_2O weighting as well as CO_2 blanking. The search was conducted in the 3900 – 650 cm^{-1} regions, with the region between 2500 – 1900 cm^{-1} containing -0.01 – 0.1 baseline noise excluded because it may still be contaminated by small features from atmospheric absorptions. Infrared profiles were also systematically searched (SEARCH PerkinElmer) using Euclidian distance against a commercial available NICDOCOM IR spectral library (Polymers and Additives; NICODOM Ltd., Czech Republic) to evaluate whether the treated microplastics could be confidently identified as PS post-digestion. A percentage match greater than 70% is considered acceptable for accurate polymer identification[87,197], and therefore, acted as the lower threshold of spectral change for this study. ^{1}H-NMR of the control and treated microplastics were recorded as solutes in $CDCl_3$ at 298K on a Bruker 400 MHz NMR spectrometer using standard Bruker pulse sequences and were referenced to the residual solvent peak ($CDCl_3$, δ 7.26; Figure S2.3). Only peaks diagnostic of bonds susceptible and indicative of polymer reactivity (i.e., aromatic and aliphatic bonds) were monitored[198]. Finally, photoluminescence spectroscopy was used to measure the fluorescence intensity of Nile Red pre- and post-digestion. Microplastics (n = 3) were dissolved in THF in a ratio such that the optical density (OD) of the solution was 1 ± 0.01 at an absorbance wavelength of 257 nm. The OD was measured on a Shimadzu UV-2600 spectrophotometer using Shimadzu UV probe 2.61 software. The photoluminescence intensity of Nile Red in solution was measured using a Shimadzu RF-600 spectrofluorophotometer with an excitation wavelength of 530 nm, emission ranges between 545 – 800 nm and an emission slit bandwidth of 10 nm. The emission peak intensity at 677 nm was compared in all samples. All spectral profiles were consistent with those of pure polystyrene and Nile Red reported in literature[90,111] (Figure 2.2 – 2.4 and Figure S2.1 and S2.3).

Molecular Weight Analysis

Weight-average molecular weight (*Mw*: the average molecular weight of the polymer in g/mol) and the polydispersity index (PDI; polymer length heterogeneity) gives information regarding the molecular weights distribution of a polymer, and was used in this study to estimate the extent to polymer degradation. Changes in *Mw* and PDI serve as chemical indicators of polymer degradation because they reflect the breakdown of long-chain polymers into smaller and lower molecular weight constituents (i.e., oligomers and monomers). Changes to the *Mw* and PDI of PS pre- and post-digestion was evaluated using GPC. Microplastics (2 mg) were dissolved in THF (1.5 mL) and injected (50 µL) into a 1260 Infinity II Multi-Detector GPC (Agilent Technologies) equipped with an ultraviolet (UV) absorbance and refractive index detector. The two PLgel 5 µL MIXED-C columns (300×7.5 mm) (Agilent Technologies) were calibrated using PS narrow standards (470 – 482k g/mol) in THF at 35°C. The *Mw* of the microplastics was 174k g/mol with a PDI of 2.5.

Microscopy

A Leica MZ26A microscope fitted with a Leica DFC 600 camera was used to assess any discolouration and/or changes to the microplastic morphology post-digestion. For samples exhibiting significant physical alteration, the surface morphology was further examined using a Jeol Superprobe JXA-8200 SEM at a working distance of 11364.21 µm. For these analyses, microplastics were first coated with platinum to improve surface conductivity and improve image resolution. Microscopy revealed that all untreated microplastics were irregularly shaped and within their respective size category (Figure 2.1 and Figure S2.2).

Data collation and statistical analysis

Standard deviations for OD were calculated in Microsoft Excel. Standard error margin for GPC was calculated in Microsoft Excel, as a sum of the instrument and experimental error. Statistical differences between mean *Mw*, PDI and photoluminescence intensity among chemical digestion treatment and microplastic size was calculated using pairwise t-test in Microsoft Excel at a $p\text{-value} < 0.05$.

Results

Alkaline digestions

Microplastics were exposed to alkaline media (1.8 M KOH and 10 M NaOH) at 30, 60 and 90°C for 12, 24 and 48 hours. Spectroscopic, chromatographic and microscopy analyses of the fragments post-digestion did not reveal any significant alterations the chemical or physical properties of the microplastics when compared to the controls (Figure 2.1 – 2.4). Infrared spectral comparison was above 95% for all treated microplastics when compared to the untreated, control fragments, and similarly, all treated polymers returned a confident match to PS against the spectral library (Polymers and Additives; NICODOM Ltd., Czech Republic) (Figure 2.3 and Table S2.1). Additionally, the ^{1}H-NMR and photoluminescence profiles were consistent with the control (Figure S2.3 and Figure 2.4), and no morphological changes during 30 and 60°C treatments was observed. However, microplastics exposed to 90°C alkaline digestion conditions saw fragments expand in size after 48 hours, with enlarged pores and pitting on the surface (Figure 2.1).

Oxidative digestions

Microplastics were exposed to oxidative media (9.8 M H_2O_2) at 30, 60 and 90°C for 12, 24 and 48 hours. While no significant changes to the ^{1}H-NMR and photoluminescence profiles of treated plastics was observed (Figure S2.3 and Figure 2.4, respectively), there was some minor changes in the infrared profiles and molecular weights information, particularly during prolonged and high temperature treatments (Figure 2.2 and 2.3). FT-IR revealed structural changes to PS through the formation of a broad hydroxyl (-OH) band at 3364 cm^{-1}, emerging and increasing in intensity during prolonged 90°C treatments (Figure S2.4). However, these spectral changes not did significantly impact polymer identification, with treated microplastics returning between 86 to 91% similarity to the control plastics, as well as to PS in the spectral library database (Table S2.1). Similarly, GPC analyses showed a small decrease in *Mw* from 174k to 171k g/mol after treatments at 90°C for 48 hours ($p > 0.05$). Finally, while no physical alterations consistent with polymer degradation were observed, microplastics digested at 90°C also displayed thermal expansion behaviours with enlarged pores and pitting on the surface (Figure 2.1).

Acidic digestions

Microplastics were exposed to acidic media (15.8 M HNO_3) at 30 and 60°C for 1, 2 and 12 hours. The caustic nature of these conditions, particularly during prolonged (2 or more hours) and high temperature (30 and 60°C) treatments, resulted in significant chemical and physical changes to microplastic consistent with polymer degradation. The ^{1}H-NMR spectra revealed a downfield shift of characteristic aromatic peaks at approximately 7.70 ppm (Figure S2.3), and, as evidenced by the integration of these regions (7.30 - 6.25 and 1.95 - 1.15 ppm)[111], increased in intensity with digestion temperature and time (Table 2.2 and Figure S2.5). Comparison of these data to the infrared spectra also revealed enhanced reactivity within the aromatic region of PS, with the introduction of novel peaks at 1680, 1650, and 1330 cm^{-1}, indicative of the formation of aromatic-N-O and C-N bonds (Figure 2.3). These changes were supported by a decreased infrared spectral similarity and match to the library database, with both assessments receiving less than a 75% match (Table S2.1). Analyses of the molecular weights distribution also saw significant decreases in *Mw* from 174k g/mol to 153k g/mol (30°C for 12 hours; $p < 0.05$), 134k g/mol (60°C for 2 hours; $p < 0.05$)) and 48k g/mol (60°C for 12 hours; $p << 0.05$) (Figure 2.2). Similar trends in PDI consistent with polymer degradation were also observed, with an increase from 2.5 to 5.7 after treatment at 60°C for 12 hours ($p < 0.05$; Figure S2.6). These chemical indicators of degradation were also mirrored in the morphological characteristics of the microplastics post-digestion, through the observation of significant yellowing, embrittlement and surface cracking (Figure 2.1). The severity of these changes increased upon exposure to prolonged and high temperature treatments (30°C for 12 hours and 60°C for more than 1 hour) (Figure 2.5). Moreover, in line with the degradation rate of the polymer, photoluminescence spectroscopy revealed diminished fluorescence of Nile Red during all 60°C treatments (Figure 2.4). In fact, after 12 hours at 60°C no significant fluorescence of Nile Red was observable, with fluorescence counts decreasing from 1125 to 54 at 677 nm ($p < 0.05$).

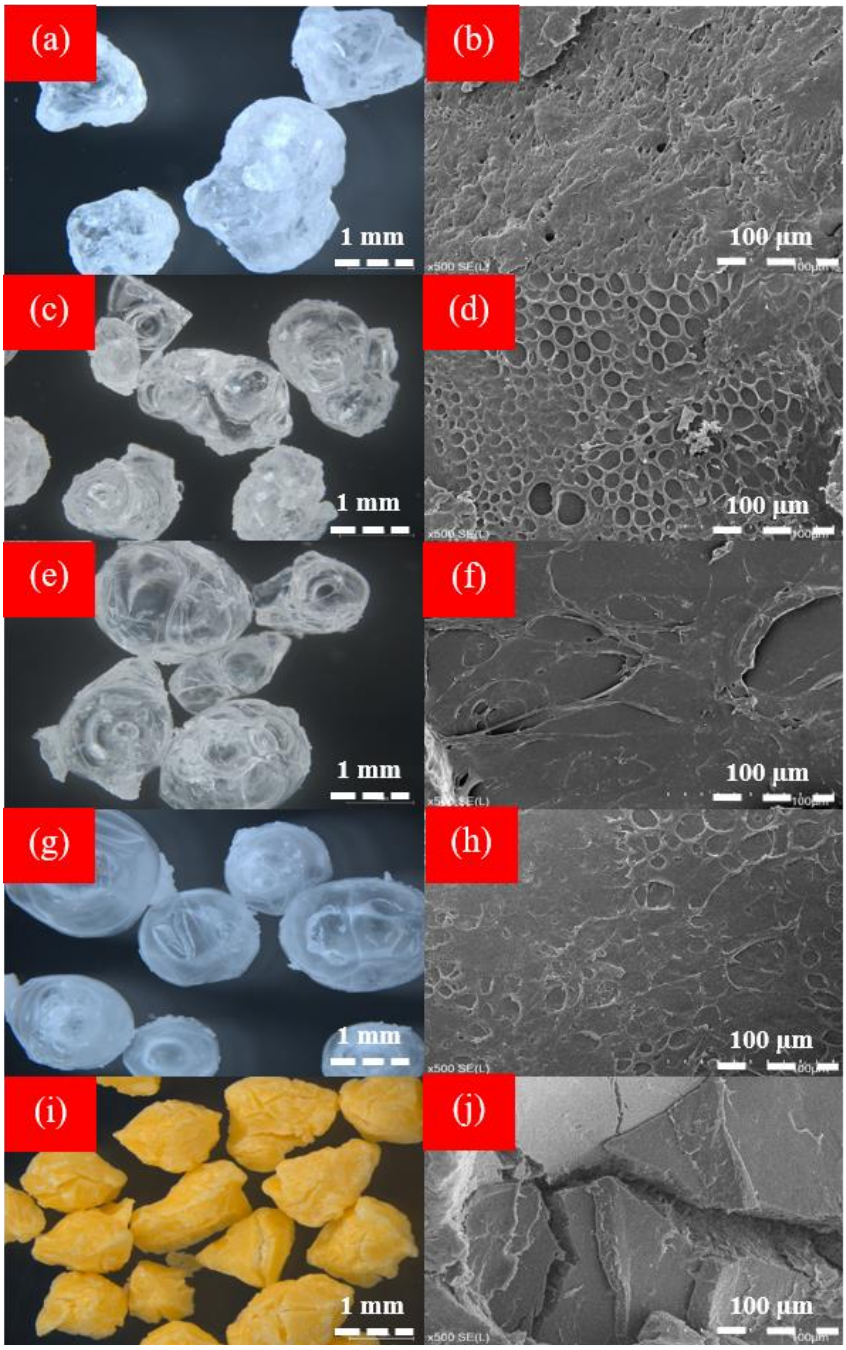

Figure 2.1. The microscope images of the (a, b) control, untreated microplastics, as well as those treated for 48 hours at 90°C with (c, d) 1.8 M potassium hydroxide, (e, f) 10 M sodium hydroxide, (g, h) 9.8 M hydrogen peroxide, and for 12 hours at 60°C with (i, j) 15.8 M nitric acid. All images in the left hand column were taken at 2.5X magnification using optical microscopy and all images in the right hand column were taken at 500X magnification using a standard electron microscope.

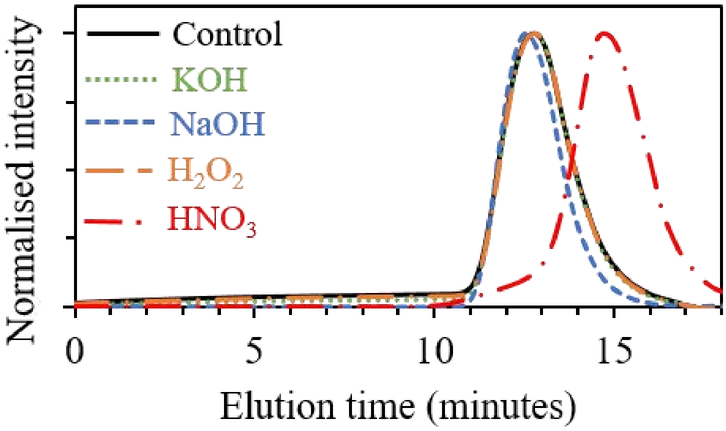

Figure 2.2. Gel permeation chromatography traces for the control, untreated polystyrene (control; solid black), as well as those treated for 48 hours at 90°C with 1.8 M potassium hydroxide (KOH; dotted green), 10 M sodium hydroxide (NaOH; dashed blue), 9.8 M hydrogen peroxide (H_2O_2; dashed orange), and for 12 hours at 60°C with 15.8 M nitric acid (HNO_3; dashed red).

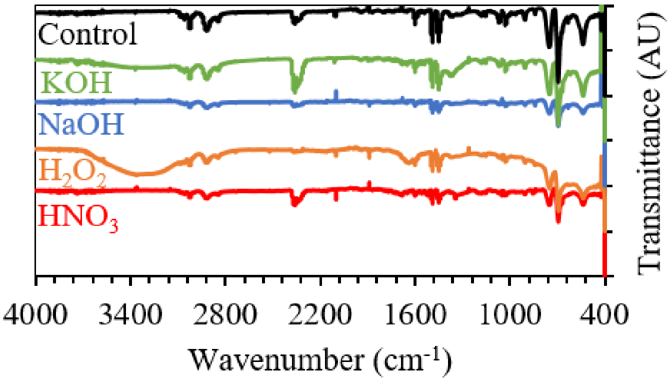

Figure 2.3. Infrared spectra of the control, untreated polystyrene (control; solid black), as well as those treated for 48 hours at 90°C with 1.8 M potassium hydroxide (KOH; solid green), 10 M sodium hydroxide (NaOH; soild blue), 9.8 M hydrogen peroxide (H_2O_2; solid orange), and for 12 hours at 60°C with 15.8 M nitric acid (HNO_3; solid red).

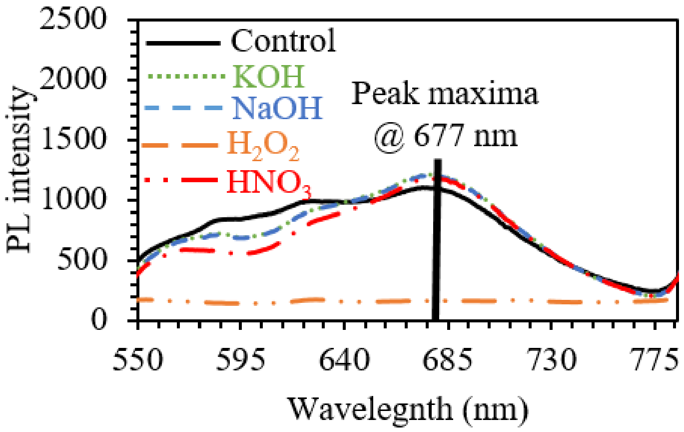

Figure 2.4. Photoluminescence (PL) intensity for the control, untreated polystyrene (control; solid black), as well as those treated for 48 hours at 90°C with 1.8 M potassium hydroxide (KOH; dotted green), 10 M sodium hydroxide (NaOH; dashed blue), 9.8 M hydrogen peroxide (H_2O_2; dashed orange), and for 12 hours at 60°C with 15.8 M nitric acid (HNO_3; dashed red).

Table 2.2. The degree of aromatic nitration (Ar-NO_2) of polystyrene treated with 15.8 M nitric acid (HNO_3). Ar-NO_2 (%) was calculated by comparing the integrated resonances from 7.30 – 6.25 ppm and 1.95 – 1.15 ppm[111]

HNO_3 treatment	**Ar-NO_2 inclusion (%)**
30 °C for 1 h	No nitration
30 °C for 2 h	No nitration
30 °C for 12 h	2.5
60 °C for 1 h	No nitration
60 °C for 2 h	1.5
60 °C for 12 h	6

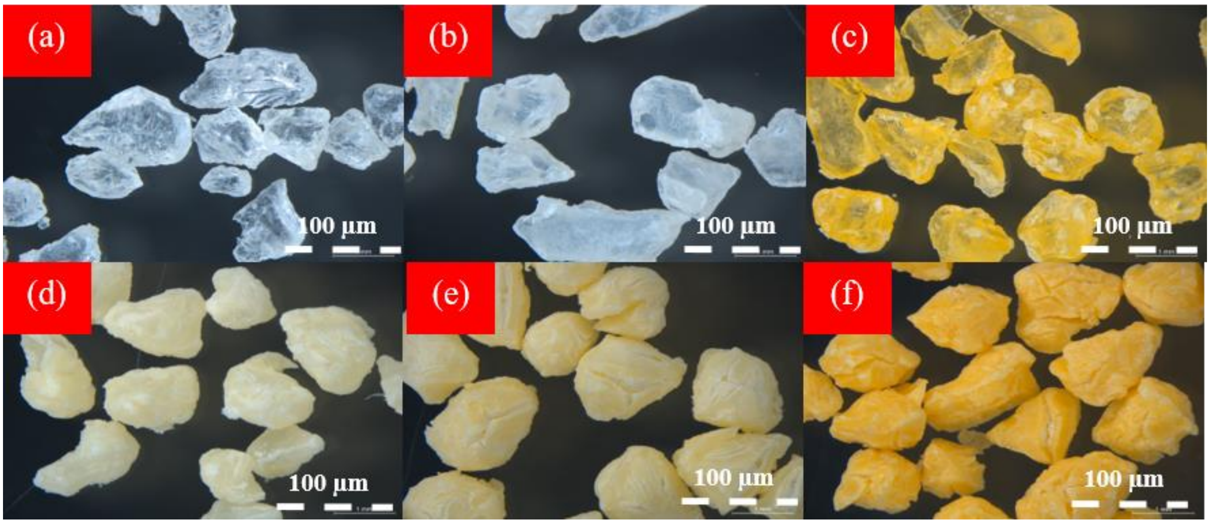

Figure 2.5. Microscope images of microplastics treated with 15.8 M nitric acid at 30ºC for (a) 1 hour, (b), 2 hours and (c) 12 hours, and at 60ºC for (a) 1 hour, (b) 2 hours and (c) 12 hours. Magnification 2.5X.

Size dependence

Small (200 to 300 µm in diameter), medium (400 µm to 1 mm in diameter) and large (5 mm in diameter) PS microplastics were exposed to alkaline (1.8 M KOH and 10 M NaOH), oxidative (9.8 M H_2O_2) and acidic (15.8 M HNO_3) media at 30 and 60ºC for 24 hours. While no significant changes to the chemical and physical properties of all microplastics treated under alkaline and oxidative conditions were observed (Figure S2.2, S2.7 and S2.8), significant deteriorations during HNO_3 were observed with respect to microplastic size. Analyses of the infrared and ^{1}H-NMR signatures of acid treated fragments indicated enhanced reactivity in the aromatic region of PS, increasing with microplastic size: small > medium > large (Table 2.3; Figure S2.8). In line with this, GPC results demonstrated significant polymer degradation associated with microplastic size, with the small, medium and large plastics showing significant decreases in *Mw* after 60ºC digestions (from 174k to 100k, 49k and 41k g/mol, respectively; $p < 0.05$) (Figure S2.9). Moreover, severe physical deterioration of medium and small plastics was observed, including discolouration, size reduction and fragments melding together (Figure 2.6). Further assessment of the surface morphological of these fragments using SEM also revealed deep surface cracking and fractures (Figure S2.10).

Table 2.3: The degree of aromatic nitration (Ar-NO_2) of small (200 – 300 µm), medium (400 µm – 1 mm) and large (5 mm) polystyrene microplastics treated with 15.8 M nitric acid treatments for 24 hours. Ar-NO_2 (%) was calculated by comparing the integrated resonances from 7.30 - 6.25 ppm and 1.95 - 1.15 ppm[111]

Acid treatment	**Ar-NO_2 inclusion (%)**
30°C, large	No nitration
30°C, medium	No nitration
30°C, small	4
60°C, large	No nitration
60°C, medium	6
60°C, small	12

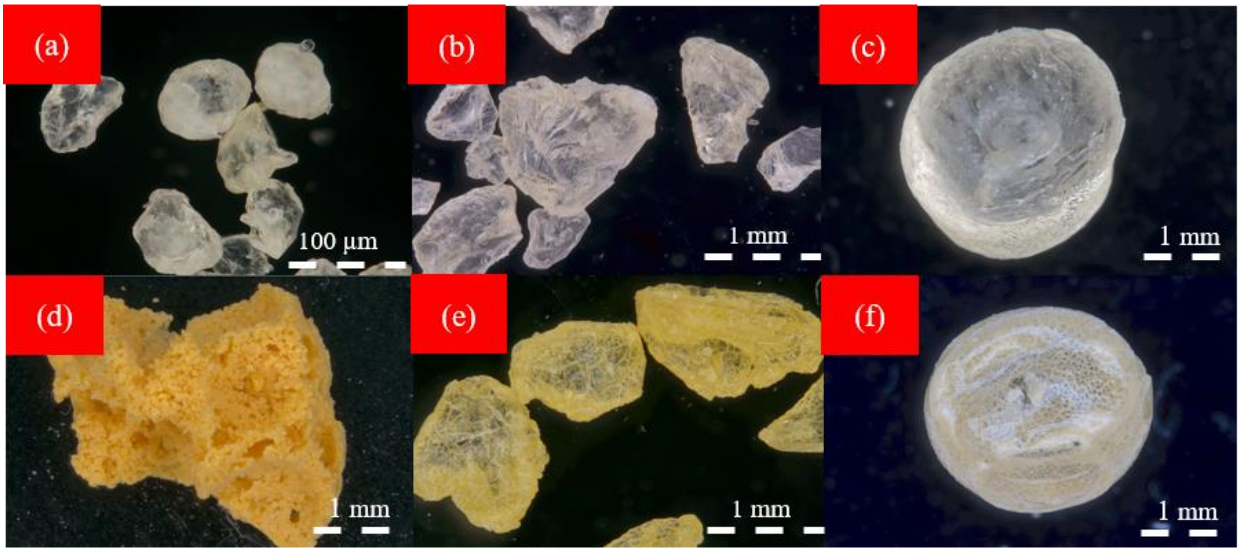

Figure 2.6. Microscope images of small (200 to 300 µm), medium (400 µm – 1 mm) and large (5 mm) microplastics treated with 15.8 M nitric acid at 60°C for 24 hours at (a – c) 30°C and (d – f) 60°C. Magnification 2.5X.

Discussion

This chapter tested the suitability of four routinely applied digestion conditions, KOH, NaOH, H_2O_2 and HNO_3 for the non-destructive isolation of PS microplastics. Table 2.4 summarises these findings and suggests that alkaline and H_2O_2 treatments up to 90°C for 48 hours minimally impact the physiochemical properties of PS. For HNO_3 digestion, prolonged and high temperature conditions were destructive for all microplastics tested, both chemically and physically. Where applicable, the use of non-destructive reagents (KOH, NaOH and H_2O_2) and conditions (<60°C) should therefore be considered in preference to HNO_3 for the isolation of PS microplastics from environmental sample matrices.

Table 2.4. Overall findings and suitability of potassium hydroxide (1.8 M KOH), sodium hydroxide (10 M NaOH), hydrogen peroxide (9.8 M H_2O_2) and nitric acid (15.8 M HNO_3) as digestive reagents at 30, 60 and 90°C for the non-destructive isolation of polystyrene microplastics (200 µm – 5 mm in diameter). Suitability ratings were characterised as follows: **good** – the treatment must not have significantly impacted any property of the polymer, **fair** – there were minor alterations to the physical and/or chemical properties of the microplastic, however they did not impede accurate characterisation, and **bad** – there were significant chemical changes and physical deterioration impeding accurate identification and characterisation of the microplastic.

Digestion Temperature	**Reagent**			
	KOH	**NaOH**	**H_2O_2**	**HNO_3**
30°C	**Good** No polymer reactivity or fragment deterioration	**Good** No polymer reactivity or fragment deterioration	**Good** Minimal polymer reactivity or fragment deterioration	**Fair** Significant polymer and reagent reactivity after 12 hours; decreased spectral similarity/match and severe mechanical damage
60°C	**Good** No polymer reactivity or fragment deterioration	**Good** No polymer reactivity or fragment deterioration	**Good** Minimal polymer reactivity or fragment deterioration	**Bad** Significant polymer and reagent reactivity at all exposure times; infrared spectral dissimilarity/match and severe fragment deterioration
90°C	**Fair** No polymer reactivity, but significant morphological changes	**Fair** No polymer reactivity, but significant morphological changes	**Fair** Minimal polymer reactivity but significant morphological changes	-

Alkaline digestions using NaOH and KOH

Alkaline digestion methods using either NaOH, KOH or a combination, are commonly employed for isolating microplastics from organic matter in water and soil matrices[66]. These protocols can also yield high matrix removal and recovery efficiency in biological samples containing soft and epithelial tissues (> 98% recovery[86,88]), however, they may not be as effective for digestion of corals, sponges and whole organisms containing high levels of skeletal and GIT content (< 23% efficiency[88]). Despite this, alkaline reagents are reported to cause the least plastic damage and thus can be employed with great success in certain studies[104,191,192]. Of the four digestion reagents investigated in this study, alkaline treatments had the least effect on the chemical and physical properties of PS. This lack of reactivity and degradation was evidenced through spectroscopic (^{1}H-NMR, FT-IR and photoluminescence) and chromatographic (GPC) analyses. Furthermore, while no physical alterations consistent with polymer degradation was observed (e.g., fragmentation, cracking), alkaline treated fragments at 90°C were noticeably swollen after 24 hours. Given that the chemical properties of PS remained unchanged, the swelling behaviours observed could be associated with the polymer's thermal stability (glass transition temperature; $Tg \approx$ 90 – 110°C[199]). Coupled with the porous nature of the fragments (Figure 2.1), exposure to temperatures approaching the *Tg* of a porous polymer has been known to result in thermal expansion (i.e., blowing)[200]. To support this theory, non-porous microplastics were analysed under the same temperature regimes with no thermal expansion observed, indicating that the blowing agent (i.e., NaOH or KOH) could not enter these microplastics. The impact of surface porosity on the physical properties of microplastics exposed to high temperature, alkaline digestion procedures could have profound effects on the accurate characterisation of degraded polymers recovered from the environment. Aged plastics often experience size reduction, surface pitting and enhanced porosity upon exposure physical weathering and biodegradation processes[183,201], therefore, it is recommended that high temperatures be applied with caution when extracting soft plastics using chemical digestion (e.g., foamed or expanded PS[70]), as well as when processing samples contaminated with smaller micro- and nano-sized plastics[194].

Oxidative digestions using H_2O_2

Oxidative digestion protocols are commonly used in studies requiring robust clarification and removal of microplastics from complex sample matrices[86]. In many cases, H_2O_2 protocols can be applied with much shorter digestion times to achieve high removal efficiency and recovery rates (85 – 100%[86]). However, as a strong oxidising reagent, H_2O_2 has been known to discolour[202] and physically degrade plastics[109]. In this study, all 30°C H_2O_2 treatments up to 48 hours were suitable for the non-destructive isolation of PS microplastics (200 µm ~ 5 mm in diameter), however, upon increasing digestion temperature beyond 60°C, a broad –OH band in the infrared profile emerged and increased in intensity alongside thermal expansion. Given that no changes in the polymer structure was found using all other spectroscopic techniques (^{1}H-NMR, GPC and photoluminescence), it is unlikely these changes in the infrared profile is related to PS degradation, rather the degradation products of H_2O_2 at high temperatures (i.e. water)[203]. During thermal expansion (i.e., blowing; Figure 2.1), excess water formed during H_2O_2 degradation was able to easily infiltrate the polymer matrix, explaining the increased intensity of the –OH peak at high temperatures (Figure 2.3). In line with data from alkaline treatments, increasing digestion temperature and a decline in microplastic size may enhance polymer reactivity and degradative susceptibility, indicating that prolonged and high temperature H_2O_2 digestion conditions should be implemented with caution during sample processing.

Acidic digestions using HNO_3

Owing to its strong acidic and oxidising power, many studies favour HNO_3 chemical digestions to accelerate the removal of complex organic tissues (> 95% efficiency)[88,102,104,190]. While HNO_3 digestions have been used at temperatures up to 100°C and for more than 24 hours[102], significant polymer degradation and loss of plastic material is often observed[104,187,202]. Thus, a balance between matrix removal, microplastic recovery and minimal polymer reactivity must be attained. The chemical and physical data obtained in this study suggests that HNO_3 protocols can have dramatic impacts on the plastic. Alterations to the chemical structure of PS was evidenced in all chemical techniques (FT-IR, ^{1}H-NMR, GPC and photoluminescence), highlighting changes to the hydrodynamic radii of PS (increased PDI), bond cleavage (decreased *Mw*), novel bond formation (aromatic nitration) and fluorescence quenching of Nile Red. Chemical indicators of aromatic nitration was observed through the downfield shift of aromatic protons and the introduction of C-N and N-O bonds in the ^{1}H-NMR and infrared spectra, respectively. These bonds may have been formed through electrophilic aromatic substitution of PS, as well as during the production of dinitrogen tetroxide (N_2O_4) at high temperatures[204]. N_2O_4 is a colourless gas produced during thermal decomposition of HNO_3, however, in a closed reaction vessel (as was used in this study), dissolution of N_2O_4 into an aqueous solution and the subsequent formation of nitrogen dioxide (NO_2; red/yellow-brown in colour) could have contributed to the inclusion of these bonds into the spectral profiles of treated PS. As well, this was likely the cause of the yellowing discolouration of fragments treated under these conditions (Figure 2.5 and 2.6)[205], as microplastic degradation and the mechanism of polymer yellowing can take more than several months *in-natura*[111]. Conversely, the deep surface cracking and embrittlement of fragments observed using microscopy was representative and consistent with polymer degradation, indicating prolonged and high temperature HNO_3 digestion conditions are destructive to PS microplastics. Overall, while HNO_3 is often the reagent of choice to digest complex biological sample matrices, it should be used with caution when attempting to recover microplastics, particularly aged plastics, smaller particles and those polymers highly susceptible to reactivity[109].

Detection of microplastics using Nile Red staining

Nile Red staining techniques and photoluminescence spectroscopy/microscopy are often used in combination with chemical digestion to facilitate the detection and quantification of microplastics from environmental sample matrices[90–92,96,100]. However, the dyes fluorescence intensity can diminish during chemical digestion and thus leaving researchers vulnerable to inaccurate count and contamination assessments[100]. In this study, the photoluminescence intensity of Nile Red was unchanged during all alkaline and oxidative treatments, however, diminished intensity was observed upon exposure to HNO_3. The most significant change in Nile Red photoluminescence was recorded during these high temperature acid treatments and was observed in line with the enhanced reactivity and degradation of the polymer[206,207]. Significant fluorescence quenching during HNO_3 treatments indicates that the incorporation of fluorescent dyes into microplastic polymers may not be beneficial if implemented alongside acidic digestion protocols. Furthermore, a comparison between the *Mw* values and fluorescence count of Nile Red indicates that fluorescence intensity cannot be used a definitive measure of polymer degradation during digestion[90,100], and was highlighted during 30°C treatments for 12 hours (Figure 2.7). While fluorescence dye indicators are promising techniques to facilitate the detection and quantification microplastics, results here indicate that significant degradation and quenching of the dye may occur when exposed to acidic media.

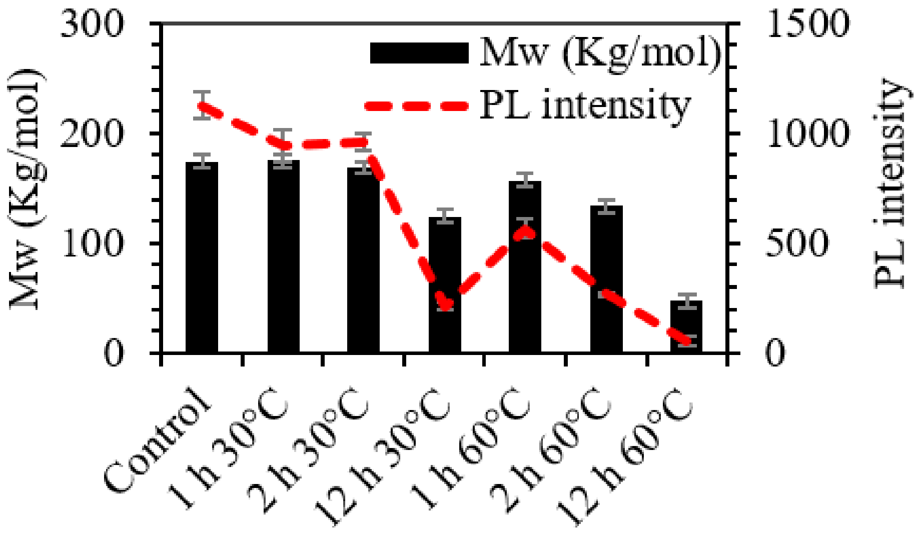

Figure 2.7. A comparison between the weight-average molecular weight (*Mw*) of polystyrene and the average photoluminescence (PL) intensity of Nile Red (dashed red) after treatments with 15.8 M nitric acid. Data is presented as the mean ± standard deviation.

Conclusion

Isolating and extracting microplastics from environmental sample matrices is a critical aspect of plastics research. While there have been many studies that outline the impact and suitability of a range of chemical digestion protocols for different sample and polymer types[86–88], many of these outcomes and recommendations were based largely off of qualitative data. Without comprehensive chemical and physical characterisation of the polymer post-digestion, the suitability of each digestion protocol cannot be confidently assessed. Using spectroscopic (FT-IR, ^{1}H-NMR and photoluminescence), chromatographic (GPC) and microscopy (optical and SEM) techniques, this study applied a molecular approach to analyse the effects of KOH, NaOH, H_2O_2 and HNO_3 chemical digestion procedures on PS based microplastics. It was found that alkaline and oxidative reagents were non-destructive (chemically and physically) for the isolation of PS up to 60°C. At 90°C these reagents can also be used with minimal alterations, however, care should be taken when also employing longer exposure times (>24 hours), as well as with porous and aged microplastics. However, HNO_3 can begin to the degrade microplastics after only 1 hour at 60°C, with reaction susceptibility and degradation rate increasing with exposure time, temperature and the decreasing size of the microplastic. Where possible, high temperature HNO_3 conditions should be avoided, as it is likely to result in some level of degradation that may impact characterisation post-digestion. Overall, this study provides a comprehensive basis for understanding the reactivity of PS under common chemical processing conditions, however, future investigations should aim to characterise these behaviours from plastics with different polymer-additive composition (e.g., plastic additives such as plasticisers). Still, these data will ensure the integrity and accuracy of studies implementing chemical digestion protocols during sample processing and is vital for accurate risk analysis of microplastics in the environment.

Chapter 3: Understanding plasticiser leaching behaviours from polystyrene microplastics

Gulizia, A. M.; Patel, K.; Philippa, B.; Motti, C. A.; van Herwerden, L.; Vamvounis, G. Understanding Plasticiser Leaching from Polystyrene Microplastics. *Sci. Total Environ.* **2023**, *857*, 159099. https://doi.org/10.1016/j.scitotenv.2022.159099

Introduction

Plastic polymers possess unique physiochemical properties encouraging their abundant use in society[5], however, continuing advancements in synthetic methods and manufacturing has allowed for further tailorability of plastics through the addition of chemical additives[34]. Plasticisers are some of the most common plastic additives incorporated into polymers and are used to enhance polymer malleability and flexibility. Phthalate acid ester (PAE) and diphenol based plasticisers such as diethyhexyl phthalate (DEHP), dioctyl terephthalate (DEHT), bisphenol A (BPA) and bisphenol S (BPS), respectively, contribute to over 80% of all plasticisers synthetised for plastics manufacturing[208]. Importantly, these plasticisers are endocrine disruptive chemicals (EDCs), are widely recognised as hazardous aquatic pollutants[52,56,58,209] and thus are the topic of many studies investigating their combined and/or synergistic toxicity with plastics (especially microplastics <5 mm in diameter) to wildlife[85,113–115,139,210–213]. Plasticised microplastics in aquatic environments are extremely mobile and bioavailable to organisms, facilitating the transport and release of plasticisers into unique compartments of the water table and food chain[33,61,214]. However, the mechanisms that control the leaching and subsequent release of plasticisers from microplastics in aqueous environments are poorly understood.

The potential for plastic materials to transfer plasticisers into the surrounding media and to organisms has sparked a plethora of studies investigating their leaching behaviours. To date, most of these studies have focussed primarily on quantifying and comparing leaching from consumer plastics (e.g., children's toys[215]) under standard user conditions (e.g., microwaving[216]) and/or understanding human health concerns associated with exposure to plastic leachates[43,47]. These studies have shown that chronic exposure to plasticisers/plasticised plastics via dermal and dietary sources (e.g., leaching into foodstuff[45]) are the primary mechanisms underlying plasticiser toxicity in humans[49,133,215]. However, plastic leachates are also severely toxic to aquatic organisms[85,113–115,210,211,213]; with developmental, reproductive and endocrine toxicity observed in a range of tested organisms (median 48 hour lethal dose (LC_{50}): 1.04 ng/L – 4.49 mg/mL)[211,212]. To mitigate these toxic impacts, environmental risk assessments (ERAs) of plasticised microplastics should be expanded to include the major transport routes of plasticisers into aquatic environments. Moreover, the low LC_{50}'s and rapid toxicity response data (within 48 hours) highlights the critical need to identify and characterise plasticiser leachability (i.e., rate and concentration) as well as the dominant factors influencing these behaviours (i.e., microplastic physiochemical properties, surrounding conditions)[118,126].

Several recent studies investigating the release behaviours of additives from plastics have identified a relationship between the physiochemical properties of the polymer and the surrounding abiotic conditions. The extent of additive leaching has been previously linked to the thermal properties of the polymer, including crystallinity[217] and the glass transition temperature (Tg)[34,118]. In a study performed by Bandow *et al.,* (2017), experimental photoaging of polyethylene (PE), polyvinyl chloride (PVC) and polystyrene (PS) plastics (<6.33 mm in diameter) prompted a decline in polymer crystallinity, which led to the accelerated release of additives[134]. Similarly, it was found that polymer *Tg* (i.e., more free volume[128])[118,127,134], microplastic size (i.e., diffusion length[125]), additive solubility parameters[85], and fluctuating external conditions (e.g., hydrostatic pressure, temperature, salinity and agitation) can uniquely impact leaching beahviours[85,118,126,127,218,219]. These observations indicate that factors impacting molecular diffusivity (i.e., free-volume, size, temperature) as well as other abiotic conditions such as water agitation play determining roles in the leaching behaviours of microplastics in aquatic environments[118,120,127]. However, a model that considers both molecular diffusion and the influence of external conditions on plasticiser leaching and surface partitioning (boundary layer theory[127,131]) has yet to be developed.

The combination of both molecular diffusion and boundary layer theory is a powerful and more accurate approach to understanding the leaching dynamics of plastics[118,127,130,220,221]. Henkel *et al.,* (2022) reported the molecular diffusion and boundary layer properties of DEHP and DEHT from PVC microplastics into aqueous media *in-situ*, and highlighted how changes to the polymer's physiochemical properties (PAE concentration) and external factors (additive hydrophobicity) impacted leaching curves[222]. Given the hydrophobicity of the tested additives and the calculated partition coefficients, it was concluded that DEHT and DEHP leaching was limited by their diffusion across the microplastic surface-water boundary layer. However, it is expected that changes in the chemical properties (e.g., *Tg*) of plasticised polymers will differ depending on the plasticiser type (e.g., BPA and BPS), as well as fluctuating external conditions (e.g., water agitation), both of which will promote different kinetic behaviours, and thus lead to different plasticiser leaching rates and concentrations. Exploring models that incorporate concepts related to both diffusion and boundary layer properties under environmentally relevant conditions would be useful to improve the understanding of microplastic leaching dynamics, as well as to establish the dominant wildlife exposure pathways in aquatic ecosystems vital for ERAs.

It is hypothesised that the leaching behaviours of plasticised PS microplastics will be affected by the polymer-plasticiser composition, aquatic environmental conditions and the particle size. Therefore, this chapter will investigate the impacts of changing aqueous conditions (salinity, pH, water temperature and agitation) on the leaching kinetics of DEHP, DEHT, BPA and BPS from PS based microplastics (<200 µm – 2 mm in diameter). Plasticised-PS was prepared under controlled conditions to ensure homogeneity of the polymer's physiochemical properties (e.g., *Tg*) and separated into three dominant microplastic size classes (<200 µm, 300 µm – 1 mm and 1 – 2 mm). Leaching behaviours were quantified using gel permeation chromatography (GPC), high performance liquid chromatography (HPLC) and thermal gravimetric analysis (TGA), and the experimental data used to develop a diffusion and boundary layer model. The experimental results and model outputs will allow researchers to estimate and predict the leaching behaviours of PS in different environmental (e.g., fluctuating water quality with climate change) and laboratory (e.g., mesocosm tanks) conditions. Ultimately contributing towards the development of more accurate and robust ERAs of plasticised microplastics in the environment.

Experimental Section

Materials

PS (weight-average molecular weight (*Mw*) = 192 K Daltons, 430102-1 Kg), DEHP (D201154-500 mL), BPA (133027-500 g) and BPS (103039-500 g) were sourced from Sigma Aldrich, DEHT (402492-500 mL) was sourced from Acros Organics, tetrahydrofuran (THF; HPLC grade), NaOH and HCl were sourced from Unichrom, and formic acid (90%) was sourced from Univar. All were used as received. Filtered seawater (0.5 µm; pH = 8.10, salinity ≈ 35 %) was collected from the Australian Institute of Marine Science National Sea Simulator (AIMS SeaSim). MilliQ water (pH = 6.40, salinity <3 %; used to simulate freshwater), acetonitrile (ACN; HPLC grade, Fisher) and methanol (MeOH; HPLC grade, Univar) were degassed for 20 min and filtered through a 0.22 µm GS-Millipore filter prior to use.

Microplastic preparation

Pure PS beads were dissolved in THF (1:4 w/v) under ambient conditions with constant stirring until dissolved. A plasticiser solution (DEHT, DEHP, BPA and BPS in THF; 1:10 w/v) was added (85:15 or 72:25 PS:plasticiser) with constant stirring for 4 days to ensure complete dissolution and homogeneity of the solution. Solutions were then cast on a watch glass and the residual solvent removed under vacuum at 35 – 65°C to form a brittle, plastic membrane. The dried membranes were then processed using a Magic Bullet – Nutribullet® 900 Series blender and sieved over a stainless-steel screen sieve (Glenammer Sieves) to afford irregularly shaped microplastics; small (<200 µm, median size = 136 µm), medium (400 µm – 1 mm; median size = 593 µm) and large (1 – 2 mm; median size = 1.4 mm) (Figure S1a, c, e). Size distribution analysis of the fragments was undertaken according to the supplementary methods using a Leica MZ26A (for small size fragments) and a Nikon Eclipse 50iPOL (for medium and large size fragments) microscopes. These PS-plasticiser compositions are representative of common plastic products (PAE and diphenol incorporation ranges between 10 – 70 wt. %[37]), which are widely distributed in aquatic environments[61,77,223,224], and thus, leaching from these microplastics represents a significant ecotoxicological hazard[33].

Particle size statistics were obtained using image segmentation through Matlab's Colour Threshold application (vR2022a). The microscope images and resulting binary image masks (Figure S3.1b, d and f) were denoised using image morphology (open and close operations using a 5-pixel diameter circular structuring element), and any detected objects that touched the boundary of the image were removed using the *"imclearborder"* function in Matlab. These machine-generated binary masks were then manually reviewed and correct by hand in paint.net (v4.3.12) to ensure accuracy of the image segmentation. Finally, particle statistics were determined using the *"regionprops"* function in Matlab (Figure S3.2 and Table S3.1). A limitation of this imaging approach is that when multiple particles overlap, they are detected as a single layer object, therefore the distribution occasionally counts objects larger than the corresponding size range.

Chemical characterisation of prepared plastics

The chemical properties of the plastics pertaining to leaching (i.e., plasticiser concentration and thermal properties) were characterised using GPC, TGA and DSC. GPC was used to measure the concentration (wt. %) of plasticiser incorporated into the microplastics, and was calculated using prepared calibration curves of PS, DEHT, DEHP, BPA and BPS in THF from 0 mg/mL – 1.05 mg/mL ($r^2 = 0.99$; Figure 3.3). For these analyses, microplastics (3 – 5 mg) were dissolved in THF (1.5 mL) and 50 µL aliquots were injected into a 1260 Infinity II Multi-Detector GPC (Agilent Technologies) equipped with a UV absorbance and refractive index detector. Two PLgel 5 µL MIXED-C columns (300 × 7.5 mm; Agilent Technologies) were calibrated using PS narrow standards (Agilent EasiVial PS-M; 470 – 482k g/mol). GPC confirmed successful incorporation of plasticisers with an average of 15.78 ± 1.31 wt. % (herein 15 wt. %) and 24.45 ± 2.75 wt. % (herein 25 wt. %). TGA was performed on a TA SDT 650 instrument at a heating rate of 10°C/min up to 500°C under a constant flow of nitrogen (50 mL/min) to confirm samples were free of solvent impurities and to establish the plasticiser concentration in the plastics (Figure S3.4). The relevant thermal properties of the plastic and the polymer-plasticiser interactions pertaining to leaching (*Tg*[225–227]), was characterised using DSC (TA DSC250). Plastics (2–6 mg) were subjected to two cooling and heating cycles from −20°C – 250°C at a rate of 50°C/min under a constant flow of nitrogen (50 mL/min). The thermograms generated after the second heating step are presented in Figure S3.5. According to these thermal analyses, virgin PS had a thermal decomposition temperature of 371°C and a *Tg* of 116°C.

Leaching experiments

To understand the leaching dynamics and mechanism(s) driving plasticiser leaching, prepared plasticised microplastics were exposed to environmentally relevant, aqueous conditions (water temperature, salinity, pH and agitation) according to Table 3.1. These conditions are representative of the range of aquatic ecosystems contaminated with microplastics (e.g., acidic/alkaline lakes, shoreline wave action, open ocean and etc.[9,228,229]), and therefore are susceptible to the long-term exposure consequences of plasticised-microplastic leachates. For these leaching studies, prepared microplastics (15 mg in a 20 mL glass scintillation vial) were immersed in excess aqueous media (10 mL) to ensure complete leaching of plasticisers. By leaching in excess solution, this eliminated any influence by, or dependency on, the dissolved plasticiser concentration (i.e., rate limiting or accelerating factors). Samples (*n* = 12 per plasticiser type, size class and condition, with *n* = 3 removed at each time point) were maintained under constant conditions using a temperature-controlled water bath (Grant Instruments for static conditions) and a temperature-controlled-shaking water bath (ThermoFisher for agitated conditions). After leaching, microplastics were filtered (0.5 µm filter paper; Whatman), rinsed with deionised water to remove residual leachate, and were left partially covered, under ambient conditions until dry. Leachate solutions were stored in airtight glass vials.

Determining the concentration of plasticiser present on the surface of the prepared microplastics is important as plasticisers can often migrate and accumulate on the surface of plastic materials[220]. Knowledge of the surface plasticiser concentration will impact the microplastic surface-water boundary layer properties, and thus also facilitate determination of leaching kinetics (i.e., limited by molecular diffusivity or surface partitioning)[118,127]. Quantifying the amount of plasticiser accumulating on the surface of the plastic prior to leaching was achieved by undertaking a rising experiment using additional plasticised microplastics (15 wt. %; 75 g, *n* = 3 per plasticiser type, *n* = 12 total) in filtered seawater (50 mL; pH 8.1) with constant stirring under ambient conditions for 1 minute. This was to ensure all surface plasticiser was adequately removed from the microplastic, leaving only the leachable component available during the experiment.

Table 3.1. The leaching conditions investigated in this chapter.

Exposure Condition	Plasticiser conc. (wt. %)	Media Type	Temperature (^{o}C)	Agitation	pH	Microplastic size (mean of maximum Fever diameter)	Sampling time
Temperature	15	Seawater	5	Static	8.1	Small (136 µm)	2 & 24h, 7 & 21 d
	15	Seawater	25.5	Static	8.1	Small (136 µm)	2 & 24h, 7 & 21 d
	15	Seawater	60	Static	8.1	Small (136 µm)	2 & 24h, 7 & 21 d
	15	Seawater	5	Static	8.1	Medium (593 µm)	24h, 7, 14 & 21d
	15	Seawater	25.5	Static	8.1	Medium (593 µm)	24h, 7, 14 & 21d
	15	Seawater	60	Static	8.1	Medium (593 µm)	24h, 7, 14 & 21d
	15	Seawater	5	Static	8.1	Large (1.4 mm)	24h, 7, 14 & 21d
	15	Seawater	25.5	Static	8.1	Large (1.4 mm)	24h, 7, 14 & 21d
	15	Seawater	60	Static	8.1	Large (1.4 mm)	24h, 7, 14 & 21d
Agitation	15	Seawater	25.5	90 rpm	8.1	Small (136 µm)	2 & 24h, 7 & 21 d
	15	Seawater	25.5	90 rpm	8.1	Large (1.4 mm)	24h, 7, 14 & 21d
Salinity	15	Freshwater	5	Static	6.4	Small (136 µm)	2 & 24h, 7 & 21 d
	15	Freshwater	25.5	Static	6.4	Small (136 µm)	2 & 24h, 7 & 21 d
	15	Freshwater	60	Static	6.4	Small (136 µm)	2 & 24h, 7 & 21 d
	15	Freshwater	5	Static	6.4	Large (1.4 mm)	24h, 7, 14 & 21d
	15	Freshwater	25.5	Static	6.4	Large (1.4 mm)	24h, 7, 14 & 21d
	15	Freshwater	60	Static	6.4	Large (1.4 mm)	24h, 7, 14 & 21d
pH	15	Seawater	25.5	Static	1	Small (136 µm)	24h
	15	Seawater	25.5	Static	3	Small (136 µm)	2 & 24h, 7 & 21 d

	15	Seawater	25.5	Static	10	Small (136 µm)	2 & 24h, 7 & 21 d
	15	Seawater	25.5	Static	11	Small (136 µm)	24h
	15	Seawater	25.5	Static	3	Large (1.4 mm)	24h, 7, 14 & 21d
	15	Seawater	25.5	Static	10	Large (1.4 mm)	24h, 7, 14 & 21d
Plasticiser concentration	25	Seawater	25.5	Static	8.1	Small (136 µm)	2 & 24h, 7 & 21 d

Quantifying leaching behaviours

Leaching as a function of plasticiser type and concentration, microplastic size, abiotic condition and time was quantified by calculating the decrease in plasticiser concentration in the microplastics (GPC), as well as the increase of aqueous plasticiser in surrounding solution (HPLC). GPC data was obtained as described above. Aqueous plasticiser concentration (mg/mL) was determined using HPLC on an Agilent 1100 serious HPLC system equipped with a G1379A degassing unit, G1311A quaternary pump, G1367A auto sampler, G1316A column oven and G1315B photodiode array detector operated using Agilent ChemStation software; Agilent Technologies). An aliquot of sample (n = 3) was injected onto a reverse phase analytical Raptor ARC18 column (150 × 4.6, 5 µm, Restek) and chromatographed using a modified method based on Masse *et al.,* (2017) and Rodriguez *et al.,* (2019)[230,231] (Table S3.2). Calibration curves were generated using DEHT and DEHP standards in ACN (0.082 – 3.40 mg/mL), and BPA and BPS standards in MeOH (0.018 – 0.28 mg/mL) ($r^2 > 0.99$; Figure S3.6 and S3.7).

Numerical modelling

Leaching dynamics were modelled using a diffusion and boundary layer model[118,220,222]. A spherically symmetric microplastic of a radius r_0 was assumed, with the sphere having a spatially uniform concentration of plasticiser within its volume and some additional plasticiser on its surface[220]. Different polymer-plasticiser blends will have different chemical associations[34,120], these associations were categorised into "free plasticiser", which is the fraction that leaches via molecular diffusion, and "associated plasticiser", which is the fraction that does not leach within the experimental timeframe and conditions.

For each experimental condition, the surface plasticiser was directly measured as the loss of plasticiser concentration after rinsing. To determine the fraction of associated plasticiser, the system was allowed to fully leach and reach equilibrium. The equilibrium value was calculated as the average of the last two measurements obtained through GPC, except for DEHP and DEHT where the approach to equilibrium was slower, and so only the last measurement was used. The amount of free plasticiser was then calculated by subtracting the surface plasticiser and associated plasticiser from the initial plasticiser concentration.

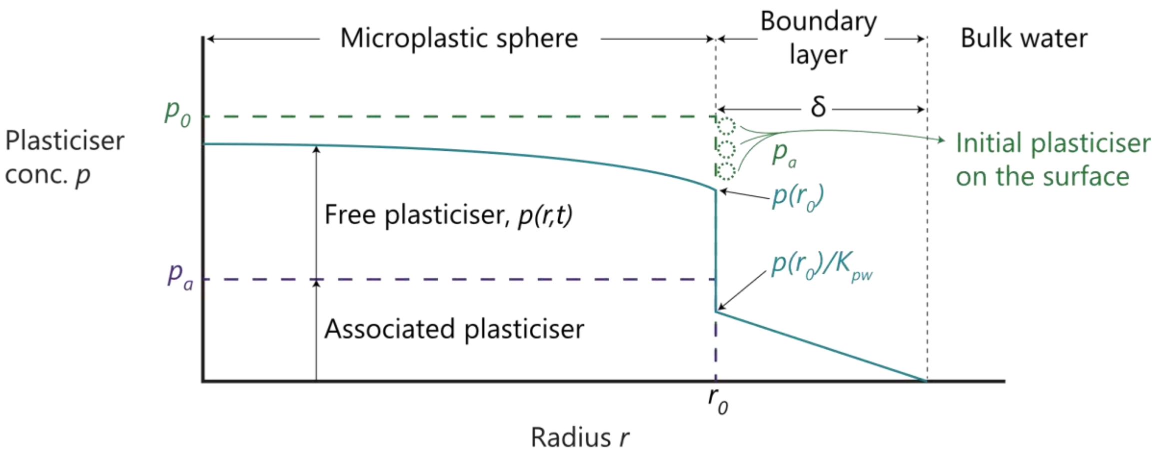

Figure 3.1. The model considers a microplastic sphere with radius r_0 that has an initially uniform concentration of free plasticiser p_0, some concentration of surface plasticiser p_s, and some concentration of associated plasticiser p_a. The free plasticiser moves through the polymer by diffusion and dissolves into the water when it reaches the surface of the microplastic. The width of the boundary layer is exaggerated for clarity.

A summary of the model is shown in Figure 3.1. The free plasticiser inside the microplastic sphere is subject to diffusion. The diffusion equation in spherical geometry is:

$$\frac{\partial p}{\partial t} = \frac{D}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial p}{\partial r}\right), \tag{1}$$

where $p(r,t)$ is the concentration of free plasticiser, r is radial distance from the centre of the microplastic sphere, t is time, and D is the diffusion coefficient. The diffusion coefficient is assumed to have an Arrhenius-like temperature dependence:

$$D = D_0 \exp\left(\frac{-E_A}{RT}\right), \tag{2}$$

with activation energy E_A, where R is the ideal gas constant and T is temperature.

The interface between microplastic surface and the surrounding aqueous solution is described using a boundary layer model as follows. The boundary layer is the thin region of water between each microplastic sphere and the bulk water[127,130]. Its behaviour is characterised by the concentration of plasticiser at its two edges. Firstly, the concentration at the inside edge (accumulating on the surface of the microplastic[220]) is proportional to the concentration inside the plastic. There is a step change in concentration across the plastic-water interface due to the hydrophobicity of the plasticiser[232]. The ratio of concentrations is given by the partition coefficient K_{pw}. Therefore, at the plastic-water interface, the concentration of plastic in the water is given by $p(r_0,t)/K_{pw}$. This concentration specifies one edge of the boundary layer. At the other side of the boundary layer, the bulk water is considered to be of sufficient volume that the plasticiser has fully dispersed and the concentration is zero. Since the boundary layer is thin, it is convenient to assume that the concentration declines linearly, as shown in Figure 3.1. This allows the use of Fick's first law to describe the flux of plasticiser leaving the microplastic:

$$J(r_0, t) = \frac{p(r_0, t)D_w}{K_{pw}\delta}, \tag{3}$$

Where D_w, is the diffusion coefficient of plasticiser in water, K_{pw}, is the plasticiser-water partition coefficient, and δ, is the width of the boundary layer.

Equation 3 allows us to determine a boundary condition for the diffusion equation at the plastic-water interface. Specifically, the flux is given by $-D\nabla p = J$ which leads to:

$$\frac{\partial p(r_0)}{\partial r} = -\frac{p(r_0)D_w}{DK_{pw}\delta}. \tag{4}$$

For the purposes of curve fitting (as described below), these coefficients were combined into an adjusted boundary layer-partition coefficient product $\delta^* = \delta K_{pw}D/D_w$. The difference between static and agitated water is assumed to be due to differences in the boundary layer. Therefore, the parameter δ^* is fitted separately for static and agitated water. The other boundary condition is $\frac{\partial p}{\partial r} = 0$ at $r = 0$ due to the spherical symmetry. This partial differential equation numerically was solved using the finite difference method.

The average concentration of all plasticisers in the sphere was calculated as the sum of the associated and free plasticiser:

$$p_{total} = p_a + \frac{1}{V_s}\int_0^{r_0} 4\pi r_0^2\, p(r,t)\, dr, \tag{5}$$

Where p_a is the amount of associated plasticiser and the integral term is the spatially averaged concentration of free plasticiser in the sphere. Here $V_s = \frac{4}{3}\pi r_0^3$ is the volume of the microplastic.

Fits to the experimental data were obtained by particle swarm optimisation using data from the small and medium sized microplastic leaching data ($r_0 \leq 1$ mm), because those sizes displayed the strongest and most distinct leaching behaviour. For each plasticiser, a simultaneous curve fitting was performed over all temperatures and agitation conditions. The four parameters obtained by optimisation were the diffusion prefactor D_0, the diffusion activation energy E_A, as well as the boundary layer properties δ^*_{static} and $\delta^*_{agitated}$ in static and agitated water, respectively.

The optimisation routine was tasked with minimising the mean squared error between the measurement and the model prediction. Since optimisation is sensitive to starting conditions, the initial estimates on subsets of the data were obtained. Specifically, fit estimates of D_0, $\delta^*{}_{static}$ and $\delta^*{}_{agitated}$ using the static and agitated data at 25.5°C were determined, followed by fit estimates of D_0 and E_a using the static data at varying temperatures, before finally performing a global fine-tuning of all four model parameters on all experimental data simultaneously.

Statistical analyses

Data were presented as the mean of triplicates with standard deviation. Pairwise analysis t-tests and analysis of variance (ANOVA for BPA and BPS and Kruskal-Wallis for DEHT and DEHP) were performed in Microsoft Excel at a p-value threshold of 0.05. Data are represented in Figure S3.8 and Table S3.3.

Results and discussion

Effect of temperature on leaching

Ambient global water temperatures fluctuate significantly seasonally, spatially and geographically. However, greater fluctuations and more extreme thermal conditions associated with climate change is a serious threat facing ecosystems and wildlife. Elevated water temperatures can also impact the long-term fate and reactivity of debris present in these environments[12], and at times, can act synergistically to enhance the toxicity of wildlife exposed to microplastics[233,234]. However, without an understanding of plastic leachates and/or the leachable properties of plasticised-microplastics, it is difficult to determine the property or component (i.e., polymer, additive and/or combination) responsible for inducing the biological response[235]. To address this, the leaching behaviours of plasticisers from microplastics (15 wt. %; small and large) were measured in marine and freshwater at different temperatures (5, 25.5 and 60°C). No temperature dependence was observed for PAE plasticisers (DEHT and DEHP) from small microplastics under all tested aqueous conditions (Figure 3.2; Figure S3.9 – S3.11). While there was an initial loss of these plasticisers after 1 minute of rinsing (2.06 – 2.54 wt. %), leaching reached equilibrium within 1 day and plateaued for the remainder of the experimental timeframe ($p > 0.05$)[127]. The *immediate* loss of PAE plasticiser during rinsing was attributed to their tendency to accumulate near the surface of plastic materials[34,220], while their high retention was attributed to their strong association with the polymer (Tg of PAE plasticised PS ≈ 65°C; Figure S4), as well as their immiscibility in water ($K_{OW} \approx 7.60$)[118,126,130,236]. In contrast, the leaching of diphenol plasticisers (BPA and BPS) followed typical temperature dependence, whereby higher temperatures were associated with the fastest leaching rates and highest leachate concentrations. These results suggested that rising and fluctuating water temperatures (both *in-situ* and *in-natura*) could alter and/or accelerate the leaching of additives from microplastics. Implying a heightened risk of toxicity from microplastics in ecosystems sensitive to climate variability (e.g., Arctic and alpine regions, tropical freshwater ecosystems[12,237]) as well as habitats with higher ambient water temperatures (e.g., coral reef ecosystems[84]), and is likely to be exacerbated with global warming.

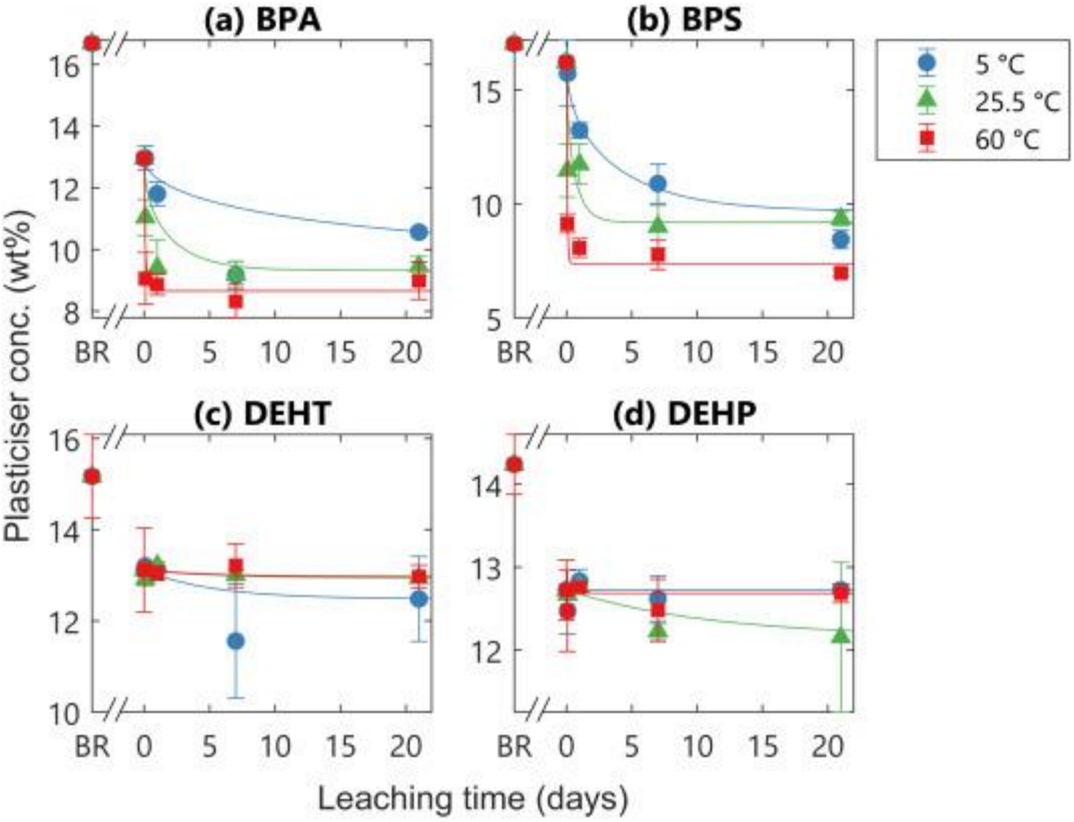

Figure 3.3. The leaching behaviours of bisphenol A (BPA), bisphenol S (BPS), dioctyl terephthalate (DEHT) and diethylhexyl phthalate (DEHT) from small microplastics (136 µm) in static seawater (pH 8.1) at 5°C (blue), 25.5°C (green) and 60°C (red) over 21 days. The plots show the starting plasticiser concentration (wt. %) before (BR) and after rinsing. The points are the experimental data ± standard deviation and the lines are the model fits.

Assessment of leaching across a range of water temperatures also allowed the fitting of the experimental data to a numerical model. Given that the leaching behaviours of PS were similar in marine and freshwater (Figure 3.2), only the modelling data of seawater is presented herein. With salinity and pH removed as affecting parameters, the modelling data can be used to describe the differences observed between the two classes of plasticiser (diphenol and PAE), applicable to both marine and freshwater ecosystems. In the case of BPA and BPS, which displayed a strong temperature dependence, leaching was limited by the diffusion of these plasticisers throughout the bulk polymer and hence was governed by an activation energy (E_A; Table 3.2). Conversely, the rate-limiting step of DEHT and DEHP leaching was the partitioning of the plasticisers across the microplastic surface-water boundary layer[127], which displayed no temperature variation. These leaching kinetics were also supported by DSC thermal characterisation, which highlighted the differences in the efficiency and strength of the polymer-plasticiser interactions (Figure S3.4)[220]. Through measured changes to the *Tg* of plasticised PS (15 wt.

%), PAE plasticisers imparted improved plasticisation and increased free volume ($Tg \approx 65^{\circ}C$) when compared to diphenol plasticisers ($Tg \approx 93^{\circ}C$)[34]. Conversely, the weaker interaction of diphenol plasticisers with PS facilitated their molecular diffusion throughout the polymer matrix and thus promoted their leaching. This further supports the concept that there are substantially different mechanisms government diphenol (diffusion limited) and PAE (boundary layer limited) leaching dynamics[118,220,222].

Table 3.1. Model fit results highlighting the different leaching dynamics of plasticisers from polystyrene (PS) microplastics. The boundary layer for both BPA and BPS was not rate limiting under any experimental conditions and therefore the reported values (< 0.1 mm) indicate the boundary layer had no impact on leaching rates. For DEHT and DEHP diffusion rate was not temperature dependent and hence diffusion kinetics (coefficient and activation energy) could not be determined.

Plasticiser	**Diffusion coefficient of plasticiser in PS at 25.5°C (cm^2/s)**	**Diffusion coefficient activation energy (kJ/mol)**	**Boundary layer coefficient (mm)**	
			Static water	**Agitated water**
Bisphenol A (BPA)	2.1×10^{-11}	70	<0.1	<0.1
Bisphenol S (BPS)	6.8×10^{-11}	60	<0.1	<0.1
Dioctyl terephthalate (DEHT)	–	–	>100*	2.3
Diethylhexyl phthalate (DEHP)	–	–	>100*	4.7

* An estimate of the lower bound of the boundary layer ($\delta*$) was determined by finding the value needed to reproduce the experiment observation of negligible leaching within 21 days.

Effect of water agitation on leaching

Exposure to agitated water could influence plasticiser leaching kinetics by accelerating the transport of plasticisers across the microplastic surface-water boundary layer[126,132]. Microplastics in aquatic systems are constantly exposed to moving and agitated waters, whereby, even the most lentic ecosystems (e.g., ponds, seasonal pools, marshes and lagoons) experience some variation in water flow[228,238,239]. Hence water turbulence should be considered when assessing microplastic leaching dynamics in environmentally relevant matrices. Here, enhanced leaching of all plasticisers was observed after exposure to agitated water for 21 days ($p < 0.05$; Figure 3.3 and Figure S3.12). Agitation most significantly impacted the release of DEHT and DEHP and accelerated leaching by up to 15% when compared to BPA and BPS, suggesting that the microplastic surface-water boundary layer properties play a significant role in leaching dynamics. Interpretation of this agitation data mirrors the temperature-dependent results, whereby the leaching of BPA and BPS is rate-limited by low diffusion within the plastic and is not significantly impacted by water movement around the boundary layer. Whereas DEHT and DEHP leaching is rate-limited by the boundary layer and is strongly impacted by water agitation[126,127]. The underpinning mechanism driving these agitation accelerating behaviours is a reduction in the thickness of the boundary layer, coming as a result of increased water flow around the microplastic fragment[131,132]. Overall, these results indicate that microplastics exposed to moving water in the environment will promote significant release of plasticisers into the surrounding water, potentially contributing to a localised decrease in water quality at the source point. This is particularly true in lotic ecosystems experiencing high fluctuations in water movement, including wave action in coastal environments and along shoreliens[12,69,240], as well as water currents in open oceans and around coral reefs[139].

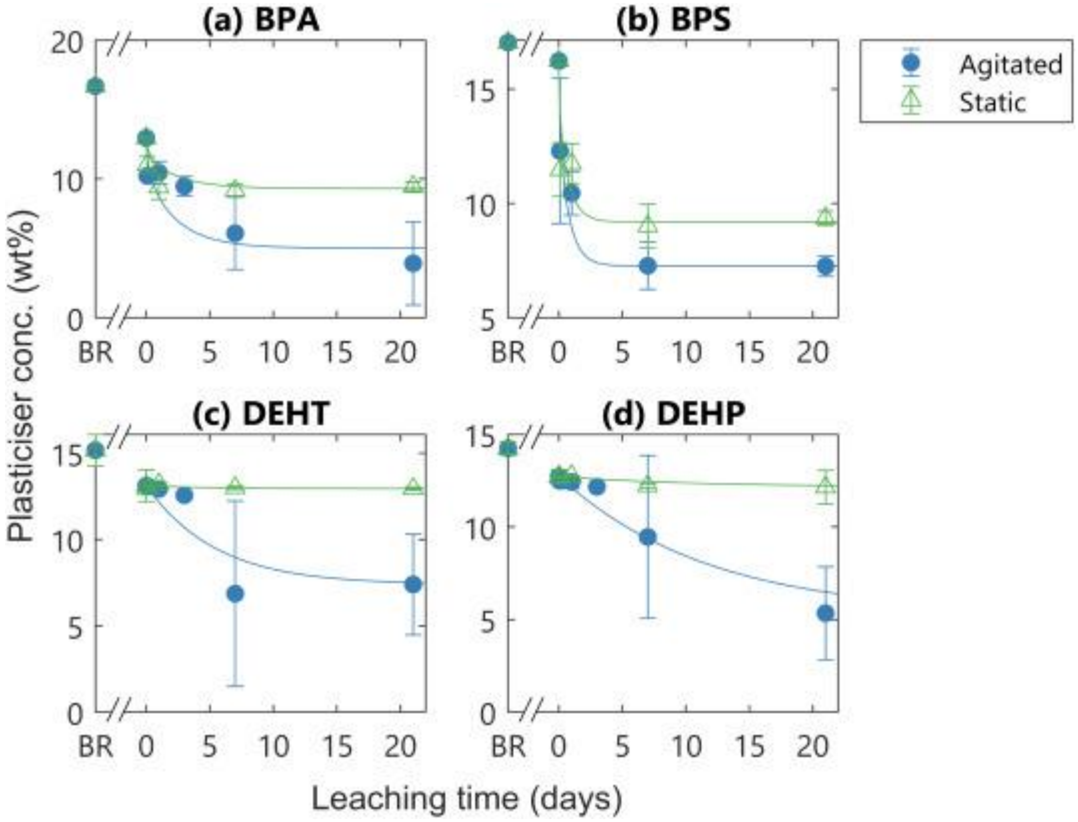

Figure 3.4. The leaching behaviours of bisphenol A (BPA), bisphenol S (BPS), dioctyl terephthalate (DEHT) and diethylhexyl phthalate (DEHT) from small microplastics (136 µm) in static (green) and agitated (blue) seawater (pH 8.1; 25.5°C) over 21 days. The plots show the starting plasticiser concentration (wt. %) before (BR) and after rinsing. The points are the experimental data ± standard deviation and the lines are the model fits.

Effect of pH and salinity on leaching

Oceanic waters are weakly alkaline with a global average pH of 8.1, while the pH of freshwater can fluctuate significantly from 5 to 9. Generally, marine and freshwater ecosystems can buffer and/or mediate small changes in water quality (e.g., pH and salinity), however, anthropogenic activity (e.g., wastewater and effluent discharge) and climate change (e.g., CO_2 concentration and precipitation) can significantly alter natural water conditions, resulting in devastating ecological consequences[241,242]. Microplastic contamination in these environments can create additional challenges for ecosystems mitigating ocean acidification and salinity changes, thus making them particularly vulnerable to the ecotoxicological hazards associated with aqueous plasticiser contamination[85]. The impacts of solution chemistry (i.e., salinity, pH, lipophilicity) on the interaction of microplastics with chemical pollutants (e.g., flame retardants) has received some research attention[31], however, how these environments regulate plasticisers leaching from microplastics is relatively unknown.

In this study, small (136 µm median diameter) and large (1.44 mm median diameter) microplastics were exposed to seawater and freshwater, as well as seawater with pH varying from 1 to 11, to explore the impacts of salinity, alkalinity and acidity on plasticiser leaching dynamics. In line with other published studies[126], no unique variation in leaching behaviours were observed in seawater or freshwater from all microplastics at ambient (pH 8.1 and 6.4, respectively), low (pH 1 and 3; water adjusted with 0.1 M HCl) and high (pH 10 and 11; water adjusted with 0.1 M NaOH) pH after 24 hours (Figure S3.13 – S3.16 and Table S3.4). While these extreme conditions (pH 1 and 11) are very rare in the environment, extrapolation of leaching experiments conducted over 21 days at pH 3 and 10, indicates that even major pH fluctuations would not significantly alter the leaching dynamics of microplastics ($p > 0.05$). Suggesting that all freshwater and oceanic environments contaminated with microplastics are equally likely to be exposed to leached plasticisers. This can be further extended to include uniquely acidic or alkaline systems, such as those in the deep sea, soda and volcanic lakes/rivers, and even mammalian digestion[85,228,243]. These results also indicate predicted changes in ocean acidification will not alter plasticiser leaching behaviours, and if considered in isolation, will not have any adverse impacts on wildlife.

Impact of microplastic size on leaching

Smaller micro- and nano-sized plastics have become more prevalent in aquatic matrices[194,244]. Continued size reduction of microplastics can facilitate their transport in the environment[6,245], increase their interaction with widlife[66,82], and via leaching processes, can facilitate size-dependent transfer of toxic plasticisers into ecosystems and the food chain[33,85]. In this study, the leaching profiles of small (136 µm), medium (593 µm) and large (1.4 mm) irregularly shaped microplastics incorporated with BPA and DEHT were analysed as a function of size (Figure 3.4). Given the similarities in leaching dynamics between the two classes of plasticiser (PAE and diphenol), the leaching profiles of BPA and DEHT generated here are also representative of BPS and DEHP under the same conditions[120,222]. Consistent with the model output, size-dependency (small > medium > large) was only observed for diffusion-limited plasticisers (diphenols; $p < 0.05$), whereby leaching rate slowed as the length of diffusion increased (i.e., the distance from the centre of the microplastic to the surface, r). Contrary, DEHT did not show any significant size variation, which was expected given that PAE leaching is limited by the boundary layer[127]. Still, there was an overall trend in smaller microplastics releasing more plasticisers, which further supports the idea that the fragmentation of both primary and secondary microplastics in the environment poses unique risks to wildlife through leaching[14,224,246]. The effects of amplified leaching from smaller microplastics can also be extended to include plasticised nanoplastics[194], as well as in organisms with filter feeding mechanisms[82,247].

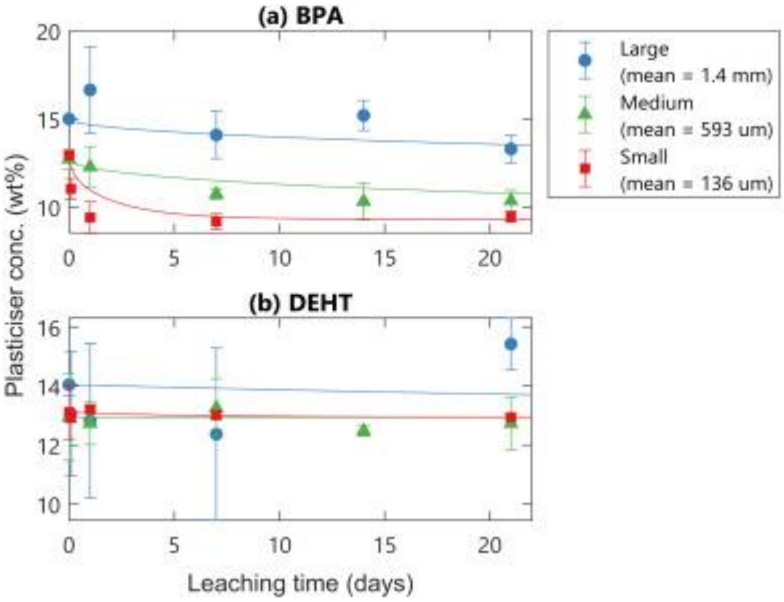

Figure 3.5. The leaching behaviours of bisphenol A (BPA) and diethylhexyl phthalate (DEHT) from small (red), medium (green) and large (blue) microplastics in static seawater (pH 8.1; 25.5°C) over 21 days. The points are the experimental data ± standard deviation and the lines are the model fits.

Model fit and analysis

The numerical model considered two steps in the leaching process: firstly the plasticisers must diffuse throughout the polymer matrix and reach the interface with the water. Following this, plasticisers accumulating on the surface of the microplastic must partition into the surrounding water by crossing the boundary layer. The experimental data was fitted to the model to obtain the solid lines shown in Figure 3.2 – 3.4 and the coefficients listed in Table 3.2. The boundary layer was not limiting BPA and BPS under any test experimental condition, and therefore it as not possible to specify the boundary layer coefficient ($\delta*$). The reported values (<0.1 mm) indicate that the boundary layer has not impact on leaching rates. Conversely, DEHT and DEHP leaching was rate-limited by the boundary layer and not impacted by temperature, and hence the diffusion coefficient could not be determined for these plasticiser. However, in the case of static seawater, an estimate of the lower bound of $\delta*$ was determined by findings the value needed to reproduce the experimental observation of negligible leaching within 21 days.

The model allows for analysis of the spatial distribution of plasticiser during the leaching processes. Figure 3.6 shows the normalised plasticiser concentration at different time steps, from $t = 0$ (i.e., when the microplastic is first immersed in water) to a time where $t = t_{\text{leached}}$ (i.e., time taken to leach 99% of free plasticiser). The figure demonstrates that BPA and BPS have qualitatively different leaching behaviours when compared to DEHT and DEHP, whereby the distinction arises because of the different rate-limiting processes (diffusion- and boundary layer-limited, respectively). Hence, BPA and BPS show a non-uniform concentration of plasticiser during leaching, while DEHT and DEHP show nearly unique concentration within the microplastic.

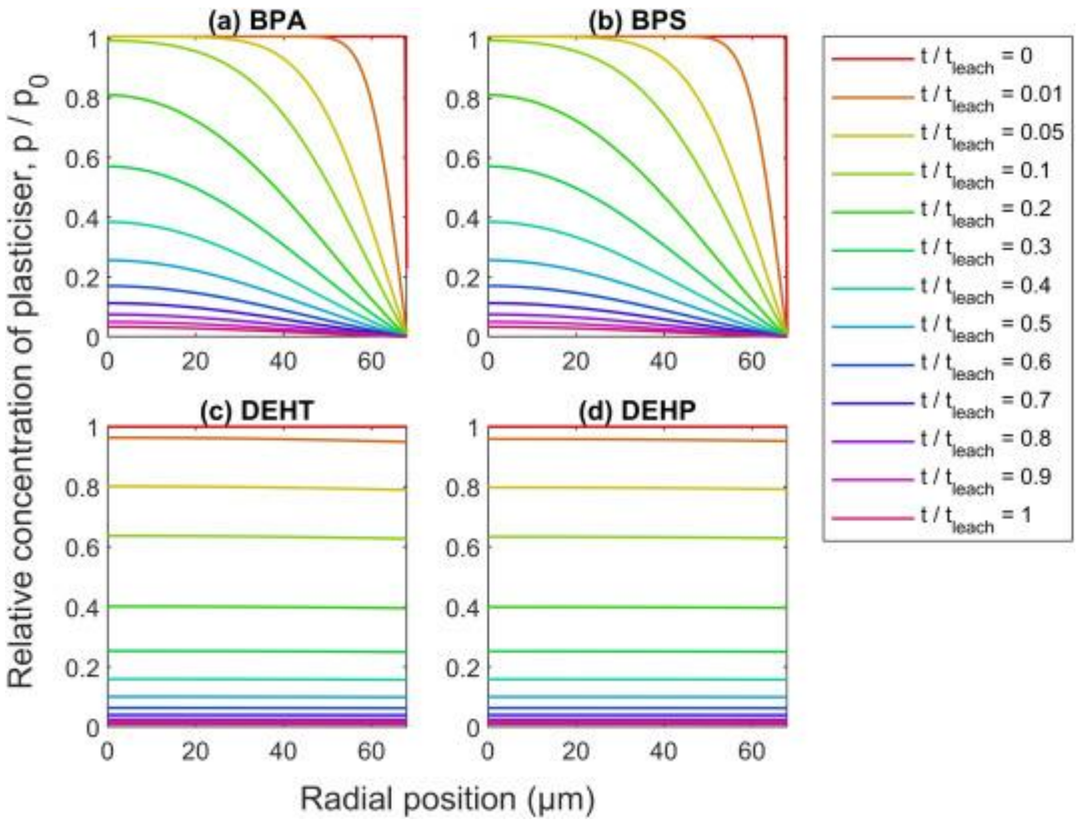

Figure 3.6. Snapshots in time of the model prediction showing the concentration of free plasticiser at various depths within a microplastic sphere of 68 μm radius, i.e. the typical size of the smaller particles in this study, under ambient agitated seawater conditions (25.5 °C, pH 8.1). The time intervals are specified relative to t_{leach}, which is defined as the time taken to leach 99 % of the free plasticiser. These results show the qualitatively different behaviours of disphenol plasticisers; bisphenol A (BPA) and bisphenol S (BPS) and phthalate acid ester plasticisers; dioctyl terephthalate (DEHT) and diethylhexyl phthalate (DEHP).

Conclusion

The leaching dynamics of tested PAE (DEHT and DEHP) and diphenol (BPA and BPS) plasticisers from PS microplastics was influenced by the polymer-plasticiser composition (polymer *Tg* and plasticiser hydrophobicity), the proportion of associated plasticiser, the size of the microplastic as well as the surrounding environmental water conditions (temperature and water agitation). While all tested conditions prompted a significant release of plasticiser into the surrounding water, differences in the rate-limited steps influenced the rate and concentration of leached plasticiser. Results here indicate that all aquatic environments are susceptible to the impacts of plasticiser leaching, especially those contaminated with high levels of small micro- and nanoplastics, those experiencing high and fluctuating water temperatures and lotic environments with significant water turbulence and wave action. The development of the diffusion and boundary layer model to describe plasticiser leaching highlights the need to comprehensively characterise the polymer-plasticiser composition impacting microplastics leaching dynamics (i.e., diffusion- or boundary layer-limited). For future studies investigating the exposure consequences of plasticised-microplastics *in-situ*, it is recommended that the leachable properties of plastics be considered during experimental design, e.g., by measuring leaching throughout the experiment, or by pre-leaching microplastics prior to exposure in order to minimise fluctuations in leachate concentration. Finally, to further strengthen this diffusion and boundary layer model, microplastics with different chemical compositions relevant to the environment should also be investigated and included to further validate the model, such as during polymer degradation. Based on the data obtained here, these considerations for future research will help to further improve ERA of microplastics in aquatic environments.

Chapter 4: Impact of molecular weight on plasticiser leaching from polystyrene microplastics

Introduction

Synthetic plastics are extremely durable and resistant to environmental stressors, and thus can persist as hazardous pollutants in the environment long-term[62]. However, ongoing exposure to degradative stressors such as UV light, physical weathering and microbial colonisation can promote the breakdown and fragmentation of larger plastic items into microplastics (<5 mm in diameter) and beyond (e.g., nanoplastics <1000 nm in diameter[194]). During degradation, microplastics can experience both chemical and physical changes, including oxidation and molecular chain scission, deteriorating thermal and mechanical properties, fragmentation and size reduction[134]. While these processes can eventually lead to the complete removal of plastics from the environment[248], in the short-term, these changes can have negative impacts on ecological processes by enhancing plastic bioavailability to biota (i.e., planktonic organisms[76,80]), increasing their mobility in tissues and organs (i.e., ability to cross the mammalian blood-brain barrier[246]) and accelerating the release of additives (e.g., plasticisers[33,117,249]). Plasticiser leaching from aged and degraded microplastics is a significant environmental concern, particularly as commonly used plasticisers such as diphenols (e.g., bisphenol A (BPA) and bisphenol S (BPS)) and phthalate acid esters (PAEs, e.g., diethylhexyl phthalate (DEHP) and dioctyl terephthalate (DEHT)) are known endocrine disrupting chemicals (EDCs) that can be highly toxic in the environment[37,250]. It is important to understand how plasticiser leaching dynamics are impacted during microplastic degradation, as these factors impact the long-term fate and exposure consequences of plasticised-microplastics in the environment and to biota.

Plasticiser leaching behaviours from aged and degraded polymers has been well documented[126,134,136,251]. In many of these studies, pristine microplastics (sourced from consumer products or a chemical supplier) containing a variety of additives (e.g., heavy metals[134], plasticisers[136], etc.,) were exposed to controlled biological, thermo- and/or photo-oxidative conditions *in-situ* to simulate the natural environment[134,136,251,252]. Degradative changes to the plastics physiochemical properties were measured using Fourier Transform-Infrared Spectroscopy (FT-IR) and/or microscopy by comparing the degree of oxidation (i.e., carbonyl index), discolouration (i.e., yellowing) and surface changes (i.e., cracking), respectively. Overall, while accelerated leaching from microplastics exposed to combined thermo- and photo-oxidative conditions has been observed, no definitive relationship between polymer degradation and additive leaching has been achieved[134,136,251,252]. Several hypotheses have been developed to explain these behaviours, which consider microplastic adsorption behaviours (i.e., of aqueous contaminants and water) as well as changes in polymeric free-volume and surface morphology. However, the severity of these physical indicators (e.g., yellowing, surface cracking and fragmentation) can vary depending on the polymer's degradative susceptibility[111,150,253], therefore, without implementing quantitative techniques that assess specific polymer properties related to degradation (e.g., weight-average molecular weight (Mw)[111,254]), it is difficult to isolate and accurately determine how, and by what mechanism(s) polymer degradation influences additive leaching dynamics[118,135,255,256]. To that end, the fragmentation pattern and size distribution of degraded plastics should also be considered when designing leaching experiments, especially because molecular diffusivity and the diffusion length (i.e., size/thickness) has been identified as the rate-limiting step in the release of many additives[125,221,257] (including plasticisers[127,220,255]) from microplastic particles[249]. Considering these factors in isolation will enable a more comprehensive and long-term environmental risk assessment (ERA) of the behaviours and ecological fates of plasticised-microplastics in aquatic ecosystems.

Therefore, this chapter applied a diffusion and boundary layer approach to characterise the leaching kinetics of PAE (DEHP and DEHT) and diphenol (BPA and BPS) plasticisers from polystyrene (PS) based microplastics with controlled aging properties (i.e., *Mw*) under simulated marine conditions. With the aim to reveal *Mw*-dependent leaching, virgin PS microplastics with different *Mw* (35k, 192k and 350k g/mol) and size distributions (43 – 199 µm) were prepared and characterised, and the leaching behaviours quantified using gel permeation chromatography (GPC) and thermal gravimetric analysis (TGA). The results were then applied to a numerical model to explain the leaching kinetics of plasticisers from degraded PS. It is hypothesised that declining polymer *Mw* and the size of the microplastics will impact leachability (i.e., the rate and concentration of plasticiser available to leach), particularly for plasticisers with previously reported diffusion-limited behaviours (i.e., BPA and BPS; Chapter 3). These data and model predictions will enable isolation of the dominant force(s) influencing plasticiser leaching from aged microplastics (i.e., secondary microplastics) in oceanic ecosystems, ultimately improving risk analysis and predictions of their long-term behaviours in environmentally relevant scenarios.

Experimental Section

Materials

Low- (L*Mw*; *Mw* = 42k g/mol; 331651-500 g), medium- (M*Mw*; *Mw* = 192k g/mol; 430102-1 Kg) and high-*Mw* (H*Mw*; *Mw* = 350k g/mol; 441147-1 Kg) PS, DEHP (D201154-500 mL), BPA (133027-500 g) and BPS (103039-500 g) were sourced from Sigma Aldrich, DEHT (402492-500 mL) was sourced from Acros Organics, and tetrahydrofuran (THF; HPLC grade) was sourced from Unichrom. All were used as received. Filtered seawater (0.5 µm; pH = 8.10, salinity ≈ 35 %) was collected from the Australian Institute of Marine Science National Sea Simulator (AIMS SeaSim).

Microplastic preparation

To understand the implications of polymer degradation on the leaching behaviours of plasticisers from microplastics, virgin PS beads with low (L*Mw*; 42k g/mol), medium (M*Mw*; 192k g/mol) and high (H*Mw*; 350k g/mol) *Mw* were incorporated with plasticisers (DEHT, DEHP, BPA or BPS) according to altered methods developed and described in Chapter 3. Virgin, low (1:3), medium (1:5) and high

(1:6) *Mw* PS beads were dissolved in THF under ambient conditions and with constant stirring, after which a plasticiser solution (DEHT, DEHP, BPA or BPS in THF; 1:10 w/v) was added (85:15 PS:plasticiser) with constant stirring for a further 4 days to ensure complete dissolution and homogeneity of the solution. Solutions were then cast onto a watch glass and the residual solvent removed at room temperature form a brittle, plastic membrane. The dried plastic membranes were processed using a Magic Bullet – Nutribullet® 900 Series blender and sieved over a stainless-steel screen sieve (Glenammer Sieves) to afford irregularly shaped microplastics <200 μm in diameter, with the low, medium and high *Mw* having a mean maximum diameter of 144, 126 and 157 μm, respectively. Particle sizes were analysed using optical microscopy (Nikon Eclipse 50iPOL; 4 images per polymer-plasticiser composition) and Matlab's Colour Threshold application (v R2022b) according to methods described in Chapter 3. Microscope images, corresponding binary image masks and particle size statistics are presented in Figure S4.1 – 4.15 and Table S4.1.

Chemical characterisation of prepared plastics

The chemical properties of the plastics pertaining to leaching were characterised using TGA and GPC. GPC was used to measure the concentration (wt. %) of plasticiser incorporated into the M*Mw* and H*Mw* microplastics and was calculated using prepared calibration curves of H*Mw* and M*Mw* PS, DEHT, DEHP, BPA and BPS in THF from 7.4 mg/mL – 126 mg/mL ($r^2 = 0.99$; Figure S3.3). For these analyses, microplastics (3 – 5 mg) were dissolved in THF (1.5 mL) and 50 μL aliquots were injected into a 1260 Infinity II Multi-Detector GPC (Agilent Technologies) equipped with an UV absorbance and refractive index detector. Two PLgel 5 μL MIXED-C columns (300 × 7.5 mm; Agilent Technologies) were calibrated using PS narrow standards (Agilent EasiVial PS-M; 470 – 482k g/mol). Given the bimodal distribution of the L*Mw* PS (Figure S4.16), GPC could not be used to accurately separate or clarify the polymer and plasticiser peaks, and thus, TGA was used to measure plasticiser concentration in the microplastics (wt. %). TGA (TA SDT 650) of the L*Mw* microplastics containing DEHP or DEHT (30°C/min ramp speed), and BPA or BPS (10°C/min ramp speed) were performed under constant flow of nitrogen (50 mL/min) up to 530°C (Figure S4.17). GPC and TGA values aligned (Table S4.2) and confirmed successful incorporation of plasticisers with an average of 21.08 ± 5.28 (H*Mw*), 15.78 ± 1.31 wt. % (M*Mw*) and 14.27 ± 2.89 wt. % (L*Mw*).

Leaching experiments

Plasticiser leaching dynamics were investigated from PS microplastics with varying *Mw* (simulating controlled chemical degradation) and size distribution (representative of fragmentation) to understand how polymer degradation impacts leaching[134]. Prepared microplastics (15 mg in a 20 mL glass scintillation vial) were immersed in excess filtered seawater (10 mL, pH = 8.1) and maintained at 25.5°C using a temperature-controlled water bath (Grant Instruments). Samples (*n* = 12 per plasticiser with *n*

= 3 removed a each time point) were taken after 1, 7, 14 and 21 days for H*Mw* PS, and after 2 hours, 1, 7 and 21 days for the M*Mw* and L*Mw* samples. After leaching, residual leachate was filtered (0.5 µm filter paper; Whatman) and stored in airtight glass vials, and the remaining microplastics were rinsed with deionised water and allowed to air dry under ambient conditions. Leaching behaviours were quantified by calculating the plasticiser concentration remaining in the microplastics (wt. %) using GPC (H*Mw* and M*Mw*) and TGA (L*Mw*), as described above. Seawater was chosen as oceanic environments represent the largest accumulation zone of microplastics[10], with PS microplastics[9,61,70], PAE and diphenol plasticisers detected in high concentrations worldwide[52,59].

Numerical modelling

Leaching was modelled using a similar approach to previous work[255]. Briefly, a spherically symmetric microplastic particle was assumed. The total concentration of plasticiser was partitioned into "free plasticiser", whose movement is described by the diffusion equation, and "associated plasticiser", which is bound to the polymer and unable to move under the given experimental conditions. The plastic-water interface was described by a boundary layer model, whereby the concentrations of plasticiser on the water and plastic sides of the interface are related by a partition coefficient. In the boundary layer model, there is an assumed linear decrease in plasticiser concentration with increasing distance into the water, away from the microplastic edge. The bulk water is assumed to have zero concentration of plasticiser.

The model previously developed in Chapter 3 was extended here to consider the distribution of particle sizes. Particle sizes obtained by optical microscopy were grouped into 15 histogram bins based upon their equivalent radius (Figure S4.13 – S4.15), which is the radius of a sphere whose area is the same as the irregularly shaped microplastic. To account for the distribution of plasticiser mass across the differently sized particles, each histogram bin was weighted in proportion to their volume, $V = \frac{4}{3}\pi r^3$, where r is the midpoint of the histogram bin. The results, given in Figure S4.18, show the relative proportion of plasticiser in the samples at different sizes. The numerical model was then run for each of these size categories. The overall results are reported as the weighted sum of each individual model run.

For DEHP and DEHT that the rate-limiting step occurs at the boundary layer rather than in the plasticiser bulk[255]. Therefore, the model was simplified to include only the boundary layer step. Under this assumption, the flux of plasticiser leaving the microplastic particle is proportional to its surface area. If the boundary layer is the rate-limiting step, then this implies rapid resupply of plasticiser from within the microplastic back to the edge. Hence, the average plasticiser concentration decreases at a rate inversely proportional to the particle's volume. Therefore, the average free plasticiser concentration p is given by:

$$\frac{\partial p}{\partial t} = -\frac{pAD_w}{K_{pw}V\delta}, \tag{1}$$

where A is the surface area of the particle, D_w is the diffusion coefficient of plasticiser in water, K_{pw} is the plastic-water partition coefficient, V is the volume of the plasticiser, and δ is the boundary layer thickness. Assuming spherical microplastic particles and solving the differential equation, an exponential trend is predicted, given by:

$$p = p_0 \exp\left(-\frac{3D_w t}{K_{pw} r\delta}\right) = p_0 \exp\left(-\frac{t}{\tau}\right), \tag{2}$$

where p_0 is the initial concentration of free plasticiser and $\tau = 3D_w/K_{pw}r\delta$ is the time constant. If D_w (the diffusion coefficient of plasticiser in water) is approximately independent of temperature within the experimental range, then the entire expression will have negligible temperature variation. Therefore, this model predicts that the time constant τ will be approximately independent of temperature, as was observed for the DEHP and DEHT data.

Finally, the total plasticiser concentration was measured as the sum of the free and associated plasticiser:

$$p_{total} = p + p_a = p_0 \exp\left(-\frac{t}{\tau}\right) + p_a, \tag{3}$$

where p_a is the concentration of associated plasticiser.

It was found that Eq. (3) was a good fit for DEHP and DEHT but not for BPA and BPS. Therefore, the more sophisticated model that includes the diffusion through the bulk polymer was used for BPA and BPS[255].

Statistical analyses

Data were presented as the mean of triplicates with standard deviation. Pairwise t-tests were performed in Microsoft Excel to evaluate the differences between the leaching behaviours of plasticisers from PS with different *Mw* at a p-value threshold of 0.05.

Results and discussion

Plastics in the environment are exposed to environmental stressors such as UV light, physical weathering and microbial colonisation that contributes to their chemical and physical degradation[183]. These pathways are responsible for the production of the bulk of microplastic contamination detected in the ocean (secondary microplastics[64]). However, the physiochemical properties of degraded microplastics, including *Mw*, size, surface morphology (e.g., swelling) as well the adsorption of aqueous contaminants (e.g., heavy metals) and water into the polymer matrix can impact the leaching of additives[134,136,251,252]. While many studies have demonstrated accelerated leaching from aged and degraded polymers[126,134,136,252], no specific property of the plastic has been identified as a significant contributor to these behaviours. Thus, to deconstruct these behaviours and isolate the dominant factor(s) contributing to the accelerated leaching behaviours, the leaching dynamics of diphenol (BPA and BPS) and PAE (DEHP and DEHT) plasticisers were measured experimentally from PS microplastics with different *Mw* ranges (used to simulate controlled polymer degradation) in ambient seawater for 21 days (25.5°C, pH = 8.1). In this study, significant leaching of all plasticisers was observed within 2 hours of immersion in seawater, albeit at different rates and concentrations (Figure 4.1 and Table 4.1). The bulk of BPA and BPS leaching occurred within 24 hours ($D_w = 2.51_E^{-9}$ and 7.04_E^{-10} cm^2/s, respectively), reaching equilibrium after ~5 days once 43 - 53% of plasticiser was released. Whereas DEHP and DEHT leaching occurred within 2 hours of immersion in seawater (3 – 12% leached), and did not vary significantly thereafter for the remainder of the experimental timeframe (t = 21 days). While fluctuations in leaching concentrations at each time point was observed with respect to polymer *Mw*, these variations were extremely minor and fell within error margins for all plasticisers: BPS (47 – 50%), BPA (55 – 57%), DEHT (88 – 89%) and DEHP (87 – 97%) (Figure 4.1). Therefore, no direct correlation between declining *Mw* and accelerated plasticiser leaching was observed. These *Mw*-independent behaviours suggested that polymer *Mw* does not play a determining role in plasticiser leaching, and instead suggests that other changes to the microplastic during degradation may influence leaching more profoundly (e.g., fragmentation and size distribution).

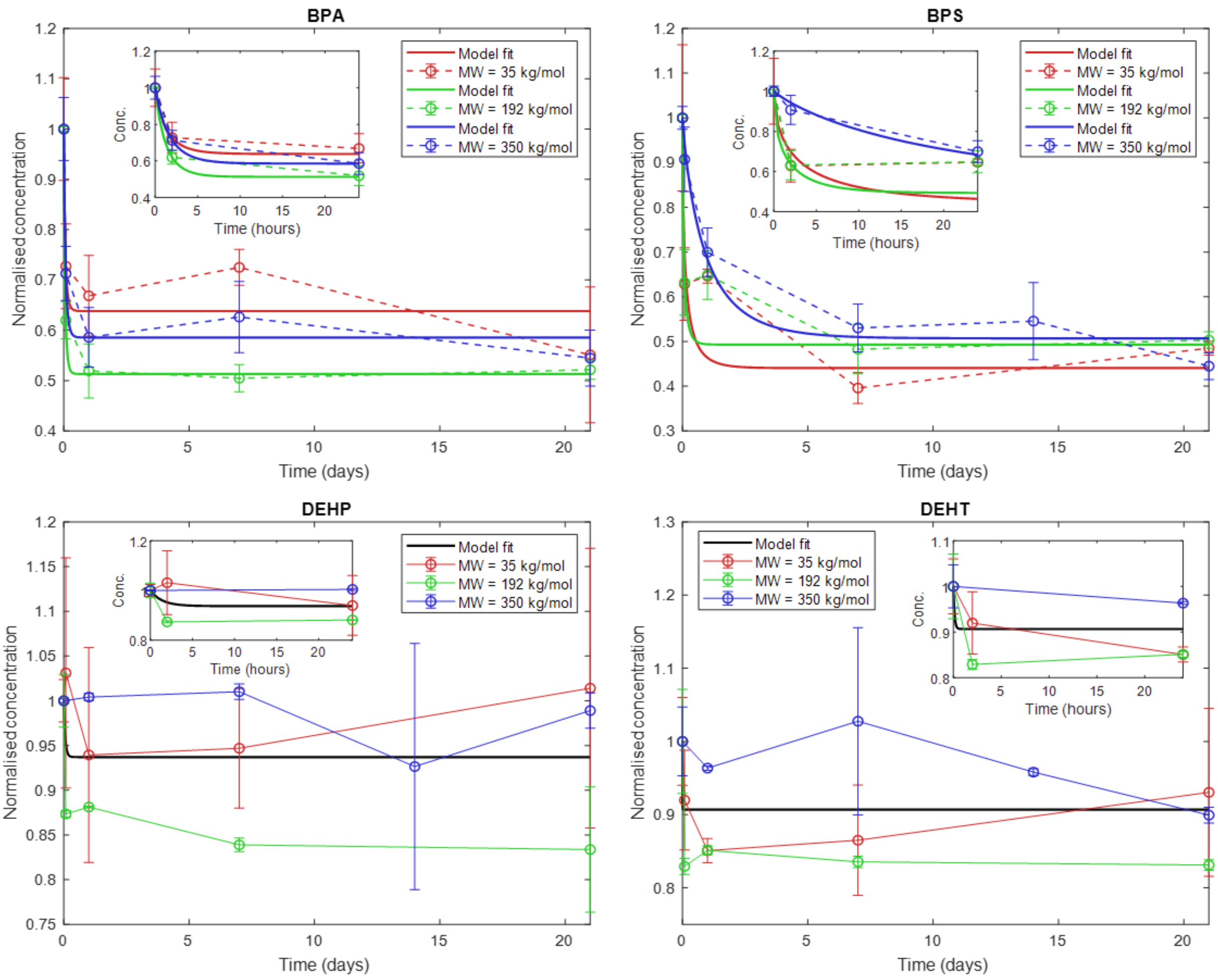

Figure 4.1. Leaching behaviours of bisphenol A (BPA), bisphenol S (BPS), diethyhexyl phthalate (DEHP) and dioctyl terephthalate (DEHT) in seawater from polystyrene microplastics with different weight-average molecular weight values (*Mw*; L*Mw* = 35k g/mol, M*Mw* = 192k g/mol, H*Mw* = 350 k g/mol). The inset shows the leaching behaviours in the first 24 hours. The plots show change in normalised plasticiser concentration (wt. %), whereby the points are the experimental data ± standard deviation and the solid lines are the model fits.

Table 4.1. Model fit results highlighting the different leaching dynamics of bisphenol A (BPA) and bisphenol S (BPS) from polystyrene microplastics with low (35k g/mol), medium (192k g/mol) and high (350k g/mol) molecular weight. The boundary layer for both BPA and BPS was not rate-limiting under the experimental conditions and therefore reported values less than 0.1 mm indicate the boundary layer had no impact on leaching rates. For diethylhexyl phthalate (DEHP) and dioctyl terephthalate (DEHT) the diffusion and boundary layer coefficients could not be determined using the simplified model.

Plasticiser	Molecular weight (g/mol)	Diffusion coefficient (cm^2/s)	Boundary layer coefficient: δ^* (mm)
BPA	35,000	4.87_{E}^{-10}	0.0008
	192,000	2.18_{E}^{-09}	0.0397
	350,000	4.86_{E}^{-09}	0.0913
BPS	35,000	1.63_{E}^{-09}	0.0625
	192,000	4.48_{E}^{-10}	0.0001
	350,000	3.92_{E}^{-11}	0.0009

Size is a well-established variable that significantly impacts leaching[125,221,255,258], particularly for additives governed by molecular diffusion-limited leaching kinetics such as BPA and BPS[255]. The microplastics employed in this experiment encompassed a variable size range dependent on the plasticiser (i.e., diphenol or PAE class) and the polymer's *Mw*, with the mean Feret diameter of the low, medium and high *Mw* microplastics ranging from 53 – 76, 43 – 71 and 86 – 130 µm for BPA, 49 – 73, 67 – 104 and 99 – 150 µm for BPS, 93 – 139, 83 – 137 and 99 – 113 µm for DEHP, and 99 – 144, 115 – 191 and 140 – 199 µm for DEHT, respectively (Table S4.1). It is possible that the individual influence of *Mw* on plasticiser leaching fluxes, when considered in isolation, was only relatively minor, and thus the contribution of each microplastic size (particularly smaller microplastics <100 µm[120]) may have masked the impacts of *Mw* on leaching. However, it is important to note that the bimodal distribution of the L*Mw* PS indicted a wide range of molecular weights within this polymer sample. These varying chemical properties introduced a potential confounding variable in the study, and thus further research should aim to confirm plasticiser leaching behaviours, especially from low molecular weight polymers. Furthermore, other degradative stressors such as physical weathering, UV exposure and elevated temperatures can also significantly impact leaching during polymer degradation[126,134]. Therefore, when investigating the leaching behaviours of aged microplastics, it is important to consider all degradative impacts, including *Mw* and size distribution, as their combined effects and/or synergistic relationship may contribute to the overall behaviours[134].

Conclusion

In this study, the leaching of diphenol (BPA and BPS) and PAE (DEHP and DEHT) plasticisers were measured experimentally from PS microplastics with different *Mw* values (35k, 192k and 350k g/mol) in static and ambient seawater and modelled using a diffusion and boundary layer model. While all examined microplastics released significant plasticiser under the experimental conditions, no correlation between polymer *Mw* and leaching was observed on this timescale. The *Mw*-independent behaviours indicated that polymeric chain scission (i.e., reduction in *Mw*) does not directly impact leaching, and instead suggests that other physiochemical changes initiated during degradation (e.g., size reduction and oxidation) may have more substantial impacts on plasticiser leaching dynamics. Regardless, employing controlled conditions to simulate microplastic aging was valuable to deconstruct the properties that contribute to the accelerated leaching of additives from aged polymers, and underscores the importance of considering multiple variables when investigating leaching from degraded microplastics. Further development of the predictive diffusion and boundary layer leaching model to consider the many aspects of polymer degradation, both individually and in combination, should be expanded upon, especially with respect to microplastic size. Nonetheless, the findings here are critical for understanding the long-term consequences of microplastics in the environment and are vital to improve ERAs.

Chapter 5: Plasticiser leaching from polyvinyl chloride microplastics

Gulizia, A. M.; Philippa, B.; Zachurak, J.; Motti, C. A.; Vamvounis, G. Plasticiser leaching from polyvinyl chloride microplastics and the implication for environmental risk assessment. Mar. Poll. Bul. 2023, 195, 115392. https://doi.org/10.1016/j.marpolbul.2023.115392

Introduction

Plastics are pervasive and ubiquitous environmental contaminant that have been detected in terrestrial, aquatic and biological matrices surveyed. Microplastics (<5 mm in diameter) are an especially hazardous by-product of plastic's manufacturing, and can originate in the environment as either primary (i.e., purposefully manufactured at microscopic size) or secondary (i.e., fragmentation products during degradation of larger items) debris[62]. Microplastics are diverse in their polymeric origin, with polyethylene (PE), polypropylene (PP), polystyrene (PS) and polyvinyl chloride (PVC) comprising the bulk of all microplastics detected in oceanic environments worldwide[9,23,61]. The chemical composition of microplastic dictates their density and determines their position in the water table (i.e., buoyancy), and thus influences their interaction with different biota (e.g., benthic or pelagic). PVC is one of the most prevalent high density commodity polymers ($\rho = 1.38$ g/cm^3), and thus is one of the few negatively buoyant plastics detected in the ocean[127,259,260]. Sinking and sedimentation of PVC microplastics can enhance their bioavailability to low trophic level and benthic organisms[66,218,261], and can influence leaching behaviours of incorporated additives[120,127,262]. Plasticisers leaching from PVC microplastics represents a significant environmental stressor[127,222,262], and given the unique position of PVC in the water table, this may accelerate the introduction of toxic leachates into unique compartments of the ocean[66,218,261]. These leachates can also act synergistically with other environmental stressors to amplify toxicity response(s), e.g., by decreasing surrounding water quality and altering microbiomes[25,33,55,85,126,210,255]. However, to fully clarify the ecological impact of PVC microplastics and their leachates, a comprehensive understanding of their leachable properties is required.

Owing to their excessive annual production and high additive composition (10 – 70% by weight (wt. %)[37]), PVC leaching is a significant pathway for additives into aquatic ecosystems[127,222]. While the unique carbon-chlorine backbones gives virgin PVC high tensile strength and rigidity suitable for application in building and construction industries, most PVC products require flexibility and malleability that can only be achieved through the incorporation of plasticisers[5,34]. Common plasticisers such as diethylhexyl phthalate (DEHP) and bisphenol A (BPA), respectively, are produced annually in high concentrations (>10 million tonnes) mainly for the manufacturing of flexible PVC products (80 – 95%)[34,263]. Moreover, they are known as endocrine-disrupting chemicals (EDCs) with severe toxic consequences for wildlife[53,59], thus the leaching of these plasticisers from PVC is an ongoing ecotoxicological concern[249]. Plasticiser leaching behaviours in fluctuating environmental conditions as well as from aged and degraded microplastics has been well described in previous chapters of this thesis, as well as in literature from PVC microplastics with different additive compositions in publications by Henkel *et al.,* (2022), Yan *et al.,* (2021) and Suhrhoff *et al.,* (2016)[126,127,251]. Combined, these data indicate that factors impacting molecular diffusivity (e.g., polymeric free volume), as well as the plastic surface-water boundary layer properties (e.g., plasticiser solubility) are determinate of the kinetic behaviours of plasticiser leaching from microplastics[127]. However, unlike other major microplastic polymers, i.e., PS[255], leaching behaviours of PVC into environmentally relevant matrices has seldom been investigated. Thus, predictive leaching models are not yet applicable for PVC microplastics present in aquatic environments worldwide[126,255].

To better inform environmental risk assessments (ERAs) of PVC microplastics, factors impacting molecular diffusion (e.g., water temperature, microplastic size, polymeric free-volume) and boundary layer properties (e.g., water agitation, plasticiser hydrophobicity/solubility) need to be investigated in combination when developing an holistic leaching model[255,264]. Moreover, when applying the diffusion and boundary layer model to plasticiser leaching, consideration should also be given to the size of the microplastic, as well as the specific polymer-plasticiser chemical interactions, as this will impact molecular diffusivity and microplastic leachability[118,120,125,127,255]. Recent studies predominately assess and quantify leaching from plastics with unknown chemical compositions and profiles (i.e., commercially available plastics[262]), or under specific experimental conditions (i.e., infinite-sink approach[222]). Implementing both a controlled experimental and mathematical modelling approach using a combined diffusion and boundary layer model under environmentally relevant conditions will allow for kinetic assessment of PVC leaching behaviours relevant to the environment. This will enable predictions of plasticiser leaching from microplastics into different waterways (i.e., seawater[255]), under fluctuating conditions (e.g., global warming[234,265]) and with different size distributions (i.e., during fragmentation[194]).

Therefore, this study applied a diffusion and boundary layer approach to characterise the leaching kinetics of DEHP and BPA from PVC based microplastics in aquatic environments. Virgin PVC microplastics with known plasticiser concentrations were prepared and characterised, and exposed to fluctuating marine conditions that impact both molecular diffusion (water temperature) and boundary layer properties (water agitation). Leaching behaviours were quantified using gel permeation chromatography (GPC) and thermal gravimetric analysis (TGA), and the results applied to a numerical model to explain the leaching kinetics of plasticisers from PVC[255]. It is hypothesised that the specific polymer-plasticiser interactions (i.e., *Tg*) will impact the amount of "associated plasticiser" (i.e., the concentration of plasticiser able to leach), while fluctuating water temperature and agitation will impact molecular diffusivity and boundary layer properties. These data and model predictions will also allow for extrapolation of PVC leaching behaviours in other aqueous conditions (e.g., freshwater), and ultimately contribute towards to generation of comprehensive and accurate ERAs of plasticised-microplastics.

Experimental Section

Materials

PVC (389239-500), DEHP (D201154-500 mL), BPA (133027-500 g) and deuterated chloroform ($CDCl_3$; 99.8 atom %) were sourced from Sigma Aldrich, tetrahydrofuran (THF; HPLC grade) was sourced from Unichrom and filtered seawater (0.5 µm; pH = 8.10, salinity ≈ 35 %) was collected from the Australian Institute of Marine Science National Sea Simulator (AIMS SeaSim). All were used as received.

Microplastic preparation

Throughout this thesis, a robust and controlled method for understanding and modelling the leaching behaviours of microplastics has been developed. These protocols were underpinned by the controlled preparation and chemical characterisation of the microplastics prior to leaching, which requires that the polymer be both soluble at room temperature (i.e., for GPC analysis) and rigid enough to allow for mechanical processing into microplastics. These considerations ensure that the chemical and physical properties of the prepared microplastics are known and controlled, and thus parameters affecting leaching (i.e., polymeric free-volume, plastic size/thickness) are understood and comparable across all published and unpublished chapters of this thesis. In this chapter, a work-flow approach outlined in Table S5.1 was used to determine the suitability of plasticised-PVC microplastics for leaching. PVC was chosen based on its solubility parameters in ambient conditions, the mechanical properties of the dried and casted solutions, as well as the dominance of these polymer-plasticiser blends (DEHP and BPA) in the environment[10,52,77,194,224,266].

For the preparation of plasticised-PVC, virgin PVC beads were dissolved in THF (1:7 w/v) under ambient conditions with constant stirring until dissolved. A plasticiser solution (DEHT or BPA in THF; 1:10 w/v) was added (85:15 PVC:plasticiser) with constant stirring for a further 4 days to ensure complete dissolution and homogeneity of the solution. Solutions were then cast on a watch glass and the residual solvent removed through evaporation under reduced pressure and high temperature (65°C) for 1 month. Solvent removal was confirmed using TGA on a TA SDT 650 instrument at a heating rate of 10°C/min up to 500°C and under constant flow of nitrogen (50 mL/min) (Figure S5.1). The dried membranes were then processed using a Magic Bullet – Nutribullet® 900 Series blender and sieved over a stainless-steel screen sieve (Glenammer Sieves) to afford irregularly shaped microplastics $\lesssim$200 µm in diameter (median size = 159 - 223 µm). Particle sizes were analysed using optical microscopy (Leica Mz26A; 4 images per plasticiser) and Matlab's Colour Threshold application (vR2022b) according to methods described in Chapter 3 and 4. The microscope images, corresponding binary image masks and particle size statistics are presented as Figure S5.2 – 5.4 and Table 5.2.

Chemical characterisation of prepared plastics

The chemical properties of the plastics pertaining to leaching (i.e., plasticiser concentration and thermal properties) were characterised using GPC, differential scanning calorimetry (DSC) and Fourier Transform-Infrared Spectroscopy (FT-IR). GPC was used to measure the concentration (wt. %) of plasticiser incorporated into the microplastics, and was calculated using prepared calibration curves of PVC, DEHP and BPA in THF from 0.515 – 8.24 mg/mL ($r^2 = 0.99$; Figure S5.5). For these analyses, microplastics (1.5 – 3 mg) were dissolved in THF (1.5 mL) and 50 μL aliquots were injected into a 1260 Infinity II Multi-Detector GPC (Agilent Technologies) equipped with an ultraviolet (UV) absorbance and refractive index detector. Two PLgel 5 μL MIXED-C columns (300 × 7.5 mm; Agilent Technologies) were calibrated using PS narrow standards (Agilent EasiVial PS-M; 470 – 482k g/mol). GPC confirmed successful incorporation of plasticisers with an average of 13.75 ± 2.48 wt. %. The relevant thermal properties of the plastic and the polymer-plasticiser interactions pertaining to leaching (glass transition temperature; *Tg*[225–227]) was characterised using DSC (TA DSC250). Plastics (2–6 mg) were subjected to two cooling and heating cycles from −20°C – 250°C at a rate of 50°C/min under a constant flow of nitrogen (50 mL/min). The thermograms generated after the second heating step are presented in Figure S5.6. Infrared profiles of the neat plasticisers, virgin PVC and plasticised-PVC microplastics were obtained using FT-IR equipped with an attenuated total reflected (ATR) crystal diamond head attachment (Figure S5.7).

Leaching experiments

PVC leaching dynamics were investigated under agitated seawater conditions to reveal the mechanisms driving plasticiser leaching from microplastics in marine environments. As discussed in Chapter 3 of this thesis, surface plasticiser concentration (i.e., plasticiser not chemically associated with the polymer and thus not impacted by diffusion/boundary layer properties) as well as surrounding abiotic conditions (i.e., temperature and water agitation) are significant parameters impacting the leaching dynamics of plasticisers from microplastics[34,118,126,255,262]. Therefore, plasticised-PVC microplastics were rinsed for 1 minute in filtered seawater to ensure complete removal of all surface plasticiser. Rinsed plastics (10 mg) were then immersed in excess seawater (10 mL) and exposed to controlled and constant agitation (200 rpm) at 26, 43 and 60ºC using a Syncore® vortex evaporator (Buchi). Concurrent exposure to agitated seawater at various temperatures allowed for the assessment of the dominant leaching kinetics driving plasticiser leaching from PVC (i.e., diffusion or boundary layer limited). After leaching, microplastics were filtered (0.5 μm filter paper; Whatman), rinsed with deionised water to remove residual leachate, and allowed to dry under ambient conditions. Leachate solutions were stored in airtight glass vials. Leaching behaviours were quantified by calculating the plasticiser concentration in the microplastics (wt. %) using GPC, as described above.

To highlight the diffusion and boundary layer kinetics, careful consideration was given to obtain the initial leaching decay and time taken to reach equilibrium for each plasticiser. It was found that DEHP reached equilibrium within 2 hours of immersion in seawater, and thus leaching experiments were conducted over 1, 3, 5, 15, 30, 60 and 120 minutes, 0.8, 4 and 7 days (starting $n = 90$, with $n = 9$ removed at each time point). BPA reached leaching equilibrium after 4 days, and thus leaching experiments were conducted over 30, 60 and 120 minutes, 0.8, 4, 7, 14 and 21 days (starting $n = 72$, with $n = 9$ removed at each time point). Given that the prepared microplastics encompassed an average size range between 159 – 223 µm, the contribution of each size class (Figure S5.7) was also considered when developing the leaching model.

Numerical modelling

Numerical modelling followed the same procedure as Chapters 3 and 4. Briefly, the DEHP data was fit to an exponential:

$$p(t) = p_0 \exp\left(-\frac{t}{\tau}\right) + p_a, \tag{4.1}$$

where $p(t)$ is the concentration of plasticiser at time t, p_0 is the initial concentration of free plasticiser (i.e. the plasticiser available for leaching), p_a is the concentration of associated plasticiser (i.e. plasticiser not available for leaching), and τ is the exponential time constant given by

$$\tau = \frac{3D_w}{K_{pw} r \delta}, \tag{4.2}$$

where D_w is the diffusion coefficient of plasticiser in water, K_{pw} is the plastic-water partition coefficient, r is the radius of the plasticiser, and δ is the boundary layer thickness.

On the other hand, the BPA data were fitted to a diffusion and boundary layer model, which was also extended to include the experimentally observed size distribution as described in previous chapters[255].

Statistical analyses

Data were presented as the mean of triplicates with standard deviation. T-tests performed in Microsoft Excel were used to evaluate the differences between each plasticiser with temperature and over time at a p-value threshold of 0.05.

Results and discussion

The extent and effect of plasticiser leaching on ecological processes are influenced by various environmental parameters such as water temperature and agitation, as well as the physical and chemical properties of the microplastic polymer. Under the simulated conditions of agitation (200 rpm) and temperature (26 – 60°C) employed in this study, differences in the leaching kinetics of DEHP and BPA from PVC microplastics was observed. Leaching of both plasticisers commenced within 1 minute of immersion in agitated seawater (Figure 5.1), however, the leaching rates varied (Table 5.1). For DEHP, 1.1 wt. % (7% of the total leaching concentration) was leached after 1 minute rinsing at ambient temperatures, and following a further 1 minute of immersion in agitated seawater, an additional 2.6 wt. % was leached (17% of the total leaching concentration), irrespective of temperature. In total, 94% of DEHP leaching occurred within the first 2 minutes of immersion in seawater, with negligible loss (<1 wt. %; $p > 0.05$) observed thereafter for the remainder of the experimental timeframe ($t = 7$ days). These temperature-independent and fast leaching behaviours indicated that the bulk of DEHP leaching was controlled by surface rinsing. Combined, the impact of surface rinsing and temperature-independency suggested that molecular diffusion is not a determining factor in DEHP leaching, and instead suggested that DEHP leaching on this timescale is rate-limited by the boundary layer[127]. In contrast, the leaching of BPA occurred at a much slower rate, reaching equilibrium between 7 to 10 days and exhibiting a strong temperature dependency up to 60°C (Figure 5.1). Consistent with previous findings in this thesis and literature, the temperature-dependent behaviours of BPA indicated that leaching is rate-limited by molecular diffusion within the polymer matrix[118,136,220,255]. Given these diffusion-limited behaviours, it is likely that BPA leaching from PVC will also be impacted by microplastic size[125,126,255]. Considering that the leaching rate and concentration (i.e., leachability) of microplastics are importance factors influencing water quality and the plastic/plasticiser toxicity, the different kinetic behaviours of each plasticiser can be used to implicate the hazardousness and ecological fate of plasticised-PVC microplastics in aquatic environments.

Table 5.1: Parameters obtained by least squares curve fitting describing the leaching kinetics of bisphenol A (BPA) and diethylhexyl phthalate (DEHP) from polyvinyl chloride microplastics in agitated (200 rpm) and heated (26 – 60°C) seawater.

Plasticiser	Model type	Fitted parameters	
BPA	Diffusion equation and boundary layer	Diffusion coefficient at 26°C	1.0×10^{-11} cm^2/s
		Diffusion coefficient activation energy	53 kJ/mol
		Boundary layer coefficient	< 0.1 mm
DEHP	Exponential (Equation 3)	Exponential time constant	48 seconds

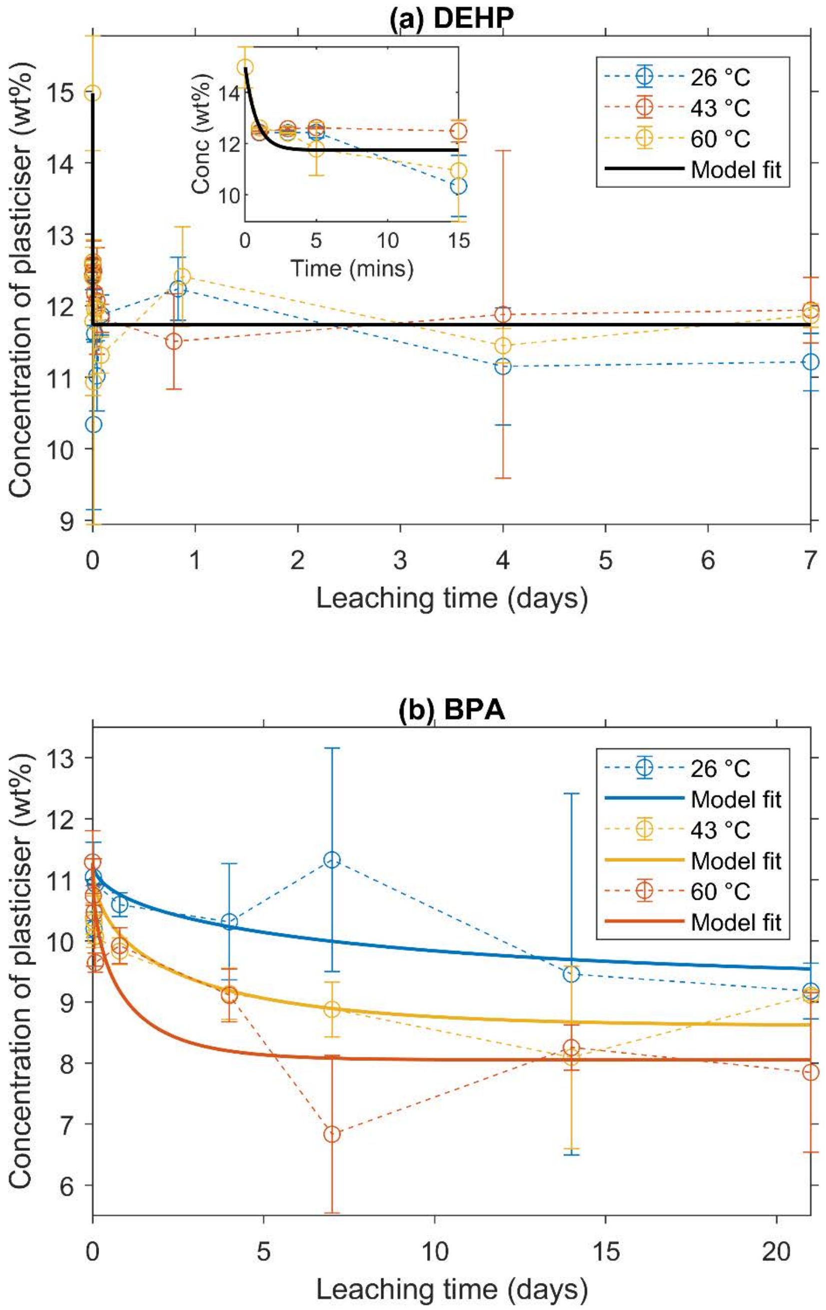

Figure 5.1. Leaching behaviours of (a) bisphenol A (BPA) and (b) diethylhexyl phthalate (DEHP) from polyvinyl chloride (PVC) microplastics in seawater at 26ºC (blue), 43ºC (yellow) and 60ºC (orange). The inset in (a) shows the leaching behaviour in the first 15 minutes. The plots show the change in plasticiser concentration (wt. %) after rinsing, whereby the points are the experimental data ± standard deviation and the lines are the model fits.

Environmental implications of plasticised PVC leaching

Plasticiser leachates possess endocrine disruptive, mutagenic and carcinogenic potential that can have devastating consequences for exposed biota[129,138,209,267]. It has been well-established that microplastic polymers can leach plasticisers into aqueous and terrestrial environments[36,120], whereby the specific polymer-plasticiser composition (e.g., plasticisation efficiency and the proportion of polymer-associated-plasticiser), size distribution and fragmentation pattern have been identified as deterministic of these of the microplastic's leachability[120,125,126,220,255]. The rate and concentration of plasticisers released from a microplastic can have implications for both short- and long-term exposure consequences[268], therefore considering the leachability of different polymer-plasticiser blends is crucial to understand the fate and behaviours of microplastics *in-situ* (e.g., laboratory exposure studies[267,269]) and *in-natura* (i.e., as aquatic contaminants[52,59]).

Size-dependency

Degradative history and the size of microplastics in the context additive leaching has been well studies in literature as well as in Chapter 4 of this thesis[125,134,135,255,258,262]. In all cases, it was observed that reducing diffusion length (i.e., decreasing size or film thickness[125,126,255]) will accelerate leaching, however these parameters have yet to be examined across narrow size ranges of microplastics in aquatic environments. For additives that exhibit diffusion-limited leaching (e.g., bisphenol analogous such as BPA and bisphenol S (BPS)[255,270]), the size distribution of microplastics will significantly influence leaching[45,118,220,255]. Therefore, in this study, model fit parameters generated from the experimental data in Table 5.1 were used to predict the leaching dynamics of BPA from microplastics ranging from 5 and 136 µm in diameter in agitated and heated seawater (Figure 5.2 and Figure S5.7 and S5.8). When leaching is limited by molecular diffusion (as was the case for BPA), the model outputs indicated that even small variations in size (52 – 99 µm) can have significant impacts on plasticiser leaching rate, with larger microplastics leaching plasticiser at a slower rate. Furthermore, at the smallest microplastic size (< 42 µm), BPA release rate was estimated to be essentially instantaneous upon immersion in water, indicating that smaller micro- and nanoplastic contamination has the potential to rapidly impact water quality at its source point. However, as larger microplastics (e.g., >136 µm) are transported and dispersed worldwide[6,245,271], they can continue to leach plasticisers from as long as 60 days. At higher temperatures these leaching behaviours are predicted to accelerate, from 20 days at 43°C and 10 days at 60°C (Figures S5.9 and S5.10). Research into the impacts of microplastics has found size to be a key factor[272], leading to a refinement of the typical microplastic size nomenclature (i.e., <5 mm in diameter) to categorise the different size classes of microplastics (small: 0.33 – 1 mm and large: 1.01 – 4.75 mm[61])[272]. However, the data presented here indicated that even small variations in microplastic size can significantly impact microplastic behaviour and leachability. Therefore, when investigating the ecotoxicological impacts of microplastics and plasticisers (both *in-situ* and *in-natura*), microplastic size distribution and their leachability should be considered during method development.

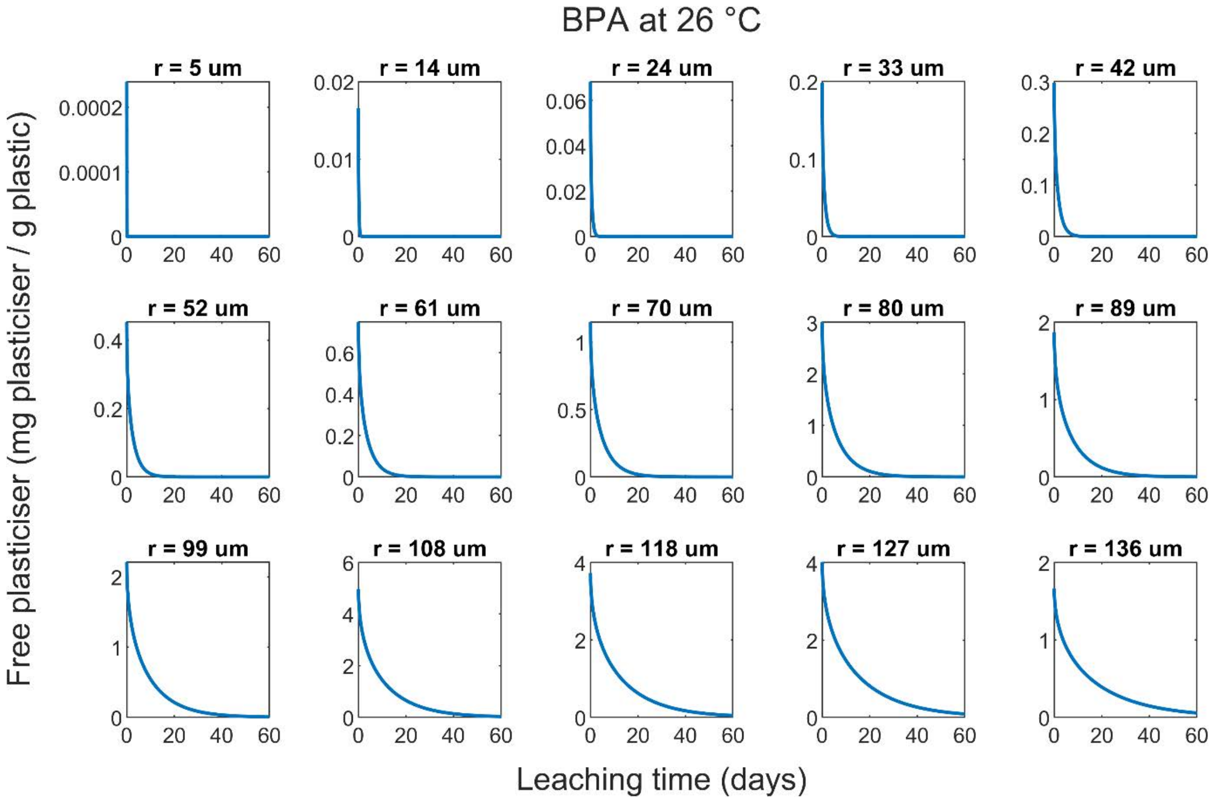

Figure 5.2. Size resolved leaching dynamics of bisphenol A (BPA) leaching from polyvinyl chloride (PVC) microplastics at 26°C in agitated (200 rpm) seawater over 21 days generated using model fit parameters in Table 1.

Leachability of PVC

The concentration of plasticiser released from a microplastic is a key factor impacting the toxicity potential of microplastic contamination. In this study, leaching experiments conducted in turbulent and heated water found only 25 – 28% (~4 wt. %) of plasticiser was released from PVC microplastics within the experimental timeframe (Figure 5.1), and suggested that the majority of BPA and DEHP incorporated into PVC will be retained within the polymer[255,262,273,274]. The leachability of microplastics is impacted by the proportion of free-plasticiser, and can be measured experimentally using DSC and FT-IR by comparing the *Tg* of the plasticised polymer and the bonding interactions between the plasticiser and the polymer backbone, respectively[34,128,255]. While both plasticisers examined in this study possess contrasting chemical properties (i.e., diphenol or phthalate acid ester (PAE) containing functional groups), they demonstrated comparable levels of plasticisation efficiency for PVC, with the *Tg* decreasing from ~80°C (virgin PVC) to 51°C (PVC-DEHP) and 49°C (BPA-DEHP). These values indicated that the proportion of free-plasticiser within each microplastic was comparable for both plasticisers, and explained the similar leaching concentration (19 ~ 26% leached)[34]. However, when comparing the leachability of plasticised-PVC to other BPA- and DEHP-plasticised polymers exposed to similar aqueous conditions (e.g., Chapter 3, Figure 3.3; PS leached 28 – 56%[255]), the release of plasticisers from PVC is significantly lower. This difference could be attributed to the bonding interactions between each plasticiser and the PVC polymer backbone[34]. Unlike PS and other high concern commodity polymers (e.g., PE), PVC-halogen bonds can form with incorporated plasticisers[273,274] (Figure S5.7), which may contribute to the high proportion of polymer-associated-plasticiser and result in a lower concentration of plasticiser being release into the surrounding water[120]. Therefore, when considering the leachability of plasticised PVC in isolation, PVC microplastics may be pose a lower hazard in aquatic ecosystems when compared to other polymers.

Implications for ERAs

PVC microplastics are widely released into aquatic environments from various sources, and especially into marine systems[5]. Thus an understanding of their leachable properties in different aqueous matrices and under different environmentally relevant conditions (e.g., predicted climate change scenarios) is important to ensure a comprehensive ERA of microplastics[181]. While DEHP and BPA plasticisers are inherently toxic[52,59], the data obtained here for PVC (and previously for PS; Chapter 3[255]) suggests that that leaching will be further exacerbated by rising and fluctuating water temperatures, such as those predicted with global warming. Similarly, exposure to water currents, including ocean upwelling from the seabed and high wave action in coastal environments is also expected to accelerate plasticised-PVC leaching[43,126,255]. However, in the context of additive leaching, the continual fragmentation of microplastics into smaller particles (i.e., nanoplastics[194]) is of the greatest ecological concern, and has the potential to acutely impact water quality at the source point. This highlights the need to manage plasticised plastics before they enter aquatic ecosystems, as well as to consider the implications of employing microplastics with broad size distributions during *in-situ* ecotoxicology/exposure studies.

Conclusion

Leaching of DEHP and BPA plasticisers from PVC microplastics follows a diffusion and boundary layer model, whereby DEHP is rate-limited by the boundary layer and BPA is rate-limited by molecular diffusion (influenced by water temperature). Exposure to agitated and heated seawater prompted the significant release of both plasticisers from PVC, confirming PVC microplastics in aquatic ecosystems contributes to aqueous plasticiser pollution. These findings are in line data obtained in previous chapters of this thesis and literature[118,127,255,262], and suggests accelerated leaching of plasticisers with rising water temperatures (i.e., climate change), in ecosystems with strong water movement (i.e., coastal ecosystems) and during the fragmentation of plastics. These kinetic behaviours impact the leachability of PVC (i.e., rate and concentration of plasticiser), which are expected to be highly localised, with immediate impacts to water quality before the material settle on the seabed. Finally, while this model has been successful at describing leaching from a number of polymers (e.g., PVC and PS[127,255]) and plasticisers (e.g., BPS and dioctyl terephthalate (DEHT)[255]), models to improve ERAs should be expanded to include the impacts of biofouling[275]. Regardless, current kinetic and chemical leaching data allows for a comprehensive assessment and extrapolation of PVC leaching behaviours in a range of environmentally relevant conditions, which are determinate of the hazardousness of microplastics in the ocean.

Chapter 6: Biofilm development on plasticised marine microplastics

Introduction

Plastics are ubiquitous and ecologically hazardous contaminants worldwide[9,10,37,84]. Owing to their diverse manufacturing, consumer and environmental histories, plastics – particularly microplastics (<5 mm in diameter) – have complex physiochemical properties pertaining to their morphology (e.g., size, shape and colour), polymer origin (e.g., polyethylene (PE), polypropylene (PP), polystyrene (PS) and polyvinyl chloride (PVC)) and additive composition (e.g., plasticisers)[62]. These chemical properties can influence the fate and toxicity of microplastics in aquatic systems, by impacting their position in the water column (i.e., density/buoyancy[22,70]), leachable properties[255,262] and bioavailability to organisms[237,276]. While these behaviours and exposure consequences in aquatic matrices and on biota have been well studied[80,115,129,277–279], the relationship between microplastics and the microbiome has yet to be fully elucidated. Microplastics resemble a durable and persistent substratum in the environment, providing colonising microorganisms with protection from natural weathering processes, enhanced dispersal opportunities and easy access to nutrients (e.g., through the incorporation of additives)[73,280]. Thus, complex and dynamic communities of microorganisms can colonise microplastics in marine ecosystems, creating biofilm communities unique from the surrounding water[73]. Biofilm formation can have both positive and negative impacts on ecological processes, by facilitating the dispersal of exotic and/or pathogenic bacteria into the food chain (e.g., *Vibrio* spp.,[145]), encouraging buoyant plastics to sink[21], as well as introducing novel ways to naturally biodegrade recalcitrant polymers (i.e., colonisation by plastic-degrading bacteria[173])[25,174]. However, to fully understand these impacts and potential solutions, understanding the factors impacting biofilm development on microplastics with different physiochemical properties is required (e.g., polymer-additive composition)[151].

Microplastics can contain a cocktail of synthetic chemical additives used to tailor product functionality for end-use application[34]. The most common chemical additives are plasticisers such as diethyhexyl phthalate (DEHP) and bisphenol A (BPA), which are used to manufacture more flexible and malleable plastics[34,117,263]. However, these plasticisers are known endocrine disruptive chemicals (EDCs), with studies revealing a wide range of toxicity consequences to both macro-[53,59] and microorgansisms[281,282]. Plasticiser leaching from microplastics into aquatic matrices has been well documented in literature and throughout this thesis (Chapters 3 – 5)[255,262], as too are the impacts of these leachates on the surrounding microbiome[78,141,142]. While many studies suggest that leached plasticisers can provide additional nutrients facilitating microbial growth, impacting bacterial adhesion and altering developing biofilm community dynamics[283], the interaction(s) differently plasticised microplastics and marine microbiomes is largely unknown. Identifying taxa that are closely and commonly associated with microplastics immersed in marine waters – irrespective of biogeography and physiochemistry – can be used to better inform environmental risk assessments (ERAs) of microplastics, and will enable easier identification of the properties that impact biofilm development (e.g., polymer-additive composition).

While generalist and opportunistic bacterial taxa involved in early biofilm formation and maturation have been commonly and abundantly detected in microplastic-biofilms worldwide[156,162,284–287], taxonomic comparison of biofilm composition has evidenced polymer- and additive-specific communities[147,286,287]. A study by Frere *et al.,* (2018) investigated the polymer-specific biofilm formation on microplastics collected from the Atlantic Ocean, and found that while there was high proportion of shared taxa, the species diversity and richness was significantly different[288]. They attributed these differences to the proportion of "rare" taxa with competitive and/or adaptive mechanisms for colonisation (e.g., for colonising hydrophobic surfaces), however, also acknowledged that without accurate and comprehensive characterisation of the polymer prior to sample processing and DNA extraction (e.g., additive composition), the specific relationship between polymer type and bacterial colonisation could not be reliably identified. Nonetheless, similar studies undertaken worldwide have identified taxonomically distinct biofilms – particularly at lower taxonomic levels – forming on different floating (e.g., PE, PP and PS) and negatively buoyant (e.g., PVC) microplastics[146,153,157]. Indicating that polymer composition can impact a developing biofilm. Dominant taxa mainly belonged to the phyla Proteobacteria (Alpha and Gammaproteobacteria), Bacteroidota, Cyanoabcteria, Planctomycetota and Verrucomicrobiota, which encompass broad metabolic capabilities, including pathogenic[145], photoautotrophic[289], carbohydrate assimilation[290] and plastic degradation[171]. Thus, understanding their attachment and long-term adhesion on microplastics can be used to promote and facilitate polymer biodegradation in the environment.

The number of microorganisms reported to degrade polymers is rapidly increasing, however, there is little evidence linking their colonisation on microplastics to the biodegradation of the polymer[171,286]. Most bacteria with these metabolic capabilities belong to five phyla; Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Cyanobacteria, with the *Pseudomonas* genus most commonly reported (7%)[171]. Quantifying biodegradation of microplastics colonised by these taxa is commonly achieved using Fourier Transform-Infrared Spectroscopy (FT-IR), by calculating mass loss, changes in mechanical properties and hydrophobicity, as well as looking at surface topography using standard electron microscopy (SEM)[146,149,177,253]. In these studies, bio-fouled microplastics have displayed a decline in crystallinity, stiffness and maximum compression as well as surface pitting conforming to the shape of colonising bacteria, suggesting active growth and assimilation of the polymer backbone[146]. Changes to polymer infrared profiles have also been observed[177,253], indicating polymer oxidation (e.g., increase in C=O adsorption bands) similar to indices reported in other plastic degradation studies[146]. However, many of these studies are conducted *in-situ* using growth conditions favourable to certain taxa[180], and thus biodegradation of bio-fouled microplastics in environmentally relevant matrices remains controversial, especially for PS and PVC[146,291]. Exploring specific microbial assemblages colonising different polymer-additive blends, particularly in the context of plastic-degrading bacteria, could help to better elucidate biodegradation pathways of marine microplastic contamination.

Therefore, in this study, it was hypothesised that microplastics with different polymer-plasticiser compositions will display unique and taxonomically distinct biofilms when immersed in marine waters. To evaluate this hypothesis, six different microplastic substrates were prepared (PS and PVC with no plasticiser (i.e., virgin), or with either BPA or DEHP), and exposed *in-situ* for 21 days to marine waters representative of the costal, central Great Barrier Reef World Heritage Area. A combined genetic and chemical approach using high-throughput DNA sequencing and spectroscopic techniques (i.e., gel permeation chromatography (GPC), thermal gravimetric analysis (TGA) and FT-IR), respectively, was used to understand biofilm community structure (alpha and beta diversity, taxon relative abundance/frequency) and polymer biodegradation as a function of time and microplastic chemical composition (i.e., polymer and plasticiser presence/type). The results provide insights into the relationship between microplastics and the marine microbiome, and can be used to better inform ERAs of microplastics, plasticisers and microplastic-associated biofilms.

Methods

Materials

PS (weight-average molecular weight; *Mw*) = 192K Daltons, 430102-1 Kg), PVC (relative *Mw* = 55K Daltons, 389239-500 g), THF (HPLC Grade), DEHP, BPA, sodium lauryl sulfate (SDS), Trizma® base (CAS 77-86-1), sodium bicarbonate ($NaHCO_3$), polyvinyl pyrrolidone (PVP), tetrasodium pyrophosphate ($Na_4P_2O_7$), Tween® 20 (CAS 9006-64-5), phenol:chloroform:isoamyl alcohol (IIA) (25:24:1, v/v) and chloroform:IAA (24:1, v/v) were sourced from Sigma Aldrich. Sodium hydroxide (NaOH), acetone, ethanol (EtOH), hydrochloric acid (HCl), potassium chloride (KCl), sodium sulfate (Na_2SO_4) and hydrogen peroxide (H_2O_2; 30% grade) were sourced from Univar. Proteinase K (20 mg/mL; recombinant; PCR Grade), Lysozyme and UltraPure™ distilled water (DNase/RNase-Free) were sourced from ThermoFisher. Sodium chloride (NaCl) was sourced from Fisher. Ethylenediaminetetraacetic acid (EDTA) and sucrose were sourced from Astral Scientific. Isopropanol (HPLC grade) was sourced from BDH Chemicals. PCR reagents including AmpliTaq Gold 360 Master Mix (2X concentrate) and GC enhancer were sourced from Applied Biosystems. Forward and reverse PCR primers were supplied by Sigma Aldrich and reconstituted to 100 µM on arrival.

Reagent preparation

Calcium and magnesium-free seawater (CMFSW) was prepared by dissolving NaCl (26.2 g), KCl (0.75 g), Na_2SO_4 (1 g) and $NaHCO_3$ (0.042 g) in Milli-Q water (1 L). 0.5 M EDTA was prepared by dissolving EDTA (18.6 g) in Milli-Q water (100 mL) at pH 8.0 (~3 g NaOH). Cell separation buffer was prepared by dissolving PVP (0.35 g) and $Na_4P_2O_7$ (0.1339 g) in CMFSW (100 mL). Once dissolved, the solution was autoclaved and Tween® 20 (0.5 mL) was added. 1M Tris-HCl was prepared by dissolving Trizma®

base (12.113 g) in Milli-Q water (100 mL) at pH 8.0 (~9 mL; 6M HCl). Sucrose lysis buffer was prepared by dissolving Tris-HCl (1M; 5 mL), EDTA (0.5M; 8 mL) and sucrose (25.6 g) in MilliQ water (100mL) before filter sterilisation (0.22 µm; Millipore). Unless specified, all solutions were autoclaved prior to use.

Preparation and characterisation of plastics

To investigate the impact of microplastic chemical composition (polymer type and additive presence/absence) on biofilm formation in marine ecosystems, PS and PVC were prepared without any plasticiser (i.e., virgin); with 15 wt. % DEHP or with 15 wt. % BPA as per methods described in Chapters 3 and 5. All three versions of PS and PVC (*n* = 6 types total) were then sorted over a stainless-steel screen sieve (Glenammer Sieves) using a mesh size < 200 µm diameter, according to the methods described in Chapter 3 – 5. GPC was used to determine the concentration (wt. %) of DEHP and BPA incorporated into the PS and PVC polymer blends, which was calculated using prepared calibration curves of PS, PVC, DEHP and BPA in THF from 0 – 1.05 mg/mL ($R^2 > 0.99$) (Figure S6.1). For these analyses, PS (3 – 5 mg) and PVC (1.5 – 3 mg) microplastics were dissolved in THF (1.5 mL), filtered through a 22 µm MS® polytetrafluoroethylene (PTFE) filter (Membrane Solutions), and 50 µL aliquots were injected into a 1260 Infinity II Multi-Detector GPC (Agilent Technologies) equipped with an ultraviolet (UV) absorbance and refractive index detector. Two PLgel 5 µL MIXED-C columns (300 x 7.5 mm; Agilent Technologies) were calibrated using PS narrow standards (Agilent EasiVial PS-M; 470 – 482k g/mol). Thermal gravimetric analysis (TGA; TA SDT 650) was performed at a heating of 10°C/min under constant flow of nitrogen (50 mL/min) using 3 – 5 mg of sample (Figure S6.2). For differential scanning calorimetry (DSC; TA DSC 250), plastics (2 – 6 mg) were subjected to two cooling and heating cycles from -20°C – 250°C at a rate of 50°C/min under constant flow of nitrogen (50 mL/min). The thermograms generated after the second heating step are presented as Figure S6.3. A Thermo Scientific Nicolet iS5 FT-IR spectrometer equipped with an attenuated total reflectance crystal head (ATR) diamond head attachment was used for infrared spectral assessment (Figure S6.4). Finally, microscopy was performed using a Leica MZ26A for size analysis and a Jeol Superprobe JXA-8200 SEM to evaluate surface morphology (Figure S6.5 and S6.6). TGA, DSC, FT-IR and SEM were used to confirm sample purity, further establish the plasticiser concentration and to characterise the size distribution and morphology of the prepared microplastics. These analyses revealed that all microplastics were irregularly shaped and sized between 88 – 157 µm, with control (i.e., 0 days exposure) PS and PVC, respectively having a relative, mean *Mw* of 171, 000 and 58, 000 g/mol; a polydispersity index (PDI) of 2.54 and 1.97; and a glass transition temperature (*Tg*) of 110 and 80°C.

Experimental setup

Biofilm formation was investigated *in-situ* as a function of polymer and plasticiser type over 21 days (Table 6.1) to reveal the underlying factor(s) impacting microbial colonisation on plastic debris in marine ecosystems. Prepared PS and PVC microplastics (PS-virgin, PS-DEHP, PS-BPA, PVC-virgin, PVC-DEHP and PVC-BPA; ~0.30 g) were placed inside empty Biotage® Sfär DVL 10 g columns fitted with a 26 µm mesh filter and a poly(methyl methacrylate) frit. The cartridges containing microplastics (*n* = 5 per polymer blend with *n* = 5 removed after 7 and 21 days, respectively) were fitted to a manifold system and immersed in a plastic Nally bin that received a constant flow of filtered seawater (1 µm; 120 ~ 550 L/hour; Figure 6.1 and Figure S6.7) from the outlets of eight mesocosm tanks for 21 days containing organisms representative of coral reef ecosystems (hard and soft corals, snails, sea cucumber, urchins, seagrass, coral reef fishes and clams[292–294]). Incoming seawater was maintained at seasonal average conditions of the coastal, central Great Barrier Reef World Heritage Area, Australia at the Australian Institute of Marine Science (AIMS) National Sea Simulator (Table S6.1). The mesocosm tank was covered with a nylon mesh to simulate natural cloud cover. The flow (mL/min) through each cartridge was monitored weekly by measuring the volume of water leaving the cartridge within 10 seconds (Figure S6.8). After bio-fouling, cartridges were collected and drained of all remaining seawater and samples were stored at -20°C until DNA extractions were undertaken.

Table 6.1: Experimental exposure conditions for polystyrene (PS) and polyvinyl chloride (PVC); virgin (i.e., containing no plasticiser) or with 15 wt. % bisphenol A (BPA) or diethylhexyl phthalate (DEHP).

Polymer type	Polymer blend	Replicates per chemical composition	Sampling time (days)	Total replicates per polymer type
PS	Virgin	5	0^a, 7 & 21	45
	BPA	5		
	DEHP	5		
PVC	Virgin	5		45
	BPA	5		
	DEHP	5		

[a] These plastics were not exposed to bio-fouling conditions and therefore served as a negative control to compare the extent of microbial bio-fouling in marine ecosystems.

Figure 6.1. Photo of the experimental setup and manifold design.

DNA extraction, sample selection and quality control

Total genomic DNA (gDNA) from the plastic associated biofilms was extracted from 0.05 - 0.105 g of bio-fouled microplastics using the DNeasy® PowerBiofilm® Kit (Qiagen) following the manufacturer's protocol, except for the following modifications. Samples were incubated at 65°C for 5 minutes following homogenisation by bead beating using a FastPrep-24™ 5G (MP Biomedicals) for 40 seconds at 40 $M.S^{-1}$. Three kit blanks containing no biofilm material were also extracted concurrently to establish environmental and laboratory contamination levels. All DNA was eluted into 50 µL of kit elution buffer and immediately frozen at -20°C until sequenced.

To establish the microbial community inherent in the seawater fed into the experiment from the SeaSim mesocosm, the incoming seawater was collected twice throughout the experiment corresponding to the sampling days in week 1 (23/06/2022) and week 3 (12/07/2022) (2 L, *n* = 3 per sampling time). Collected seawater was filtered through a Millipore Sterivex™ GP 0.22 µm filter unit using a sterilised peristaltic pump with a Masterflex easy load pump head (MilliPore XX8020ELO; 50 rpm). Filter units were immediately covered with parafilm at both ends to prevent contamination and stored at -20°C until DNA extraction. DNA was extracted from the filters by adding sucrose lysis buffer (1.8 mL) and lysozyme (100 mg/mL; 18 µL) directly into the filter units and then mixed by gentle inversions for 1 minute. Filter units were incubated at 37°C with rotation for 1 hour before proteinase K (20 mg/mL; 20 µL) and SDS (10%; 18.38 µL) were added, mixed by inversions and incubated at 55°C with rotation for 1 hour. The liquid (~2 mL) was separated into two 2 mL microtubes and extracted using equal volumes of phenol:chloroform:IAA and then chloroform:IAA (0.8 ~ 0.9 mL). Both extraction steps were mixed by inversion and then centrifuged (16,000 x *g*; ambient) for 10 minutes. The aqueous layer was recovered from each sample and the was DNA precipitated as follows: isopropanol (80%; ~0.7 mL) was added, mixed by gentle inversions and then incubated at ambient conditions for 15 minutes, followed by centrifugation (20,000 x *g*, 4°C) for 30 minutes. The supernatant was removed, and EtOH (80%; 0.5 mL) was added, mixed by gentle inversions (no incubation) before centrifugation (20,000 x *g*; ambient) for 10 minutes. The supernatant was removed and the pellets were air dried under ambient conditions until no EtOH remained (~20 minutes). Finally, dried pellets were resuspended in PCR water (25 µL) and stored at -20°C.

DNA quality (260:280 and 260:230 ratios) was determined for all samples using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific) (Table S6.2). Following NanoDrop assessment, 20 representative samples were selected across all treatment conditions and concentration ranges for further quality control checks. These representative samples were quantified using the Qubit High Sensitivity DNA kit (Invitrogen) on the Qubit 3.0 Fluorometer (Invitrogen) according to the manufacturer's instructions. PCR amplification was undertaken using the following reaction mix in 25 µL: AmpliTaq 360 PCR Master Mix (X1 concentrate; 12.5 µL), forward primer (0.4 µM; 1 µL), reverse primer (0.4 µM; 1 µL), template DNA (1 µL), GC enhancer (2-5%; 1 µL) and PCR water (8.5 µL). Cycling conditions were as follows: 7 minutes at 95°C followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 90 seconds at 72°C followed by a final extension of 7 minutes at 72°C. To visualise generated amplicon(s), each sample was run on a 1.5% agarose gel in 1x TAE buffer. Qubit concentrations were broadly aligned with NanoDrop quantification values, thus DNA samples were normalised to approximately 5 ng/µL before dispatch for sequencing (Table S6.3).

16S rDNA sequencing, bioinformatics and statistical analyses

Samples underwent 2x250bp high-throughput DNA sequencing on the Illumina MiSeq® platform at the Ramaciotti Centre for Genomics (University of New South Wales, Australia). The V4 hypervariable region of the 16S rDNA gene from bacteria and archaea was amplified with the Earth Microbial Project (EMP) universal primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′)[295,296]. The resulting paired-end demultiplexed .fastq files were subjected to quality control using FastQC (v0.11.9) and MultiQC (v1.14)[297]. After qualitative inspection of the MultiQC quality profiles, five raw samples that contained less than 2500 sequence counts were excluded from downstream analysis (<5% of dataset). The remaining high-quality sequence reads were tested for compliance with tabular bioinformatics file formats using the Keemei add-in[298], imported into QIIME2 v2022.11[299] and processed with Deblur using a trim length of 147[300]. The filtered reads were then taxonomically classified using the Silva 138 99% full-length sequences database[301]. This database was used to assign taxonomic information to the amplicon sequence variants (ASVs) identified. Sequence counts were rarefied (Figure S6.9), and a total of 5,456,975 valid sequences (i.e., reads) were obtained, representing an average of 30, 317 ± 1,137 reads per sample. The obtained reads were clustered into 11, 570 ASVs, representing an average of 471 ± 17 ASVs per sample.

Data collation, visualisation and statistical analyses were undertaken in Microsoft Excel and R v4.2.2 through RStudio v2022.12.0-353 using the *BiocManager, eulerr, phyloseq* and *vegan* packages[302–305]. Further filtering was also performed here, keeping only bacterial sequences with assignment beyond the kingdom level[306]. Global alpha diversity metrics, including ASV richness (Chao1), diversity (InvShannon) and evenness (Simpson) indices were calculated in R and plotted using Microsoft Excel[302]. Beta diversity and microbiome divergence was explored using multidimensional scaling based on the Bray-Curtis distance matrix and compared using principle coordinate analysis (PCoA)[302,307]. The amount of shared and unique ASVs between substrates over time was investigated by generating *Euler diagrams* using the *BiocManager* and *Euler* packages in R[304,305,308]. ASVs that were observed in greater than 1% abundance in more than 75% of all samples within a treatment group were included in the *Euler diagrams*[302].

Differences in global diversity metrics and taxon relative abundance among different polymer-plasticiser substrates and the surrounding seawater were investigated by applying general linear models (GLM) and pairwise hypothesis testing (p-value < 0.05) in R (Equation 6.1), as per Santana (2021) and Miller *et al.,* (2021)[185,309]. Given that the PCoA plots showed large disparity in community composition with time (Figure 6.3), for these statistical analyses, data were grouped according to exposure time to better clarify changes in biofilm community composition among different polymer-plasticiser substrates.

$$\text{indexX} = \text{ polymer type} + \text{plasticiser type (Equation 1)}$$

Where, IndexX = Chao1, Simpson's Evenness, Shannon's Diversity Index or Taxa relative abundance/frequency (%); polymer type = PS, PVC or seawater; plasticiser type = virgin (i.e., no plasticiser), 15 wt. % BPA or 15 wt. % DEHP.

Assessment of polymer biodegradation

Polymer biodegradation was assessed using GPC, FT-IR and SEM to identify changes to the chemical (e.g., spectral profiles) and physical (e.g., surface morphology) properties of control and bio-fouled microplastics, as described above[171,201,256,310,311]. For GPC, bio-fouled microplastics (n = 3 per treatment condition) were rinsed thoroughly with water, acetone and H_2O_2 to remove remaining biological material[256], allowed to air dry under ambient conditions and then under reduced pressure overnight. Statistical differences between *Mw* and PDI indices were calculated using pairwise t-tests in Microsoft Excel at a p-value < 0.05. For FT-IR and SEM representative samples (n = 1 per treatment condition) were selected at random for analyses. The presence of bacterial clades with putative degradative potential for PS and PVC polymers were identified based on recently published literature reviews[73,171,175,254,312] and cross-referenced to the sequencing data obtained in this study.

Results

Bacterial community patterns in a developing microplastic-biofilm

Diverse microbial assemblages were observed on all microplastic substrates when compared to the control plastics and surrounding seawater ($p \ll 0.05$), with variations observed among polymer type, plasticiser presence and exposure time (Figure 6.2). With time, a significant decline in ASV evenness (Simpson) was observed alongside a significant increase in ASV richness (Chao1) and diversity (Shannon) ($p < 0.001$), indicating that the complexity of the microplastic-biofilms increased with extended exposure to marine bio-fouling conditions (Figure S6.10). Significant differences in alpha diversity metrics were also observed in relation to the polymer-plasticiser composition of the microplastics, with lower ASV richness exhibited in the microbiome forming on PVC when compared to PS ($p < 0.001$), and between virgin and plasticised-polymers ($p > 0.05$). These community patterns were then further explored using beta diversity assessment, which was used to highlight community dissimilarity and taxonomic composition.

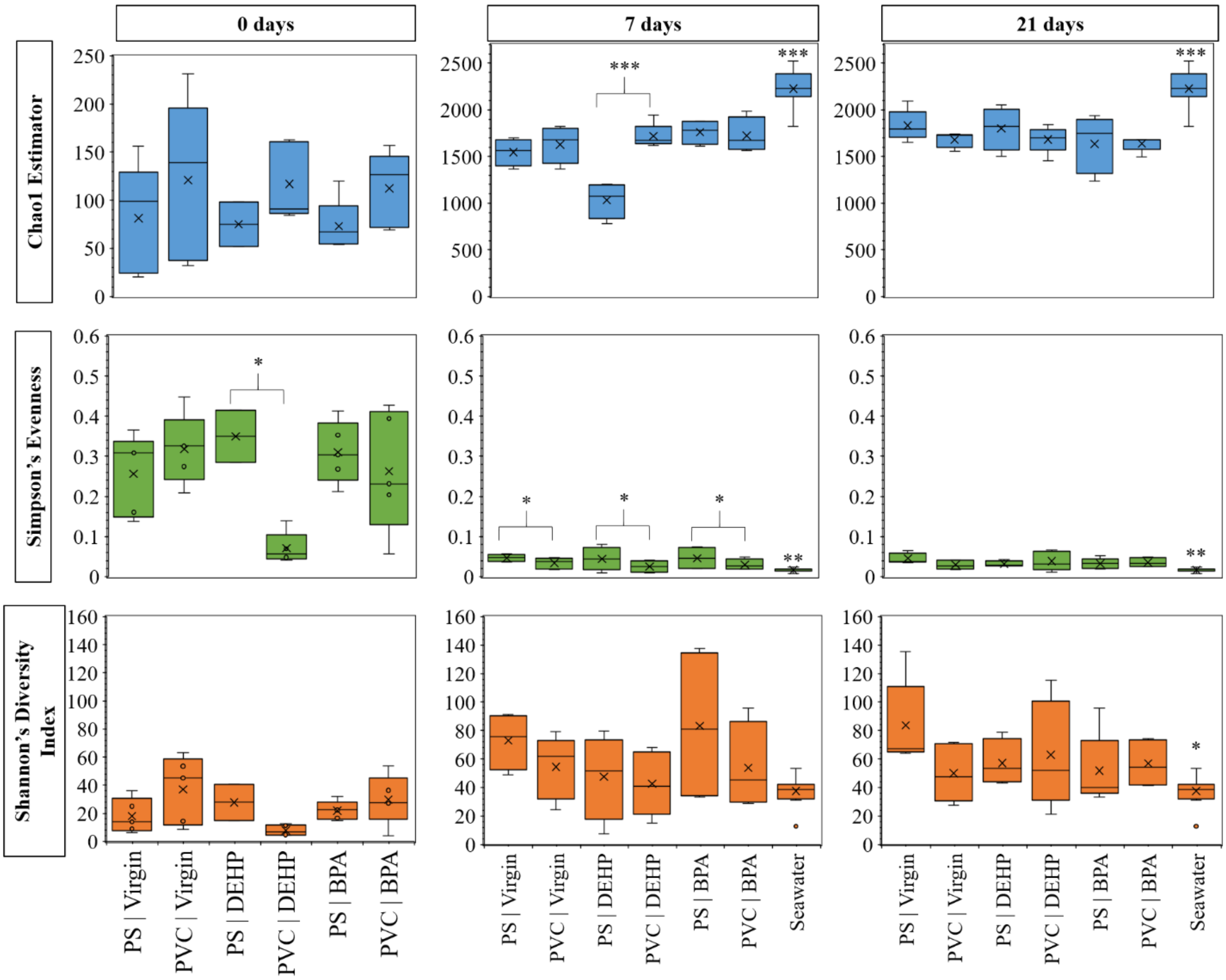

Figure 6.2. Alpha diversity metrics: ASV richness (Chao1 Estimator), Simpson's Evenness and Shannon's Diversity index for the seawater microbiome and microplastic biofilms forming on polystyrene and polyvinyl chloride; virgin (i.e., no plasticiser) and containing the plasticisers diethylhexyl phthalate (DEHP) or bisphenol A (BPA) after 0, 7 and 21 days. P-values are indicated by * (≤ 0.05), ** (≤ 0.01) and *** (≤ 0.001) and were determined by pair-wise analysis of variance using Tukey's post-hoc test.

Biofilm community dissimilarity and taxonomic composition

Overall, a total of 40 phyla were identified in the microplastic and seawater microbiome, with 23 phyla identified on the untreated microplastics (i.e., 0 days of exposure), and 34 and 37 phyla identified on the microplastics after 7 and 21 days of treatment, respectively. Ten dominant phyla were identified within all the microplastic-biofilms (>1% abundance in more than 75% of all samples within a treatment group[303]) as Proteobacteria (24 – 28%; Alpha and Gammaproteobacteria classes), Cyanobacteria (8 –

10%; Cyanobacteriia class), Bacteroidotes (8 – 8.5%; Bacteroidia class), Verrucomicrobiota, Plantomycetota, Actinobacteriota, Acidobacteriota, Myxococcota and Firmicutes (<5%, respectively), with low abundance phyla (<1% relative abundance) contributing to 24 – 28% of the microbiome (Figure S6.11), irrespective of exposure time or chemical composition. At lower taxonomic levels, differences in community composition became more pronounced, particularly after initial (t = 7 days) and prolonged (t = 21 days) exposure to experimental marine bio-fouling conditions.

Further investigation of community composition using beta diversity assessment (PCoA for dissimilarity and *Euler diagrams* to identify shared ASVs among substrates) revealed variations in taxonomic compositions with time and the microplastics chemical composition (Figure 6.3 and Figure 6.4). At 0 days, the biofilm community forming on the untreated microplastics was dominated by airborne bacterial clades and those associated with outdoor and human microbiomes[313,314], with *Corynebacteriaceae*, *Micrococcaceae*, *Sphingomonadaceae*, *Streptococcacea*, *Staphylococcaceae*, *Bacillaceae*, *Moraxellaxeae*, *Neisseriaceae*, *Pseudomonadaceae* and *Thermaceae* families belonging to the classes Actinobacteria, Alphaproteobacteria, Bacilli, Deinococci and Gammaproteobacteria dominating the microbiome (10 – 40% total relative abundance) (Figure 6.4). Upon immersion in seawater, members of these classes were significantly depleted (*Bacillaceae* and *Staphylococcaceae*) or no longer detected (*Coynebacteriaceae, Micrococcaceae, Streptococcacea* and *Thermaceae*), and thus may have contributed to the time-dependent dissimilarity in community composition observed using alpha and beta diversity assessments (Figure 6.2 and Figure 6.3). Furthermore, the absence of these bacterial clades (mainly members of Actinobacteria, Bacilli and Deinococci) in the incoming seawater throughout the experiment suggested that some airborne bacteria may remain attached to microplastics even after immersion in seawater. After 7 and 21 days of exposure to marine bio-fouling conditions, in line with early-stage biofilm formation[315], and correlating with changes in alpha diversity metrics (evenness and diversity), the biofilm community shifted with the growth of low abundance taxa (Figure S6.11), to favour *Microtrichaceae, Hyphomonadaceae, Mitochondira, Rhodobacteraceae, Flavobacteriaceae, Saprospiraceae, Bdellovibrionaceae, Chloroplast, Nitrincolaceae* and *WCHB1-41* bacterial families, belonging to the classes Acidimicrobiia, Alphaproteobacteria, Bacteroidia, Cyanobacteria and Gammaproteobacteria (10 – 30% total relative abundance) (Figure 6.4). While the complexity (i.e., ASV richness) of the microplastic-biofilms increased with exposure time, the greatest variation in taxonomic composition of the dominant taxa was observed at 7 days, which saw significant fluctuations in the relative abundance of Alphaproteobacteria, Gammaproteobacteria ($p < 0.001$) and Acidimicrobiia ($p < 0.05$) (Table S6.4). However, many of these fluctuations in relative abundance also correlated with changes in microplastics chemical composition.

While minor variations in community composition were observed with polymer and plasticiser type, the most obvious trend was observed among the virgin and plasticised substrates. The only polymer- and/or plasticiser-specific trend observed was for PS-DEHP microplastics which, after 7 days, were not only less diversely colonised (i.e., significantly lower Chao1 indicates; $p < 0.001$; Figure 6.2), but also exhibited the greatest community dissimilarity (Figure 6.3) and the shared the lowest proportion of ASVs (Figure 6.4). Changes in the relative abundance of specific families, including *Hyphomonadaceae, Rhodobacteraceae, Stappiaceae* (Alphaproteobacteria; $p < 0.001$), *Cellvibrionaceae, Comamonadaceae, Enterobacteriaceae, Nitrincolaceae, Vibrionaceae* (Gammaproteobacteria; $p < 0.01$), *WCHB1-41*, (Kiritimatiellae; $p < 0.001$), *Pirellulaceae* and *Rubinisphaeraceae* (Planctomycetes; $p < 0.05$) were associated with the dissimilarity between the virgin and plasticised-polymer microbiomes (Figure 6.4). Additionally, the plasticised polymers also exhibited depletion and/or absence of families from the classes Gammaproteobacteria and Bacilli, further distinguishing them from the virgin substrates. Moreover, depletion of *Microtrichaceae* and *Chloroplast*, coupled with the enrichment of *Rhodobacteraceae* and *Cellvibrionaceae* from the classes Acidimicrobiia, Cyanobacteriia, Alphaproteobacteria and Gammaproteobacteria, respectively, may have also contributed to the formation of taxonomically distinct biofilms.

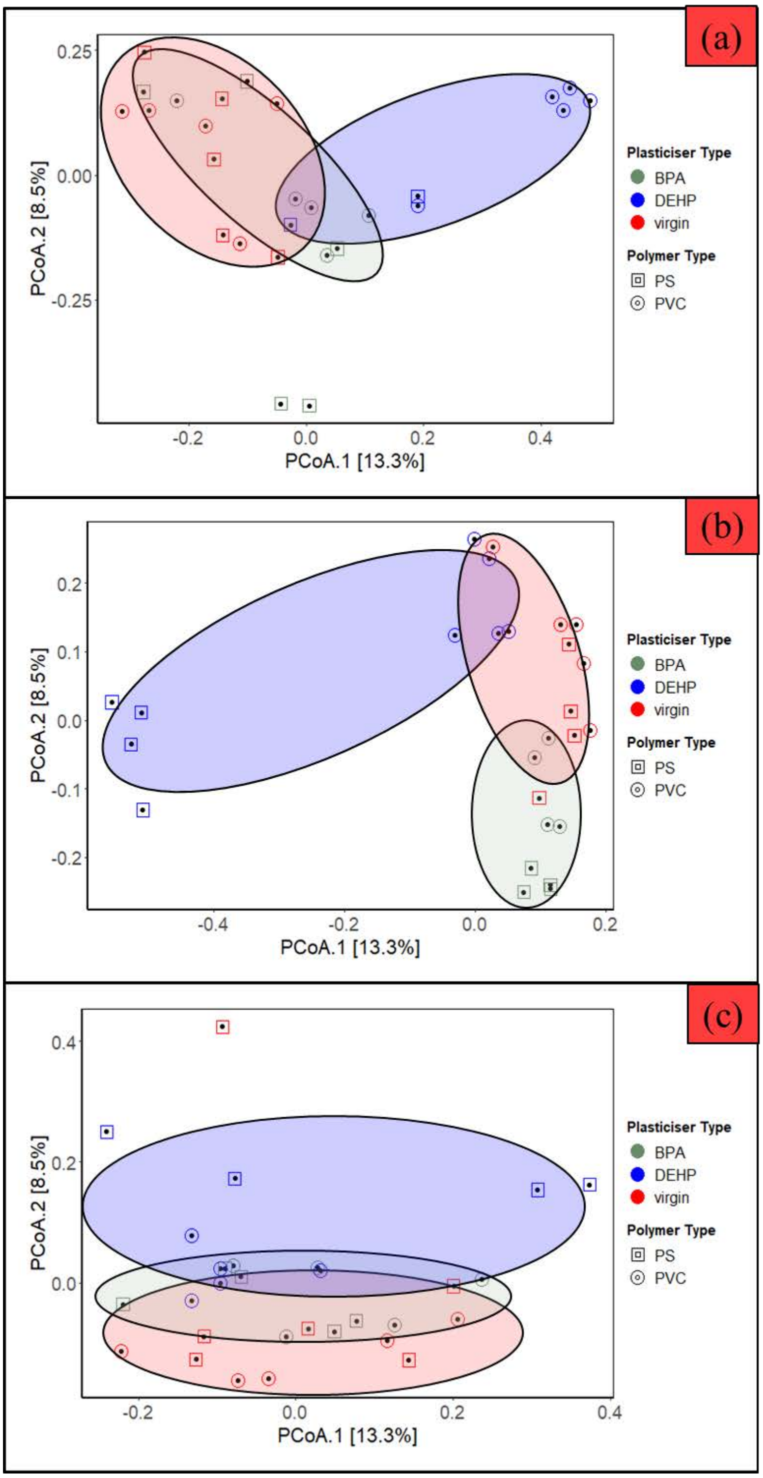

Figure 6.3. Principal coordinate analysis (PCoA) plots showing community dissimilarity of amplicon sequence variants (ASVs) from polystyrene (PS; square) and polyvinyl chloride (PVC; circle) microplastics, virgin (i.e., no plasticiser) and containing bisphenol A (BPA) and diethylhexyl phthalate (DEHP), after (a) 0, (b) 7 and (c) 21 days of exposure to marine bio-fouling conditions.

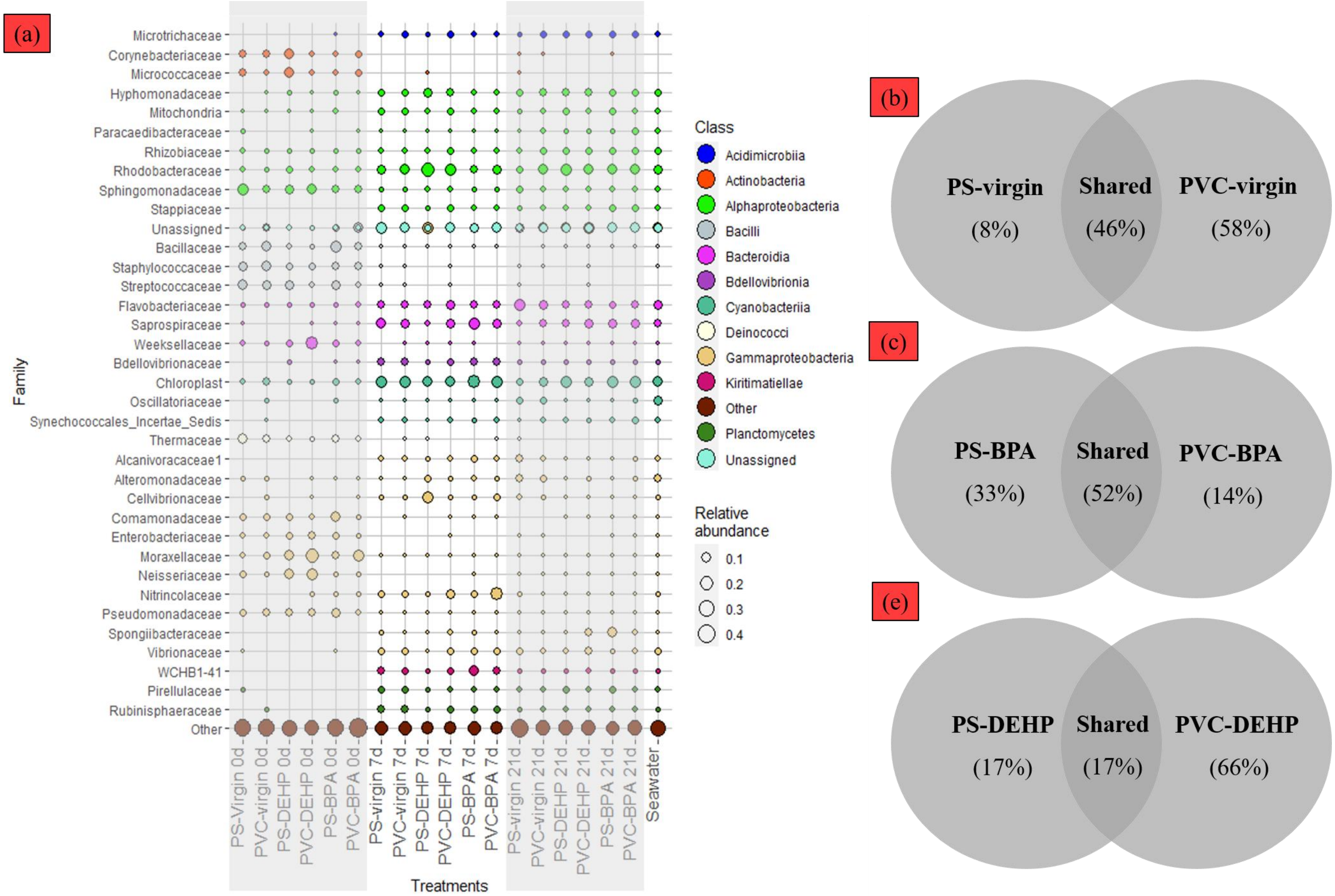
(a)
Microtrichaceae
Corynebacteriaceae
Micrococcaceae
Hyphomonadaceae
Mitochondria
Paracaedibacteraceae
Rhizobiaceae
Rhodobacteraceae
Sphingomonadaceae
Stappiaceae
Unassigned
Bacillaceae
Staphylococcaceae
Streptococcaceae
Flavobacteriaceae
Saprospiraceae
Weeksellaceae
Bdellovibrionaceae
Chloroplast
Oscillatoriaceae
Synechococcales_Incertae_Sedis
Thermaceae
Alcanivoracaceae1
Alteromonadaceae
Cellvibrionaceae
Comamonadaceae
Enterobacteriaceae
Moraxellaceae
Neisseriaceae
Nitrincolaceae
Pseudomonadaceae
Spongiibacteraceae
Vibrionaceae
WCHB1-41
Pirellulaceae
Rubinisphaeraceae
Other
Family
PS-Virgin 0d
PVC-virgin 0d
PS-DEHP 0d
PVC-DEHP 0d
PS-BPA 0d
PVC-BPA 0d
PS-virgin 7d
PVC-virgin 7d
PS-DEHP 7d
PVC-DEHP 7d
PS-BPA 7d
PVC-BPA 7d
PS-virgin 21d
PVC-virgin 21d
PS-DEHP 21d
PVC-DEHP 21d
PS-BPA 21d
PVC-BPA 21d
Seawater
Treatments
Class
Acidimicrobiia
Actinobacteria
Alphaproteobacteria
Bacilli
Bacteroidia
Bdellovibrionia
Cyanobacteriia
Deinococci
Gammaproteobacteria
Kiritimatiellae
Other
Planctomycetes
Unassigned
Relative abundance
0.1
0.2
0.3
0.4
(b)
PS-virgin (8%)
Shared (46%)
PVC-virgin (58%)
(c)
PS-BPA (33%)
Shared (52%)
PVC-BPA (14%)
(e)
PS-DEHP (17%)
Shared (17%)
PVC-DEHP (66%)

Figure 6.4. (a) Family-level taxonomic analysis of the relative abundances (%) of the most abundant bacteria forming the microbiome of seawater and the polystyrene (PS) and polyvinyl chloride (PVC) microplastics, virgin (i.e., no plasticiser) and containing diethylhexyl phthalate (DEHP) and bisphenol A (BPA) after 0, 7 and 21 days of exposure to marine bio-fouling conditions. "Other" refers to bacterial taxa with less than 1% abundance and "unassigned" refers to amplicon sequence variants (ASVs) that could not be classified. (b – e) *Euler diagrams* include all time points and show the proportion (%) of ASVs exclusive to each substrate.

Biodegradation of microplastics

While PS and PVC are highly recalcitrant to biodegradation in natural environments[180], FT-IR, GPC and SEM were used to compare biodegradation parameters (e.g., novel bond formation, molecular weights information and surface morphology, respectively[62,111,256,316]) among microplastic substrates throughout the experiment. Using FT-IR, changes in characteristic peaks commonly associated with PS and PVC biodegradation could be observed (Figure S6.4). Most noticeable was the introduction of weak, broad absorption bands at ~3380 cm^{-1} (-OH) in all microplastic profiles after 21 days, which may be indicative of biological decomposition of the polymer backbone[178,310,317]. Additionally, for PVC, the declining intensity of peaks at 827 cm^{-1} (ν C-Cl) is consistent with dehalogenation of the polymer backbone[311]. However, the introduction and/or changes in intensity of other characteristic peaks within the carbonyl region (1500 ~ 2000 cm^{-1}, i.e., oxidation[111,310]) as well as those corresponding to alkene biodegradation (600 – 1000 cm^{-1}; e.g., ν C-O-C[311]) could not be reliably identified in the infrared spectra of all plasticised-polymers, because of the overlapping BPA and DEHP bands[318,319]. Moreover, GPC was also used to measure changes in polymer molecular weights information, and highlighted a significant decrease in PS-BPA *Mw* from 171, 000 g/mol (control) to 168, 000 g/mol (3% decrease; $p > 0.05$) and 170, 000 g/mol (4% decrease; $p < 0.05$) after 0, 7 and 21 days, respectively (Table S6.5). In line with *Mw*, SEM imaging highlighted morphological changes on the surface of the PS-BPA microplastics, with the formation of deep grooves and holes (Figure 6.5). Similarly, a decline in *Mw* was observed for PS-virgin and PS-DEHP plastics (9.8 – 10.4 and 3.4 – 4.9% decrease, respectively, $p > 0.05$), with small holes appearing on the surface after 21 days. However, for all PVC substrates, no significant changes in molecular weights information or surface morphology was observed.

Using available and recent literature reviews[73,173,177,256,31], this study identified bacteria within the developing microplastic-biofilms with putative metabolic capabilities for degrading PS and PVC polymers, and their relative abundances compared across all treatment conditions using GLMs (Table S6.7). These bacteria were predominately from the phyla Pseudomonadota (57%) Firmicutes (29%) and Actinobacteria (14%), and included seven genera: *Pseudomonas*, *Cohaesibacter*, *Bacillus*, *Brevundimonas*, *Erysipelothrix*, *Corynebacterium* and *Desulfovibrio*, with the latter associated primarily with PVC degradation (i.e., dehalogenating[254]) (Figure 6.6). *Pseudomonas* and *Bacillius* species were the most prevalent bacterial genera identified in the microplastic-biofilm with plastic degrading potential and were found in 75 - 88% of all replicate samples at relative abundances greater than 0.05%. These genera, along with *Brevundimonas* and *Corynebacterium* species were abundant in the microbiome forming on PS substrates – particularly PS-BPA – suggesting a dominant role in the degradation of the polymer[320,321]. Notably, the relative abundance of these bacteria was highest on the control, untreated microplastics (i.e., 0 days of exposure) when compared to the incoming seawater, suggested that both aquatic and airborne colonisation of microplastics could promote microplastic biodegradation in the environment.

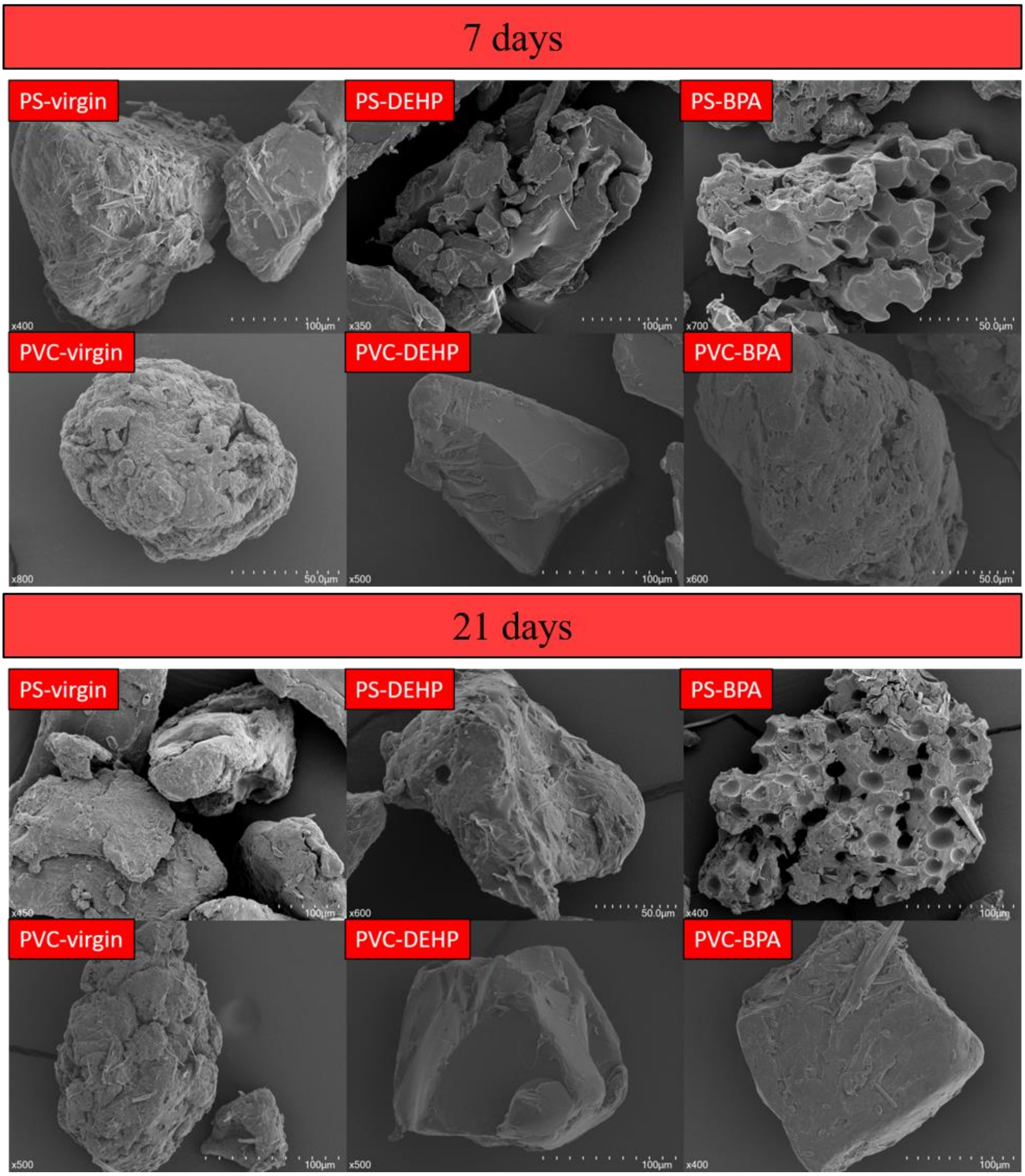

Figure 6.5. Representative images of the polystyrene (PS) and polyvinyl chloride (PVC) microplastics; virgin (i.e., no plasticiser) and containing diethylhexyl phthalate (DEHP) and bisphenol A (BPA) after 7 and 21 days of bio-fouling. Magnification and scale bars are indicated on each image.

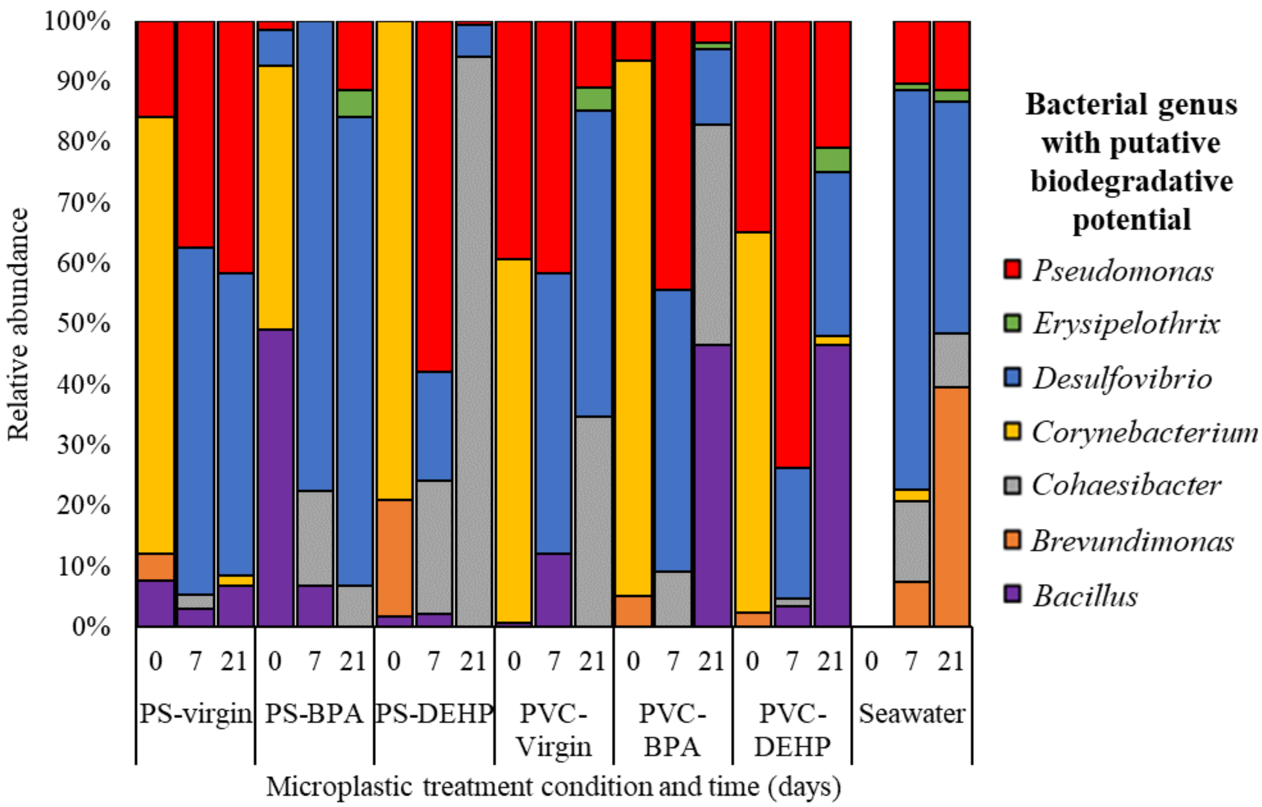

Figure 6.6. Total relative abundance (%) of bacterial genera with plastic degrading potential identified in the microbiome of seawater and on polystyrene (PS) and polyvinyl chloride (PVC) microplastics; virgin (i.e., no plasticiser) and containing diethylhexyl phthalate (DEHP) or bisphenol A (BPA) after 0, 7 and 21 days of exposure to marine bio-fouling conditions. Putative capacity of each genus to degrade PS and PVC polymers was inferred based on literature studies[73,171,175,254,312,322] and identified in the microbiome in this study.

Discussion

Microplastics and plasticisers can have widespread impacts on environmental and biological microbiomes[141,142], however, the specific influence of polymer-plasticiser composition on biofilm formation and community composition remains understudied[151]. In this chapter, microplastics were prepared under controlled conditions, ensuring homogenous plasticiser distributions (Figure S6.1) and comparable physiochemical properties (e.g., spectral profiles, thermal properties, size distribution, shape and surface morphology; Figure S6.2 – S6.6). Additionally, the experiment was conducted *in-situ* to minimise fluctuation and variation in ambient weather, water quality parameters (Table S6.1) and water flow rate (Figure S6.8); thus ensuring a comparable seawater microbiome throughout the exposure timeframe[309,323]. While research has demonstrated that different microplastic polymers can harbour taxonomically distinct biofilms, there remains a knowledge gap regarding how community structure, particularly during early colonisation stages[324], is impacted by differences in microplastic chemical composition[315]. This experiment allowed for assessment of how time and polymer-plasticiser composition impacts a developing biofilm on marine microplastic contamination.

Shared microbial community

Identifying bacteria than are closely and commonly associated with microplastic substrates will not only facilitate the development of more holistic and well-informed ERAs, but it will also enable future researchers to more easily identify factors that impact microbial colonisation and community structure in developing microplastic-biofilms[147]. *Euler diagrams* presented in Figure 6.3b - e were used to visualise ASVs that were commonly shared across all examined microplastic substrates in this study, and included species belonging to the phyla Proteobacteria, Cyanobacteria, Bacteroidotes and Planctomycetes. Members of these phyla encompass a broad range of metabolic capabilities (e.g., pathogenic, photoautotrophic[289], carbohydrate assimilation[290] and plastic degradation[171]) and are opportunistic marine colonisers identified in the microbiome of both synthetic (including PS, PVC, PE and PET microplastic[156,165,172,177,315,325]) and natural substrates worldwide[73,151,286]. Moreover, they are often involved in early biofilm formation and thus their dominance is expected during experiments conducted over short time frames[326]. These data demonstrate that there is significant overlap in the taxonomic composition of developing microplastic-biofilms, not only between PS and PVC microplastics incorporated with different plasticisers, but also among other microplastics polymers frequently detected in marine environments[145,147,286]. Characterising these shared taxa will ensure unique properties of microplastic-biofilms can be reliably identified in future studies and will facilitate more accurate assessment of how biogeography and physiochemical parameters can impact the microplastic microbiomes[143,147,327].

Changes in the microplastic-biofilm over time

Bacterial succession is a major factor influencing the development of biofilms[306] and can be impacted by the presence and growth patterns of specific taxa involved in initial attachment, early colonisation and maturation[165,315,328]. In this study, rapid bacterial colonisation on microplastics occurred within 7 days of immersion in seawater, exhibiting dynamic changes within narrow timeframes up to 21 days. The successive relationship between early marine colonisers and the background microbiome (i.e., bacteria already attached to the control, untreated microplastics at 0 days) likely contributed to the development and community composition of the microplastic-biofilms. Members of Bacteroidota pioneer biofilm formation on microplastics in seawater[329], however, they are often outcompeted by Proteobacteria and Cyanobacteria over time[306]. Statistical analyses revealed no significance changes in the relative abundances of these phyla from 7 to 21 days, indicating that the successive relationship between these early colonisers was not observed at this time scale. However, it is also possible that taxa such as *Micrococcaceae, Streptococcaceae* and Bacillus already present in the microplastic-biofilms at

0 days influenced the growth patterns of these early marine colonisers, and may have outcompeted some taxa for attachment and growing opportunities[330]. This highlights the need to consider the background microbiome when investigating developing marine microplastic-biofilms, as the origin (e.g., PVC biomedical equipment in hospital environments[43,331]) and contamination point source (e.g., microplastics emitted into urban/industrial harbours[332]) can influence the types of taxa abundant on the plastics, as well as the ecological impacts (e.g., pathogenic transmission, antibiotic resistance, etc.,). With that in mind, depletion of members of *Bacilli* – many of which have pathogenic capabilities – further indicates that microplastics may be aiding the dispersal of exotic bacteria into marine environments[145].

Changes in the microplastic-biofilm with chemical composition

Colonisation of bacteria on different microplastic polymers is strongly correlated with the morphology, particularly surface topography and hydrophilicity[165,333]. Diverse and abundant microbial communities are likely to form on hydrophilic substrates as they provide ample adhesion opportunities and easily assimilated nutrients[325,334]. In this study, differences in the hydrophobicity of the substrates may have contributed to the dissimilarity between in microbiome composition among virgin and plasticised-microplastics, and more specifically those incorporated with DEHP. While both PS and PVC polymers possess some degree of hydrophobicity[335,336], the incorporation of plasticisers (with DEHP being more hydrophobic than BPA[337]), as well as their leaching behaviours and any accumulation of plasticisers on the surface of plastics, can further enhance the hydrophobicity of the microplastic[127,255], and thus impact bacterial attachment[325]. These discrete differences in surface hydrophobicity may explain the enhanced richness and diversity of biofilms present on the DEHP-microplastics compared to those on virgin- and BPA-microplastics, i.e., the hydrophobic surface may have influenced microbial attachment and/or selected for certain primary colonisers with adapted mechanisms for colonising hydrophobic surfaces (e.g., *Pseudomonadaceae* family[338]). Surface morphology and topography of the substrate (i.e., roughness) are also important factors that can impact species-specific colonisation and influence the biofilm lifecycle[339]. Conducting more directed experiments (e.g., absolute surface roughness calculations, enrichment cultures) would be valuable to further clarify the influence of polymer-plasticiser composition on bacterial bio-fouling, and characterise the specific growth patterns of early colonising microbes, as well as those low abundant taxa, and those involved in pathogenic transmission and plastic degradation[172,340–342].

Polymer biodegradation

Microbial colonisation of microplastics holds exciting potential for synthetic polymer degradation, however, the link between bacterial colonisation and microplastic degradation remains uncertain. In this study, only PS microplastics displayed noticeable changes in molecular weights information and surface morphology consistent with polymer biodegradation. PS-BPA substrates displayed significant degradation throughout the experiment, which was evidenced by a 4% decline in *Mw* from 7 to 21 days, as well as the formation of deep holes and pits on the surface conforming to the shape of colonising bacteria[161] (Figure 6.5). Given their durable physiochemical properties and lack of microbes identified to degrade PS and PVC polymers[171], few studies have been successful at observing biodegradation of PS and PVC plastics[254,343,344]. However, studies employing long-term bio-fouling conditions (>80 days[172]) and/or utilising enrichment cultures[275,343] have observed similar changes in the microplastic's physiochemical properties consistent with biodegradation. Therefore, despite some significant indicators of PS-BPA biodegradation observed using GPC and SEM analysis, it is likely the duration of the experiment conducted here was insufficient to observe substantial biodegradation of more recalcitrant polymers (e.g., PVC and virgin microplastics[34,274,345]). Nonetheless, enrichment of bacterial clades with putative degradative potential for PS and PVC polymers were identified on microplastics exposed to marine conditions, and their varying relative abundances and activities (e.g., degradation of PS-BPA) is further evidence to support the removal of recalcitrant polymers present in oceanic environments through biodegradation[171].

Conclusion

Bacterial colonisation and biofilm community structure was influenced by exposure time and the polymer-plasticiser composition of the microplastic. All PS and PVC substrates investigated in this study shared a dominant microbiome of Proteobacteria, Bacteroidota and Cyanobacteria, indicating that microplastics immersed in marine environments may harbour similar abundant taxa, especially during early-stage biofilm formation. Furthermore, prolonged exposure to seawater prompted significant enrichment of diverse bacterial communities, whereby low abundance phyla such as Bdellovibrionota and Desulfobacterota dominating 7 day biofilms were outcompeted after 21 days by Calditrichota, Firmicutes and Marinimicrobia. Furthermore, while taxonomically distinct biofilms were formed on all microplastics with respect to time and plasticiser presence, no significant polymer-, and/or plasticiser-specific trends were observed. These unique differences may potentially be associated fluctuations in nutrient availability owing to the presence and leaching behaviours of the incorporated plasticisers as well as surface properties such as surface functionalisation and topography. This highlights the need for more directed studies in this area, such as the use of enrichment cultures that monitor growth of specific taxa on different microplastic substrates. Such research will enable more specific evaluation of the impact of polymer-plasticiser chemical properties on the growth of key bacteria, incluidng those forming the core community, as well as those involved in pathogen transmission and plastic degradation. Regardless, this study is the first of its kind to examine biofilm formation on microplastic polymers containing different plasticisers and highlights the critical need to further explore the impacts of these additives on bio-fouling.

Chapter 7: General discussion and recommendations for future work

Environmental risk assessment of microplastics

Microplastics comprise an intricate blend of polymers (e.g., polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC) and polystyrene (PS)) often manufactured in combination with a range of synthetic additives (e.g., flame retardants and plasticisers), resulting in particles with an array of shapes, sizes and colours[34,62,346]. These complex and dynamic physiochemical properties play a critical role in dictating contamination levels, transportation dynamics and exposure consequences of microplastics in the environment and to biota, motivating the need for environmental risk assessments (ERAs). ERAs play a crucial role in informing effective management decisions by providing comprehensive and realistic data on the risks associated with microplastics[181]. However, significant challenges related to the multidimensionality and complexity of microplastics – including their environmental fate, reactive properties and behaviours – hinders the development of accurate ERAs. Addressing erroneous areas of sample processing and experimental design is critical to bridge knowledge gaps essential to enhance the precision and reliability of future experiments and ERAs.

Following ERA principles, this Ph.D., thesis employed a controlled and multidisciplinary approach to deconstruct the complexity of microplastics behaviours – both in the laboratory and the environment – necessary to improve the technical accuracy of future experiments and ERAs. Specifically, the six data chapters addressed three interrelated topics across the ERA framework (Figure 7.1) to: 1) assess the suitability of sample processing techniques for method standardisation (Chapter 2), 2) understand the behaviours of plasticised-microplastics with a range of physiochemical properties and under fluctuating environmental conditions (Chapter 3 – 5), and 3) elucidate the relationship between microplastics, plasticisers and the marine microbiome (Chapter 6). Ultimately, this thesis provides vital knowledge pertaining to the behaviours of microplastics in aquatic environments and provides a necessary background contributing to the development of a holistic ERAs of microplastics.

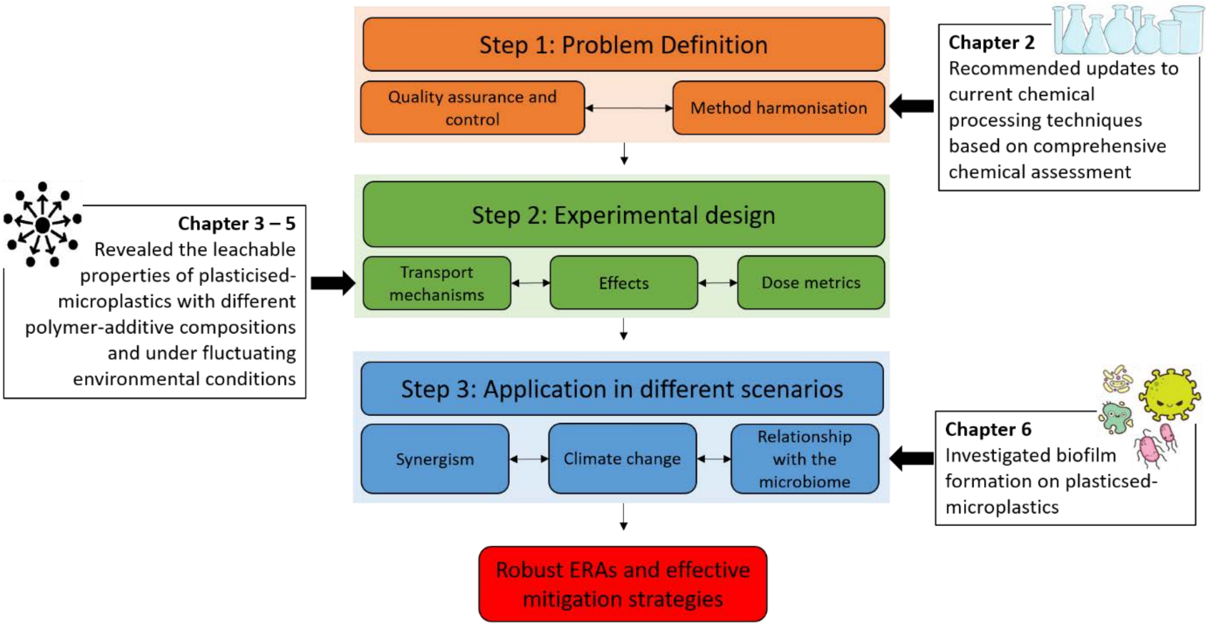

Figure 7.1. Risk analysis framework to incorporate microplastics with varying polymer-additive compositions in an environmental risk assessment (ERA), based on the workflow described by Koelmans *et al.,* (2022)[181]. Key outcomes from each chapter of this thesis relevant to the risk analysis framework are highlighted, and together contribute to the development of more robust microplastic ERAs, and ultimately more effective management strategies. Icons were sourced from ClipArt.

Standardising sample processing techniques: chemical digestion methods

ERAs require reliable data to inform subsequent environmental management decisions and as such rely on stringent quality control and assurance (QA/QC) measures. Given inconsistencies in currently applied sample processing techniques for microplastics (e.g., chemical extraction procedures) and their limited applicability to different sample matrices (e.g., aqueous vs sedimentary) and chemical compositions (e.g., polymer type, size, morphology), obtaining unrealistic estimates of microplastics loading in environmental and biological matrices remains a concern. Thus, application of this data to ERAs remain controversial and limited[87]. Hence, significant efforts to establish standardised and universal chemical digestion method(s) to improve microplastics quantification and characterisation data have become important research topics[86,88,188]. Findings reported in Chapter 2 of this thesis indicated that microplastic reactivity and degradative susceptibility during chemical digestion is reagent-, time-, temperature- and size-dependent, and confirms that extraction methodologies need to be tailored to the specific microplastic composition and sample matrix[88]. Moreover, this data contributes to the standardisation of chemical digestion protocols commonly applied for microplastics extraction and can be used as a guide for future researchers to ensure accuracy in experimental data output, particularly in studies quantifying and characterising microplastics loading in the environment. This will ensure that threatened ecosystems/species, effect and dose-response relationships can be confidently identified in ERAs, and thus policies can be developed to address these hazards[86,88,185]. Government policy changes prompted by the development of similar ERAs for macroscale plastics has led to the phase-out single-use items (e.g., bags and straws[347]), and by strengthening sample processing methodologies for microplastics, environmental management practices can now be extended to address concerns associated with microplastics contamination[181].

Ecotoxicology/exposure experiments to understand transport mechanisms: plasticiser leaching from microplastics

Despite significant research highlighting the toxicity of plasticisers (both alone and in combination with microplastics)[52,55,348], the mechanisms by which plasticisers enter aquatic matrices and affect biota is not well understood. The lack of comprehensive knowledge pertaining to microplastic leachability has meant that these behaviours are often not considered when designing exposure and ecotoxicology experiments with plasticised-microplastics[54,113–115], and thus the relationship between plasticiser leachates and biota toxicity has yet to be included in ERAs. To address this knowledge gap, Chapters 3 – 5 of this thesis investigated the leaching dynamics of diethylhexyl phthalate (DEHP), dioctyl terephthalate (DEHT), bisphenol A (BPA) and bisphenol S (BPS) from PS and PVC microplastics under fluctuating environmentally relevant, aqueous conditions (salinity, pH, temperature, wave action and during polymer degradation). Not only did these data enable determination of the rate-limiting steps of DEHP, DEHT (boundary layer-limited), BPA and BPS (diffusion-limited) leaching, predictive model outputs revealed that the plasticiser leaching from microplastics is exacerbated in environments with elevated temperatures (e.g., with climate change) experiencing significant wave action (e.g., in coastal environments), as well as those with high levels of small micro- and nano-plastic contamination (e.g., seabed). These leaching data offer valuable and necessary insights that can be used to explain toxicity response data (e.g., acute vs chronic exposure and lethal doses) by enabling researchers to predict plasticiser concentrations (both remaining in the microplastic and released into the surrounding water; *in-situ* and *in-natura*) at specific time points throughout the lifecycle of a microplastic. Incorporating the insights gained from these leaching data into ERAs will not only allow for the identification of high-concern polymer-plasticiser blends based on their leaching dynamics (e.g., rapid release; BPA and BPS vs retention; DEHT and DEHP), it can also empower government initiatives to lobby for the exclusion and/or replacement of hazardous plasticisers during manufacturing with greener alternatives. By doing so, more effective and sustainable microplastic management strategies can be implemented, safeguarding aquatic ecosystems and biota without comprising on the functionality and useability of plastic products.

Behaviours in environmentally relevant scenarios: understanding bacterial colonisation on plasticised-microplastics

Designing realistic experiments that adequately address the multidimensionality of microplastics in aquatic environments requires on a thorough understanding of their interactions with various abiotic and biotic parameters. Biofilm formation on microplastics immersed in non-sterile waters is a prevalent phenomenon[73], irrespective of whether researchers account for these behaviours in their experimental design. Chapter 6 of this thesis evidenced the formation of taxonomically distinct biofilms on PS and PVC microplastics, virgin, or incorporated with either DEHP or BPA. The study highlighted the significance of considering exposure time and microplastic chemical composition during biofilm development and highlighted that bacterial colonisation may also be impacted by plasticiser leaching dynamics. While bio-fouling plastics prior to organism exposure has become a more commonly place technique in *in-situ* experiments to emulate natural feeding conditions[84,349], differences in the physiochemical properties of the plastics has yet to be considered. Consequently, organisms may encounter different bacterial species at fluctuating abundances dependent on the microplastics properties, which may skew response data. This is especially crucial if the microplastics' polymer-additive composition facilitates the colonisation and/or selects for bacteria with pathogenic metabolic capabilities. Thus, when investigating important aspects of biota ingestion likelihood and exposure consequences vital for ERA development, it is imperative to account for the diversity of bacterial taxa forming a biofilm on different microplastics.

Concluding remarks

By advancing technical approaches used to conduct microplastics research, a more accurate and holistic understanding of their lifecycle, fate and behaviours in aquatic ecosystems can be obtained. The work outlined in this thesis contributes to our understanding of the complex behaviours of microplastics, by harmonising sample processing protocols, characterising and enabling the prediction of their leachability, and clarifying their relationship with the marine microbiome. With the results of this thesis in mind, sample processing protocols can be updated to ensure accuracy and confidence in evaluation of environmental contamination levels, ensuring threatened ecosystems with high levels of microplastic and/or plasticiser loading can be appropriately identified in ERAs and policies implemented to address these hazards. Serving as a guide to strengthen future experimental design and methodologies, which ultimately will lead to the development of more environmentally accurate ERAs of microplastics.

Recommendations for future research

Since pioneer research began on plastic and microplastic contamination in marine ecosystems[350–353], their discharge and accumulation in the environment has revealed a plethora of ecological hazards. Management and mitigation of microplastics in the environment is a collaborative and multidisciplinary effort and relies on the development of meticulous and reliable ERAs[88,181,249]. To further develop the findings outlined throughout this thesis, listed below are recommended avenues for future research. By addressing these targeted research questions, future researchers can build upon the foundational knowledge and work outlined in the thesis and begin to explore the vast and interrelated complexities of aquatic microplastic contamination.

- **Consider microplastics with diverse histories:** This thesis successfully described microplastic behaviours under simulated environmental conditions from polymers prepared under controlled conditions. However, these behaviours should also be assessed for microplastics with different ecological histories (i.e., collected from different aqueous environments and environmental compartments).
- **Investigate a range of microplastics:** The complexity of microplastics should be considered when investigating their behaviours (both *in-situ* and *in-natura*), this could include investigating different polymers (e.g., PP and PE), additive mixtures (e.g., flame retardants) and morphologies (e.g., films, primary microplastics and fragmented microplastics produced through environmental weathering).
- **Expansion of the numerical diffusion and boundary layer model:** The diffusion and boundary layer model developed in this thesis was successfully applied to explain and predict plasticiser leaching from PS and PVC microplastics under simulated environmental conditions (Chapter 3 – 5). In future studies, this model could be expanded to include a combination of conditions (e.g., eutrophication and rising water temperatures) and microplastic properties (e.g., aged microplastic exposed to biodegradation and photo/thermal oxidation).
- **Toxicity consequences and transport mechanisms:** Chapters 3 and 5 of this thesis provided a comparison between the leaching behaviours of two dominant polymers, PS and PVC. To identify major transport mechanisms and toxicity consequences of plasticised-microplastics, future studies should compare the toxicity of microplastics with different leachability over time (e.g., proportion of free-plasticiser).
- **The microbiome beyond bacteria:** While Chapter 6 of this thesis focused on bacteria, which dominate the microbiome, it is crucial to recognise the potential involvement of other microorganisms[156,160,161]. By expanding microplastic-biofilm research to the broader microbiome, a more comprehensive view of the ecological implications and potential interactions between microplastics and diverse microbial communities can be obtained.
- **Long-term monitoring of biofilms:** To gain a deeper understanding of microbial biofilm development on different microplastic substrates beyond early-stage formation, conducting

prolonged studies (over many months and years[147,162]) would be beneficial. These extended studies could help to clarify microbial succession, competition and long-term adhesion of bacterial taxa on microplastics, and potentially reveal more significant biodegradation processes.

- **Enrichment cultures:** Utilising enrichment cultures would be valuable to monitor the growth of specific taxa on different microplastic substrates. This approach will enable researchers to focus on bacterial taxa with important metabolic capabilities (e.g., pathogens and plastic-degraders) and help to better understand their interactions with microplastics.

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Supplementary Information

Chapter 1. Introduction: our world wrapped in plastic

Table S1.1. Numerical size distribution of plastics in different compartments of the aquatic environments (average number of items per study per size class (%)). Size classes were categorised according to common size nonmenclature: megaplastics (> 50 cm); macroplastics (5 – 50 cm), mesoplastics (0.5 – 5 cm) and microplastics (<5 mm))[67,194]. Only studies published after 2013, which quantify contamination levels of three of more plastic size classes by number were included. NR (not recorded) refers to studies that did not detect plastics of that size class.

Aquatic Zone	**Average number of items/study/size class (%)**				**Reference**
	Microplastics	**Mesoplastic**	**Macroplastic**	**Megaplastic**	
Shoreline	73.44	17.76	12.06	2.67	[354–363]
Tidal Zone	81.67	10.33	19.00	NR	[364–366]
Open Water	80.94	14.79	19.40	3.33	[24,61,67,367–373]
Sediment	81.83	28.30	10.25	NR	[357,369,374]
Animal Ingestion	78.43	11.00	51.48	9.50	[197,375–380]

Table S1.2. The concentration range (minimum – maximum) of diethyhexyl phthalate (DEHP), dioctyl terephthalate (DEHT), bisphenol A (BPA) and bisphenol S (BPS) detected in aquatic matrices worldwide, collated from all available data up to 2022. ND (not detected) refers to studies that investigated by did not detect any analyte (e.g., no contamination and/or below the detectable limit of the instrument) whereas NR (not recorded) refers to studies that did not investigate a specific analyte.

Geographic Location (as stated by study methodology)		**Concentration range (min. - max. where applicable)**				**Reference**
		DEHP	**DEHT**	**BPA**	**BPS**	
Oceania	Auckland Harbour, New Zealand	11500 ng/L	NR	145 ng/L	NR	[381]
	Darwin Harbour, Australia	NR	NR	0.56 ug/L	NR	[382]
	Southeast Queensland, Australia	NR	NR	0.059 ug/L	NR	[383]
	Tropical Western Pacific Ocean	9.19 ug/L	NR	NR	NR	[384]
Asia	Black Sea	NR	129 ng/L	14.8 ug/L	NR	[385,386]
	Ganga River, India	8800 ng/L	NR	4.46 ug/L	<16.70 ng/L	[387–389]
	Iranian surface waters	0.23 ug/L	NR	NR	NR	[390]
	Japanese coastal waters	98 ug/L	NR	nd - 2400 ng/L	nd - 15 ug/L	[391]
	Kaohsiung Harbour, Taiwan	21559 ng/L	NR	NR	NR	[392]
	Korean coastal waters	nd - 1.34 ug/mL	NR	1 - 1918 ng/L	nd - 42 ng/L	[393,394]
	Laguna Lake, Philippines	nd - 3 ug/L	NR	0.047 - 2 ug/L	nd - 2 ug/L	[395,396]
	Malaysian coastal waters	0.51 ug/L	NR	nd - 8.24 ug/L	NR	[397–399]
	Pearl River, China	0.08 - 91.07 ug/L	NR	4.3 - 556 ng/L	nd - 135 ng/L	[229,399–401]
	Persian Gulf	2.85 - 30.25 ug/L	NR	12.73 ug/L	NR	[402,403]
	Yellow River, China	36.30 - 2002 ng/L	NR	53.70 - 180 ng/L	NR	[401,404]
Poles	Arctic	0.024 - 3.30 ng/L	NR	NR	NR	[405]
	McMurdo Station	NR	NR	4.70 - 986 ng/L	NR	[406]
	Northern Antarctic Peninsula	NR	NR	18.74 ng/L	NR	[407]
Europe	French waters	114 - 406 ng/L	NR	1275 ng/L	21 ng/L	[408–410]

	Italian waters	NR	NR	0.99 - 244 ng/L	NR	[411]
	Mediterranean Sea	62 - 5965 ng/L	NR	3 - 350 ng/L	NR	[412]
	Nordic waters	25 - 6720 ug/L	NR	20 - 721 ug/L	NR	[413–415]
	Spanish waters	4.60 - 175 ng/L	NR	35 - 2970 ng/L	NR	[416]
North America	Canada	NR	NR	1 - 62.3 ng/L	NR	[417]
	Mississippi River	4 - 14 ug/L	NR	57.14 - 147.2 ng/L	nd - 27 ng/L	[418]
South America	Bogota, Colombia	0.28 ug/L	NR	76.82 ug/L	NR	[419]
	Brazilian waters	NR	NR	168.30 - 11725 ng/L	NR	[420,421]
	Mexico City, Mexico	NR	NR	2.282 ug/L	NR	[422]
Africa	Lake Victoria, Uganda	23,000 ng/L	NR	NR	NR	[423]
	Nigerian waters	0.18 - 480 ug/L	NR	79.40 ng/L - 52 ug/L	NR	[266,424,425]
	South African waters	1.68 - 1369 ug/L	NR	NR	NR	[426,427]

Chapter 2. Evaluating the effects of chemical digestion treatments on polystyrene microplastics

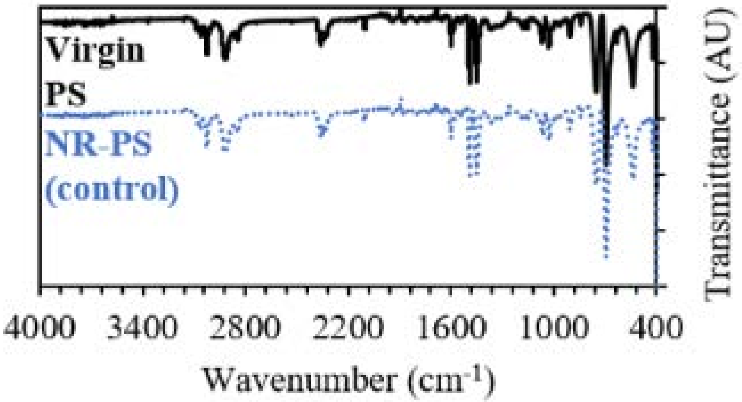


Figure S2.1. The infrared profiles of untreated, virgin polystyrene (virgin PS; solid black) and polystyrene incorporated with a Nile Red fluorescent dye (NR-PS control; blue dotted).

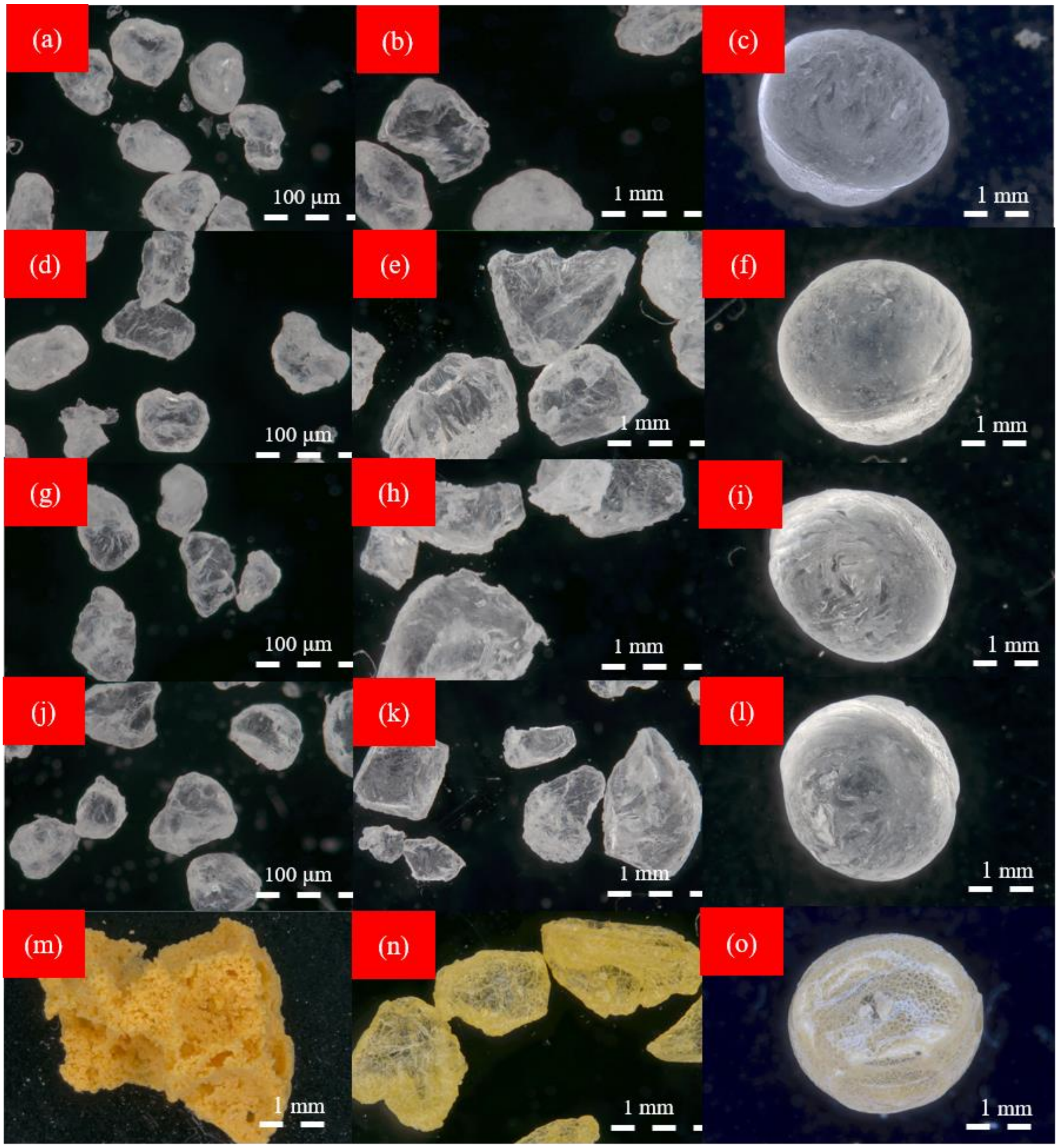


Figure S2.2. Microscope images of (a) small, (b) medium and (c) large control, untreated microplastics and those (d, g, j, m) small, (e, h, j, n) medium and (f, i, l, o) large treated at 90°C with 1.8 M potassium hydroxide, 10 M sodium hydroxide, 9.8 M hydrogen peroxide and at 60°C with 15.8 M nitric acid for 24 hours. (a, d, g, j) 8X, (b, e, h, k, n) 3.2X, (c, f, i, l, o) 1.6X and (m) 0.78X magnification.

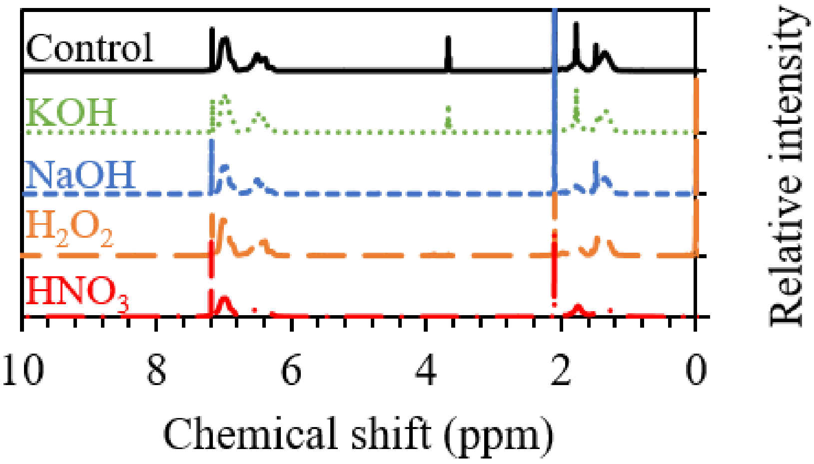


Figure S2.3. The proton nuclear magnetic resonance (NMR) spectra of control, untreated polystyrene (control; solid black), and polystyrene treated at 90°C for 48 hours with 1.8 M potassium hydroxide (KOH; dotted green), 10 M sodium hydroxide (NaOH; dashed blue), 9.8 M hydrogen peroxide (H_2O_2; dashed orange) and at 60°C for 12 hours with 15.8 M nitric acid (HNO_3; dashed red).

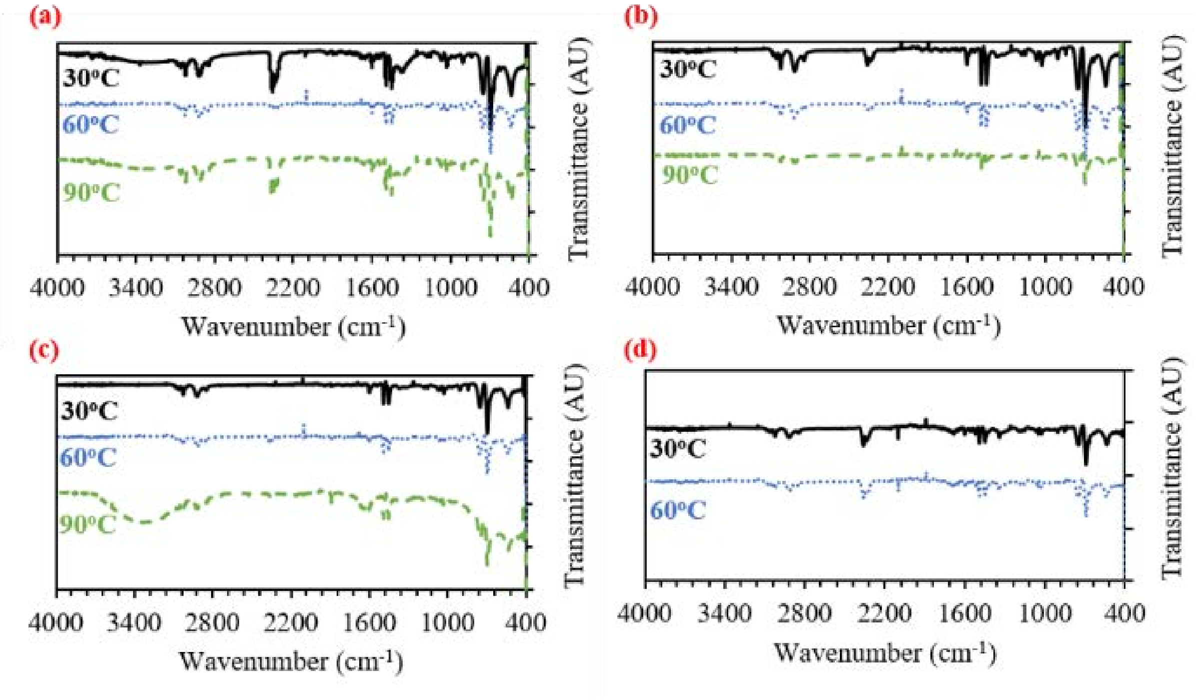


Figure S2.4. Infrared profiles of polystyrene treated at 30, 60 and 90°C for 48 hours with (a) 1.8 M potassium hydroxide solution (b) 10 M sodium hydroxide solution, (c) 9.8 M hydrogen peroxide and at 30 and 60°C for 12 hours with (d) 15.8 M nitric acid.

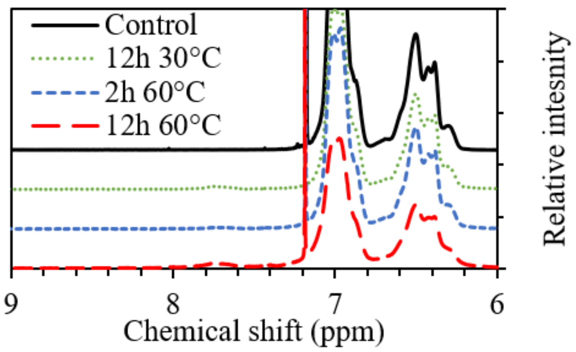


Figure S2.5. The aromatic region of the proton NMR spectra of untreated polystyrene (control) and polystyrene that was digested with 15.8 M nitric acid treatments.

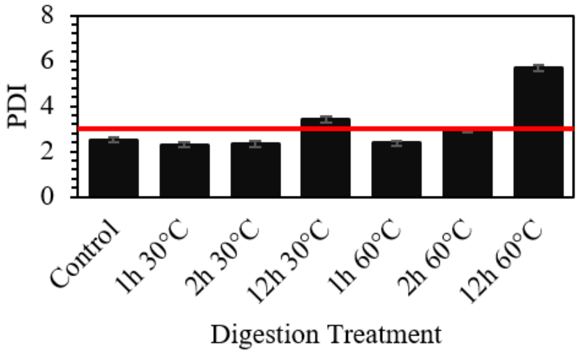


Figure S2.6. The polydispersity index (PDI) of untreated polystyrene (control) and polystyrene that was digested with 15.8 M nitric acid. Treated polystyrene with PDI values above the horizontal, red line are significantly different to the control.

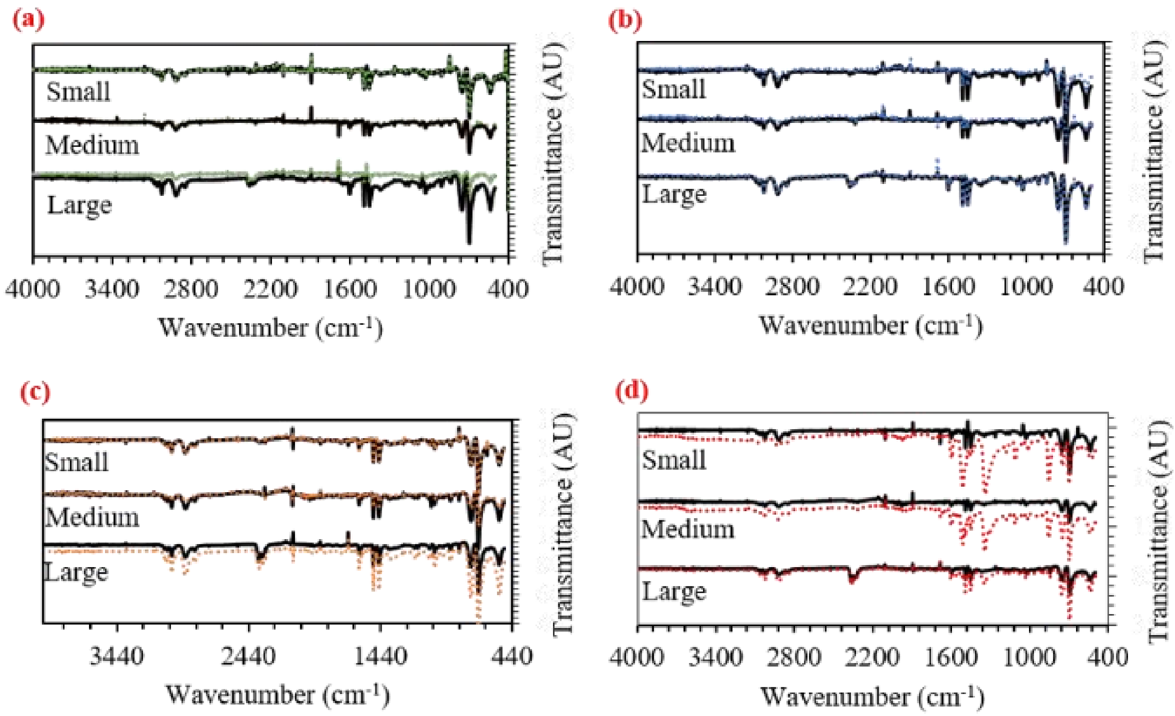


Figure S2.7: Infrared profiles of small (200 – 300 µm), medium (400 µm – 1 mm) and large (5 mm) polystyrene (PS) microplastics (MPs) treated for 24 hours with (a) 1.8 M potassium hydroxide solution at 60°C (solid black) and 90°C (dashed green), (b) 10 M sodium hydroxide solution at 60°C (solid black) and 90°C (dashed blue), (c) 9.8 M hydrogen peroxide at 60°C (solid black) and 90°C (dashed orange) and (d) 15.8 M nitric acid at 30°C (solid black) and 60°C (dashed red).

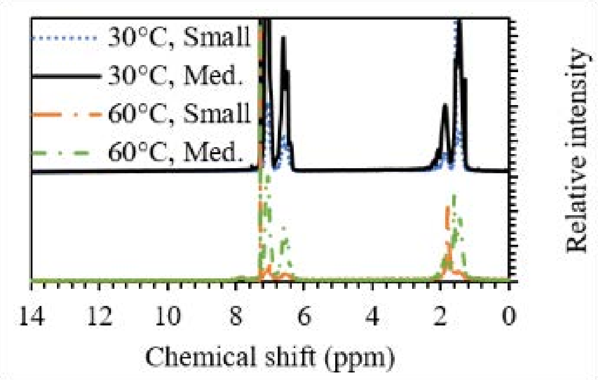


Figure S2.8. The proton nuclear magnetic resonance (NMR) spectra of small and medium polystyrene microplastics treated with 15.8 M nitric acid for 30 and 60°C for 24 hours.

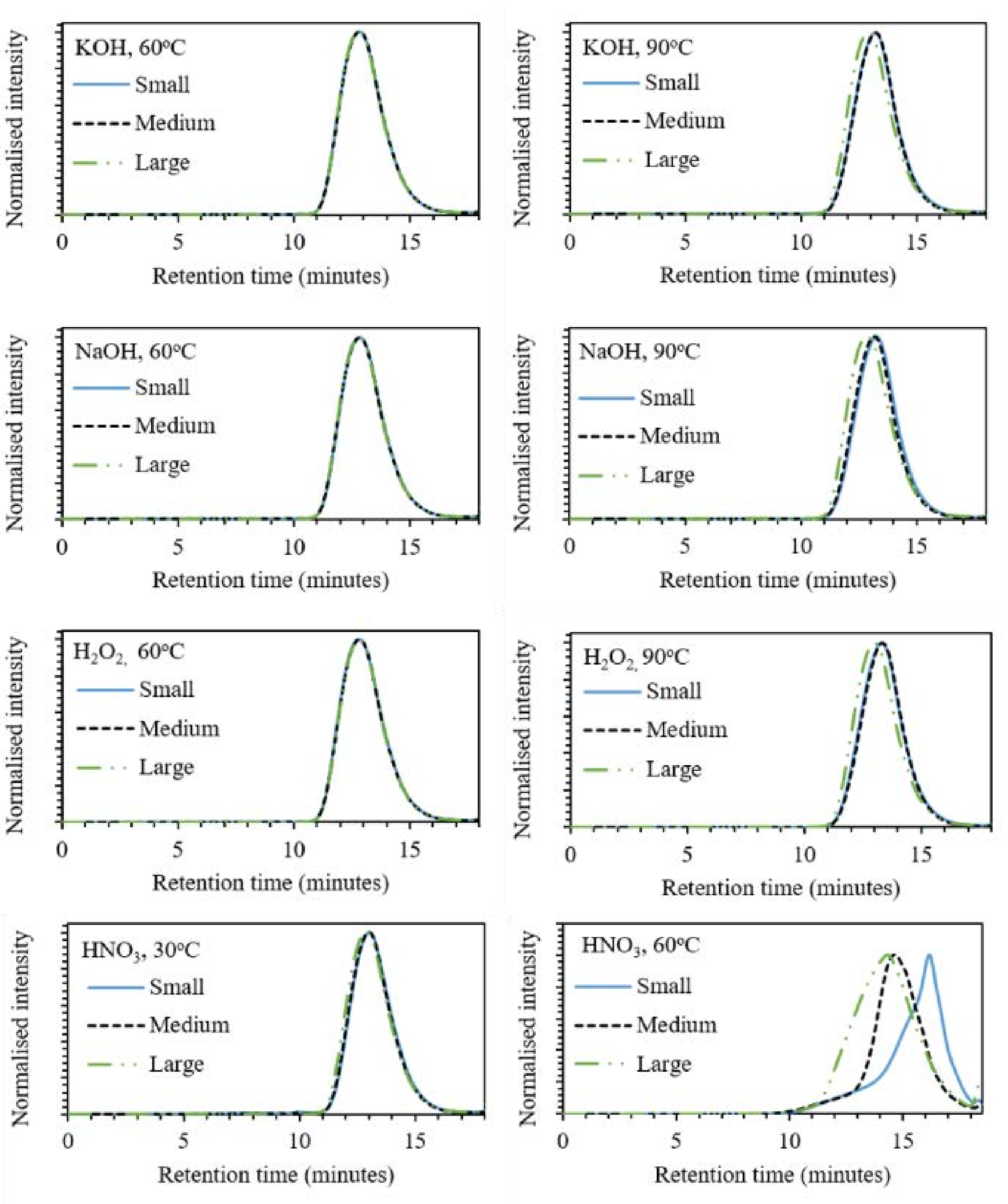


Figure S2.9: Gel permeation chromatography traces for small (200 – 300 µm), medium (400 µm – 1 mm) and large (5 mm) polystyrene (PS) microplastics (MPs) treated for 24 hours with 1.8 M potassium hydroxide (KOH) solution, 10 M sodium hydroxide (NaOH) solution, 9.8 M hydrogen peroxide (H_2O_2), and with 15.8 M nitric acid (HNO_3).

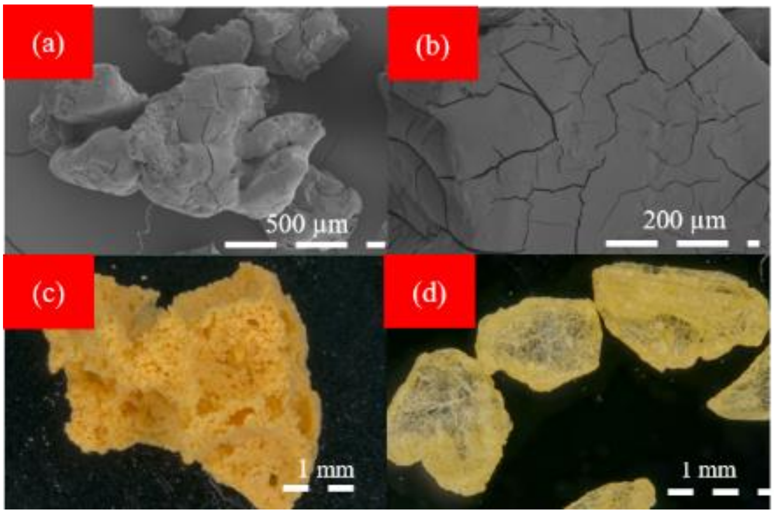


Figure S2.10: Microscope images of (a, c) small (200 – 300 µm) and (b, d) medium (400 µm – 1 mm) microplastics treated with 15.8 M nitric acid for 24 hours at 60°C at (a) 100X, (b) 250X, (c) 0.78X and (d) 3.2X magnification.

Table S2.1. The infrared spectral similarities of virgin polystyrene (PS), control Nile red (NR) stained PS (NR-PS) and PS which were treated under the following conditions: 90°C for 48 hours with 1.8 м potassium hydroxide (KOH) solution, 10 м sodium hydroxide (NaOH) solution, 9.8 м hydrogen peroxide (H_2O_2), and at 60°C for 12 hours with 15.8 м nitric acid (HNO_3).

Reagent Type	**Treatment condition**	**Average spectral similarity (%) to control PS**	**Search Score against NICDOM Ltd., spectral library (%)**[a]
Virgin PS	-	88	95
Control PS (NR-PS)	-	1	90
KOH	90°C for 48 hours	94	95
NaOH	90°C for 48 hours	86	97
H_2O_2	90°C for 48 hours	86	91
HNO_3	60°C for 12 hours	75	75

[a)] Searched using the PerkinElmer SEARCH software against NICDOCOM IR spectral library (Polymers and Additives; NICODOM Ltd., Czech Republic).

Chapter 3. Understanding plasticiser leaching from polystyrene microplastics

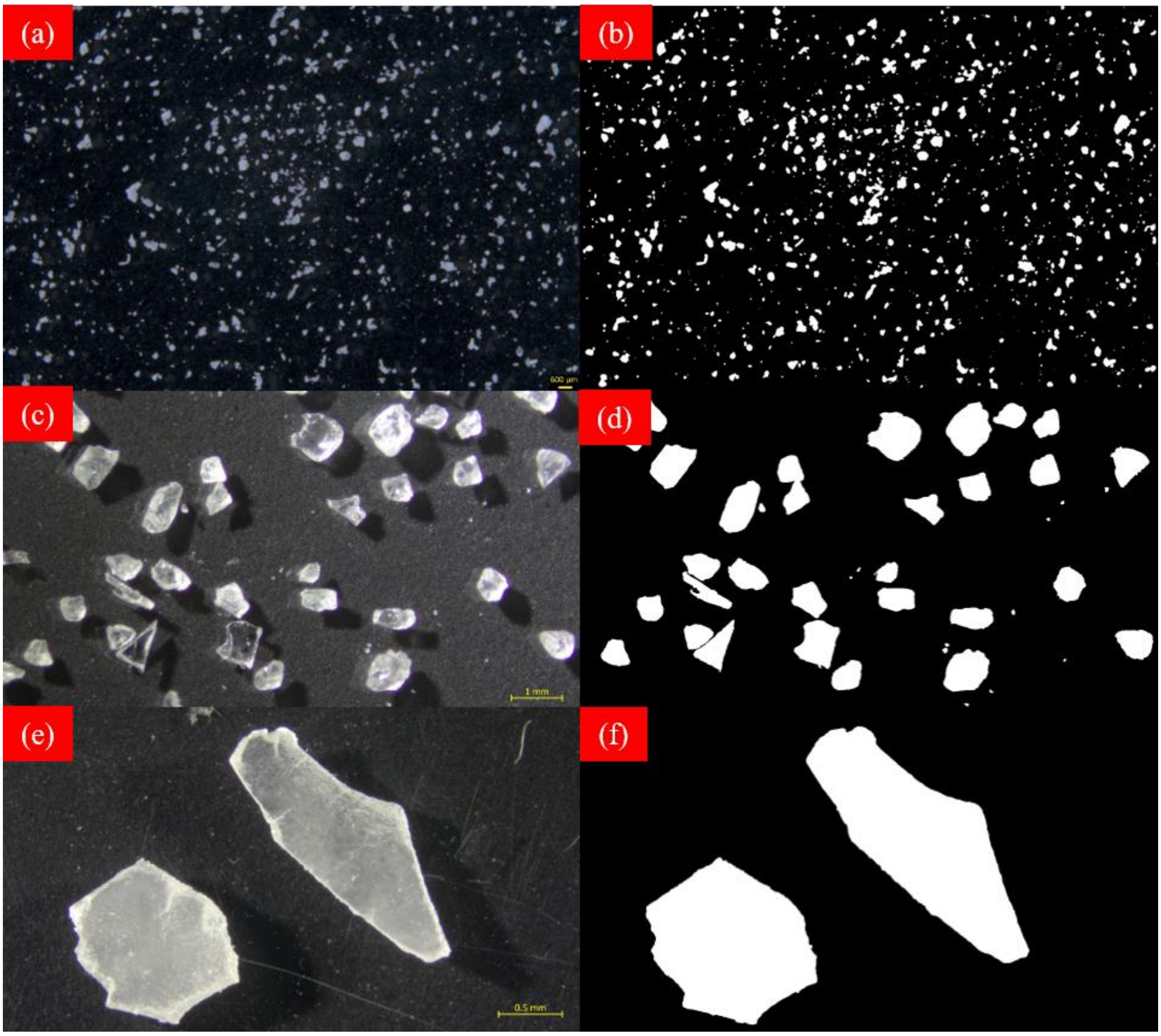


Figure S3.1. Representative microscope images of prepared (a) small, (c) medium and (e) large microplastics and the resulting binary image masks (b), (d) and (f), respectively, used from size distribution analysis in Matlab.

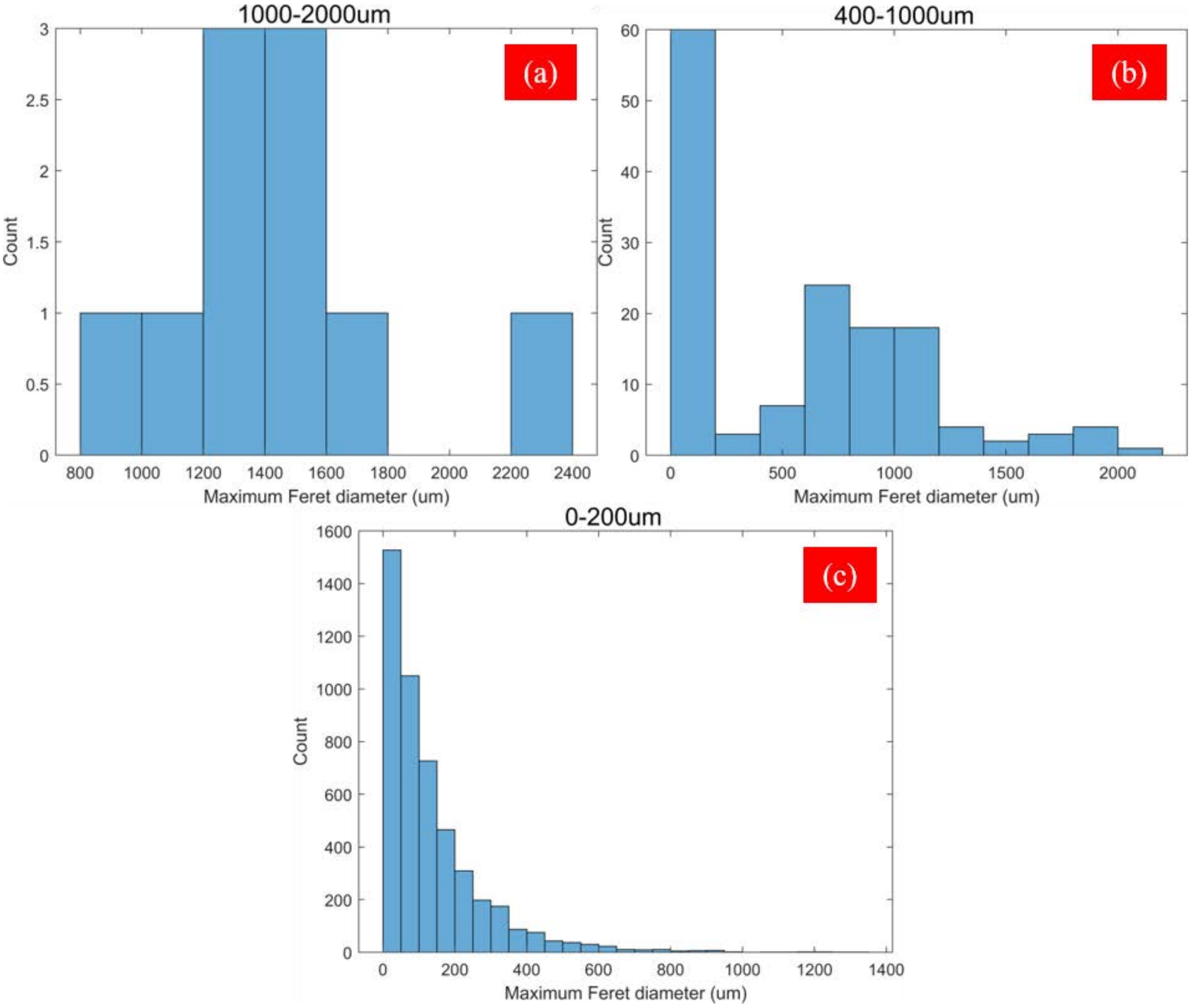


Figure S3.2. The histograms of size distribution for (a) small, (b) medium and (c) large prepared and plasticised microplastic fragments.

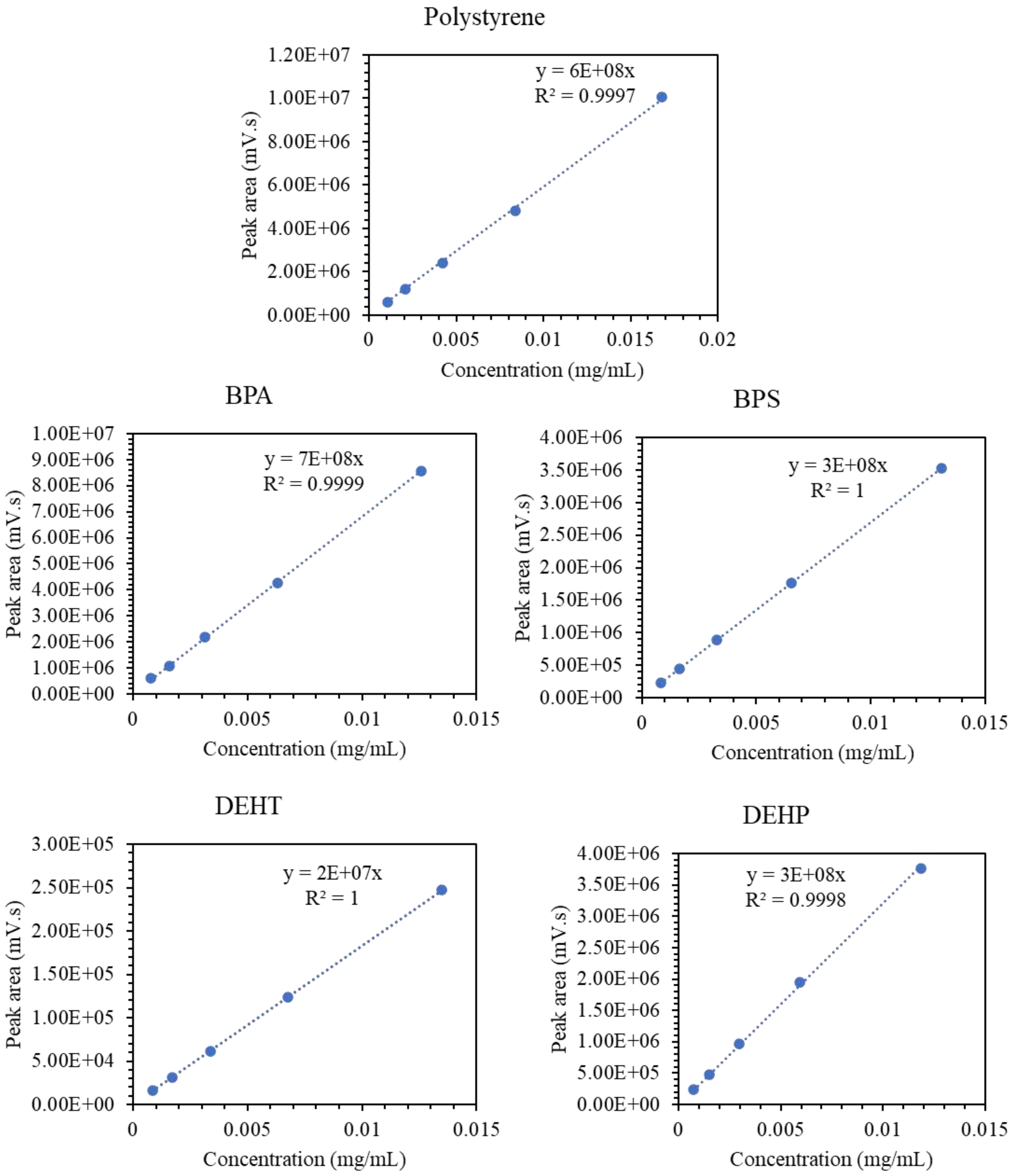


Figure S3.3. The gel permeation chromatography calibration curves for polystyrene, bisphenol A (BPA), bisphenol S (BPS), dioctyl terephthalate (DEHT) and diethylhexyl phthalate (DEHP) used for concentration determination.

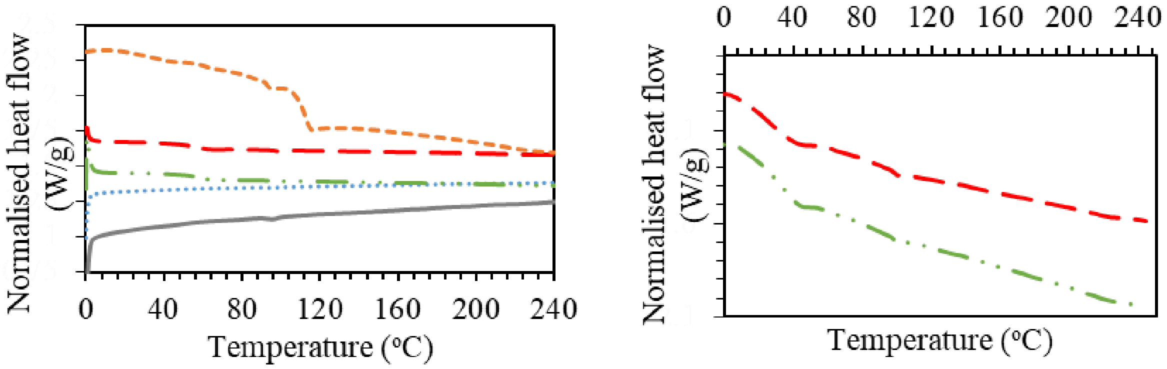


Figure S3.4. The thermal profiles of polystyrene (PS; orange dashed) containing 15% by weight (wt. %) (left) and 25 wt. % (right) dioctyl terephthalate (DEHT; red dashed), diethylhexyl phthalate (DEHP; green dashed), bisphenol A (BPA; blue dotted) and bisphenol S (BPS; solid grey).

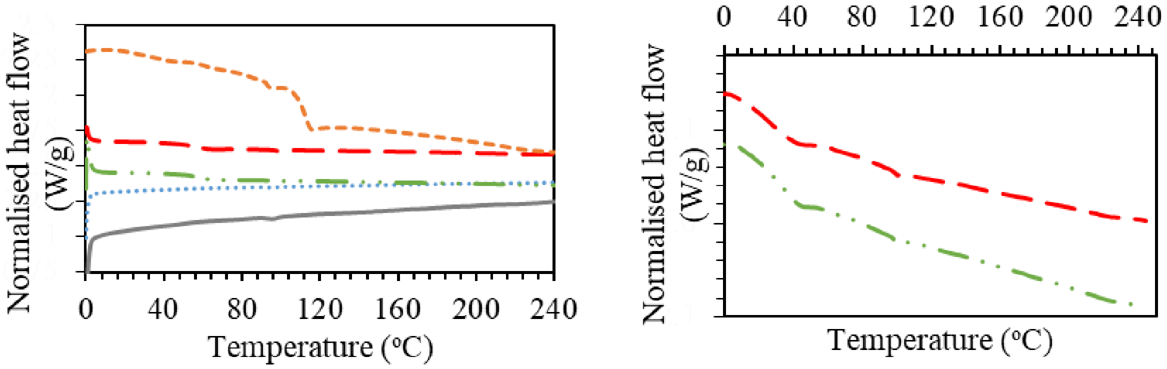


Figure S3.5. The thermal profiles of polystyrene (PS; orange dashed) containing 15% by weight (wt. %) (left) and 25 wt.% (right) dioctyl terephthalate (DEHT; red dashed), diethylhexyl phthalate (DEHP; green dashed), bisphenol A (BPA; blue dotted) and bisphenol S (BPS; solid grey).

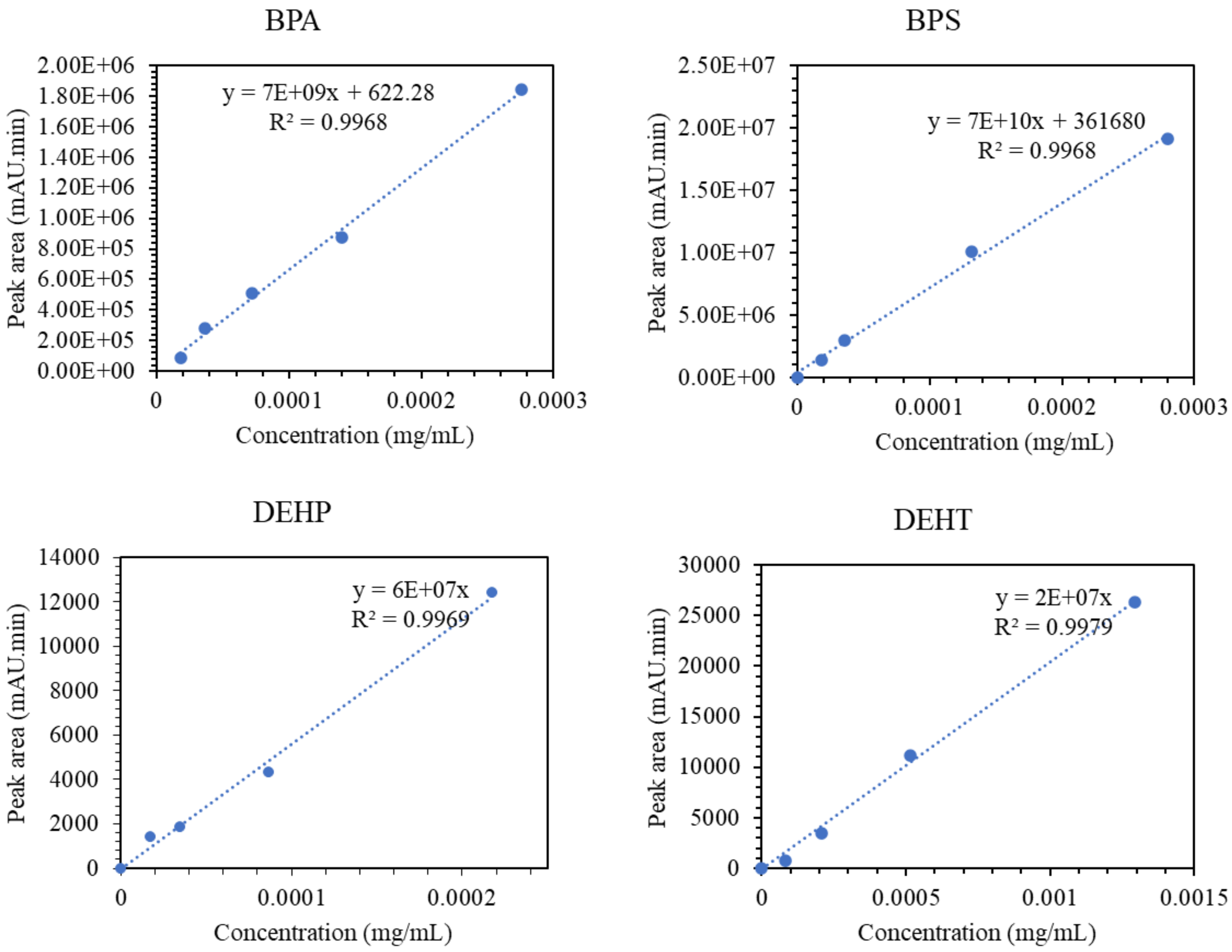


Figure S3.6. The high performance liquid chromatography calibration curves for bisphenol A (BPA), bisphenol S (BPS), diethylhexyl phthalate (DEHP) and dioctyl terephthalate (DEHT) used for concentration determination.

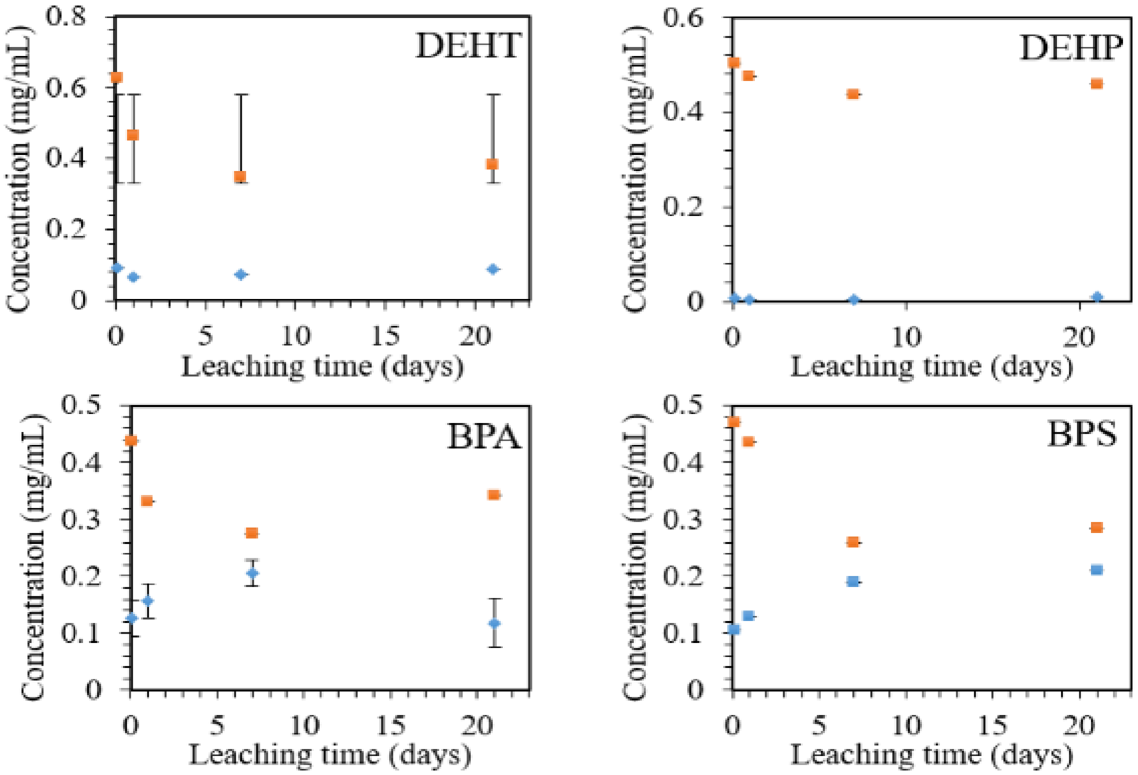


Figure S3.7. A comparison of the concentration of dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPA) and bisphenol S (BPS) associated with the plastic polymer (determined using Gel Permeation Chromatography (wt. %); orange) and in detected in the surrounding leachate (determined using High-Performance Liquid Chromatography (mg/mL); blue) from small (< 200 µm) microplastics exposed to static, ambient seawater conditions (25.5°C, pH 8.1) for 21 days. Data is presented as the mean ± standard deviation.

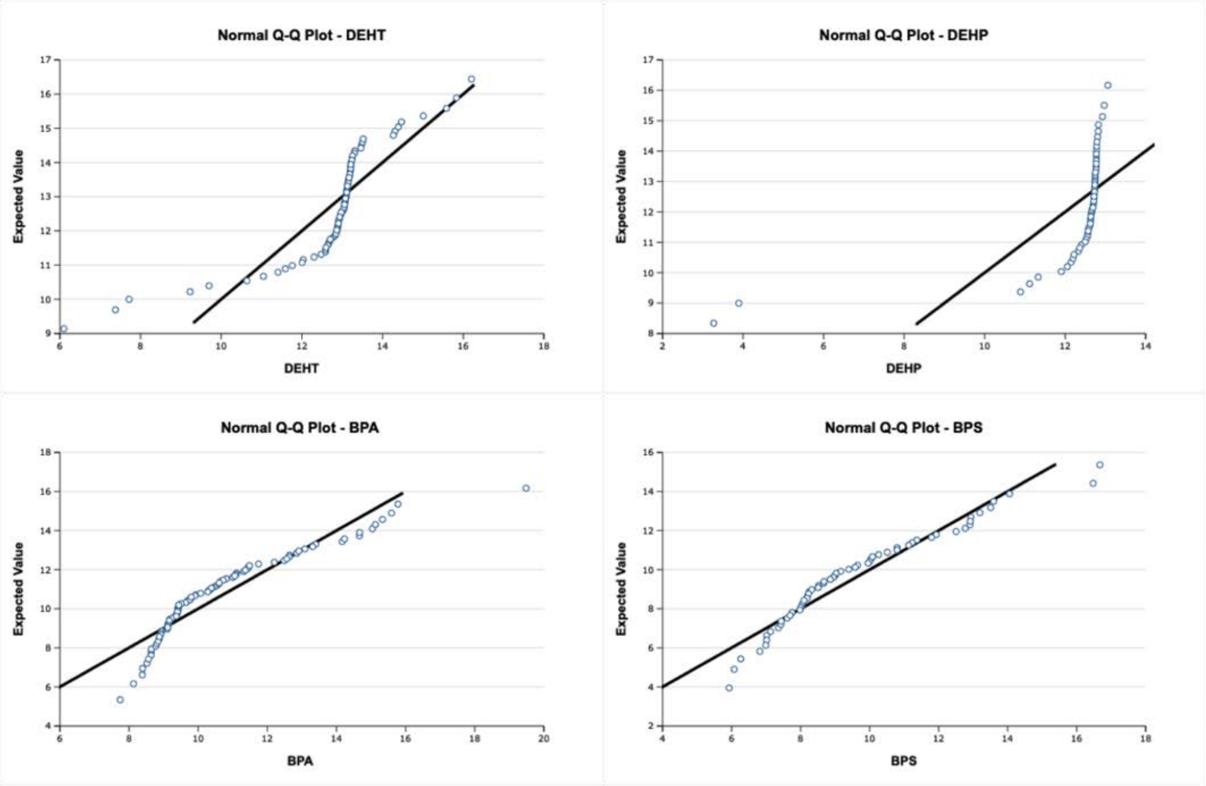


Figure S3.8. Leaching dataset distribution (Q-Q plots) of dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPA) and bisphenol S (BPS).

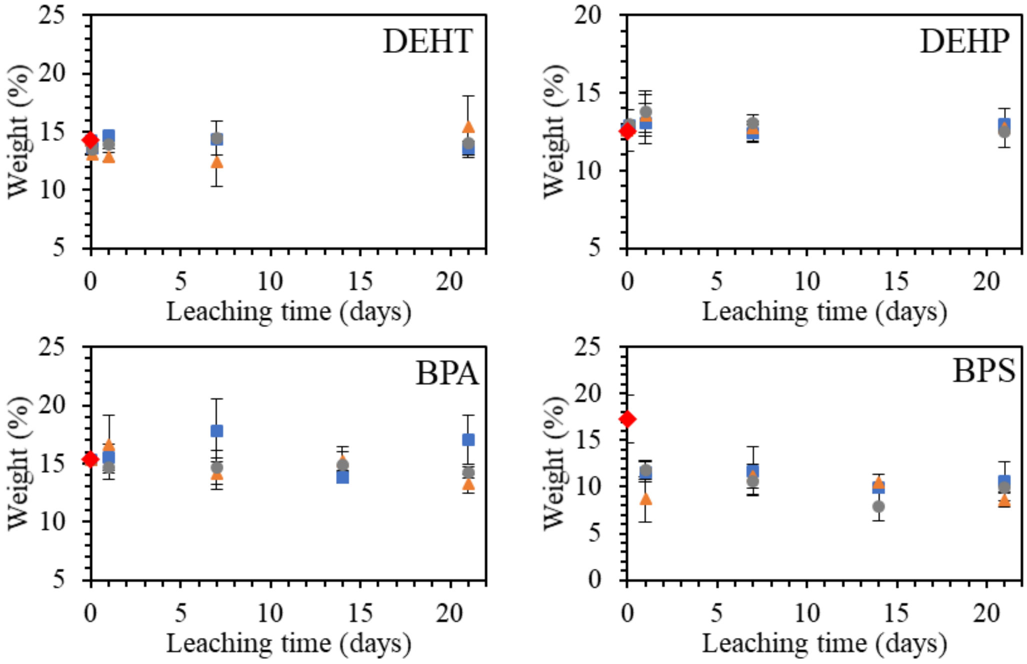


Figure S3.9. The leaching of dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPA) and bisphenol S (BPS) from large (1 – 2 mm) microplastics at 5 ºC (blue), 25.5 ºC (orange) and 60 °C (grey) in seawater (pH 8.1) over 21 days. Starting weight is shown in red. Data is presented as the mean ± standard deviation.

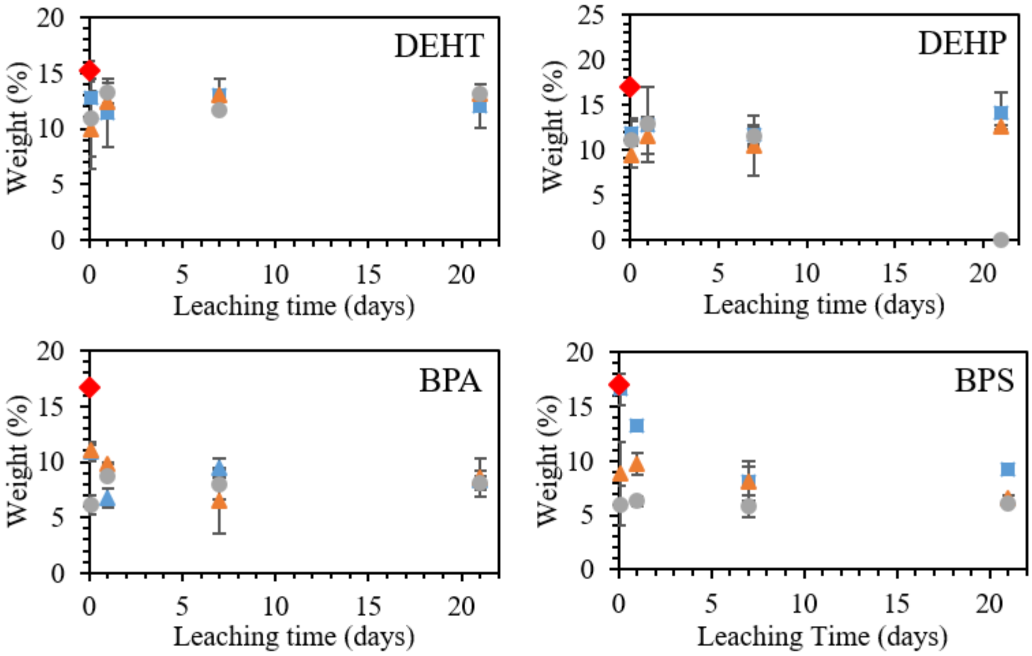


Figure S3.10. The leaching of dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPA) and bisphenol S (BPS) from small (< 200 µm) microplastics at 5 °C (blue), 25.5 °C (orange) and 60 °C (grey) in freshwater (pH 6.4) over 21 days. Starting weight is shown in red. Data is presented as the mean ± standard deviation.

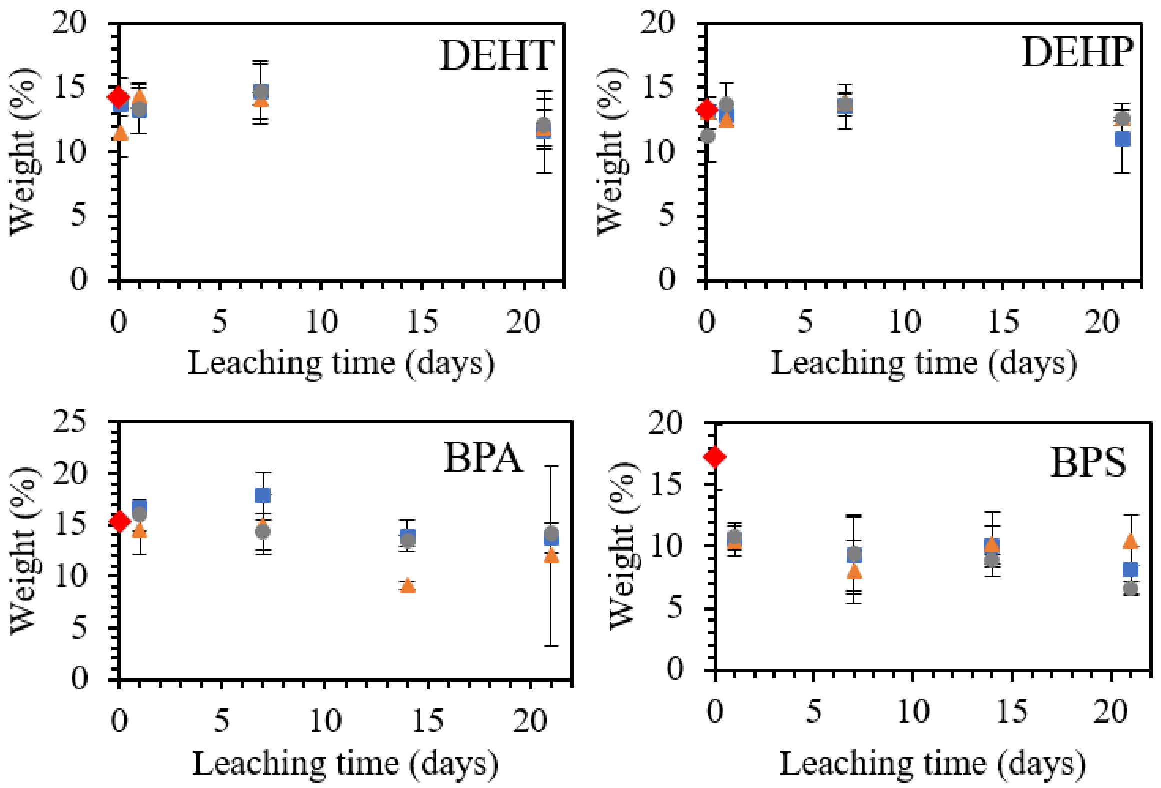


Figure S3.11. The leaching of dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPA) and bisphenol S (BPS) from large (1 – 2 mm) microplastics at 5 ºC (blue), 25.5 ºC (orange) and 60 ºC (grey) in freshwater (pH 6.4) over 21 days. Starting weight is shown in red. Data is presented as the mean ± standard deviation.

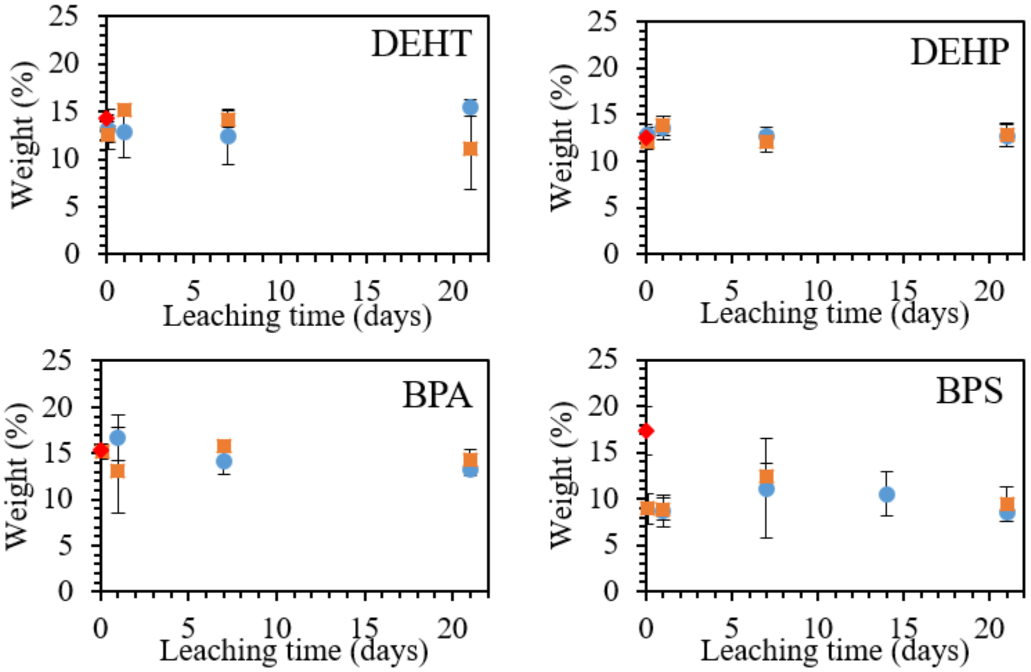


Figure S3.12. A comparison of the leaching profiles of dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPA) and bisphenol S (BPS) from large (1 – 2 mm) microplastics at

25.5 °C in static (blue) and agitated (90 rpm; orange) seawater (pH 8.1) over 21 days. Starting weight is shown in red. Data is presented as mean ± standard deviation.

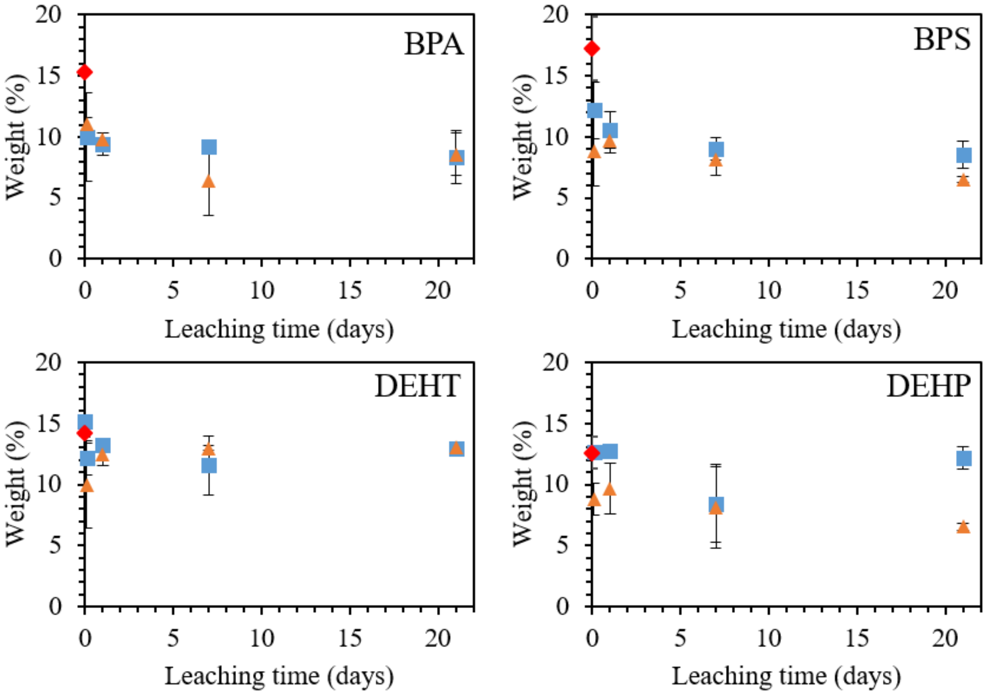


Figure S3.13. A comparison of the leaching profiles of bisphenol A (BPA), bisphenol S (BPS), dioctyl terephthalate (DEHT) and diethylhexyl phthalate (DEHT) from small microplastics (136 µm) at 25.5°C in static, ambient marine (pH 8.1; blue) and freshwater (pH 6.4; orange) over 21 days. Starting weight is indicated in red.

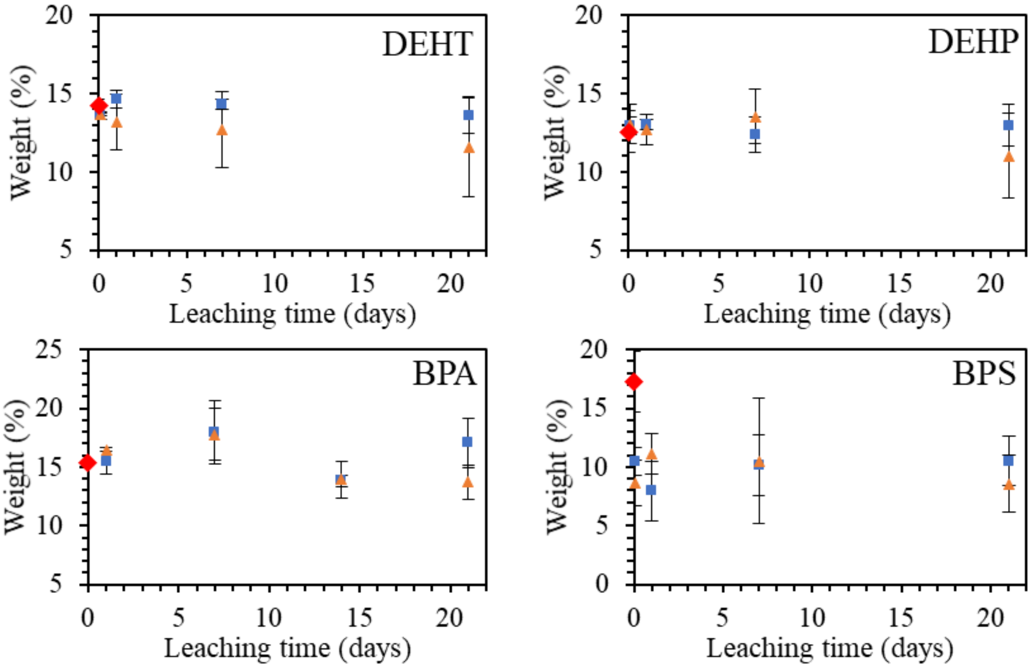


Figure S3.14. A comparison of the leaching profiles dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPA) and bisphenol S (BPS) from large (1 – 2 mm) microplastics at 25.5 °C in marine (pH 8.1; blue) and freshwater (pH 6.4; orange) over 21 days. Starting weight is shown in red. Data is presented as the mean ± standard deviation.

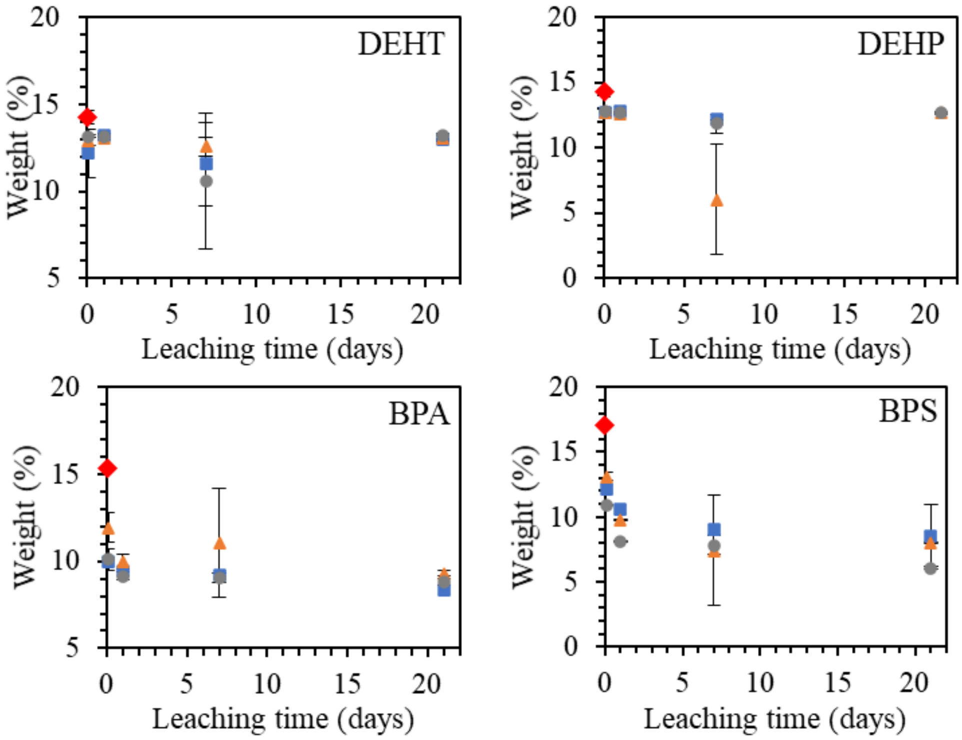


Figure S3.15. The leaching profiles of for dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPA) and bisphenol S (BPS) from small (< 200 µm) microplastics at 25.5 °C in seawater (blue) with adjusted pH of 3 (acidic; orange) and 10 (alkaline; grey) over 21 days. Starting weight is indicated in red. Data is presented as the mean ± standard deviation.

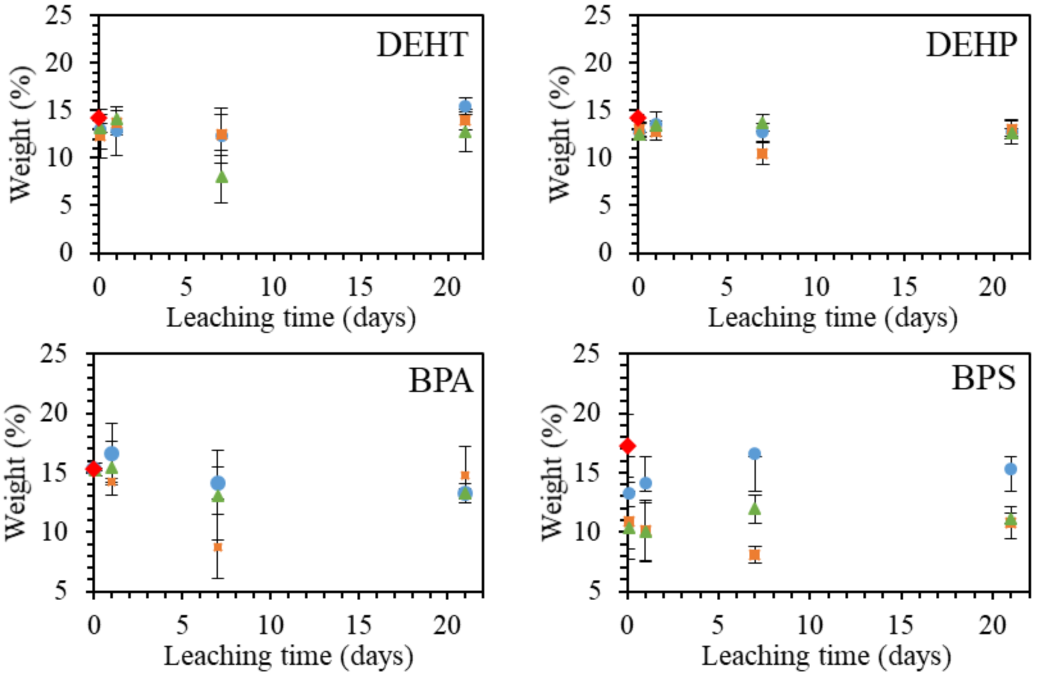


Figure S3.16. The leaching profiles of dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPA) and bisphenol S (BPS) from large (1 – 2 mm) microplastics at 25.5 °C in seawater (blue) with adjusted pH of 3 (acidic; orange) and 10 (alkaline; green) over 21 days. Starting weight is shown in red. Data is presented as the mean ± standard deviation.

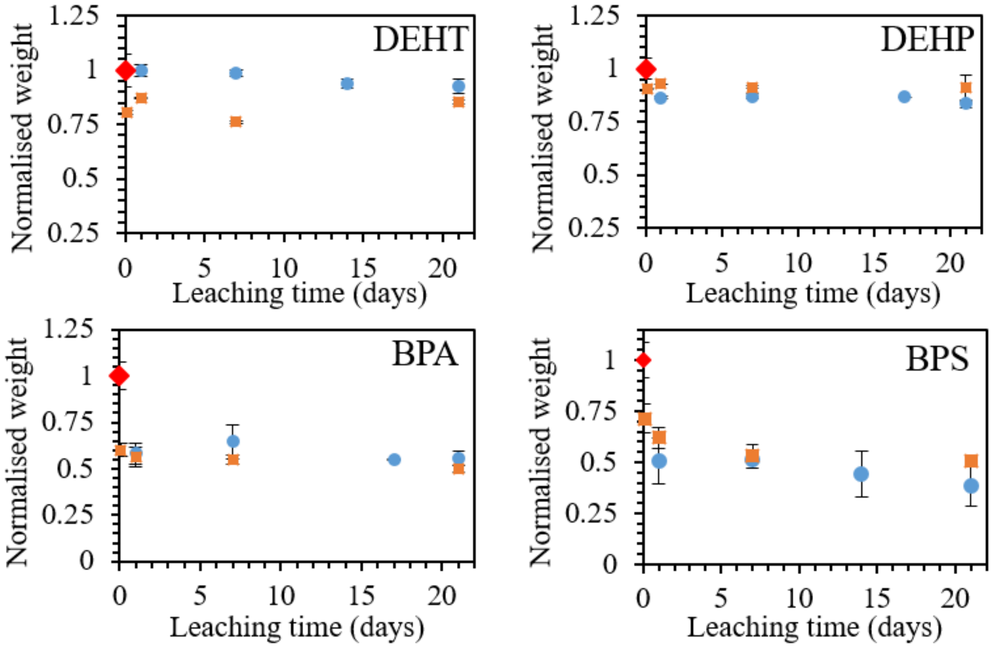


Figure S3.167. A comparison of the leaching profiles of small (< 200 µm) microplastics in ambient seawater conditions (25.5 °C, pH 8.1) over 21 days containing 15 wt. % (orange) and 25 wt. % (blue) dioctyl terephthalate (DEHT), diethylhexyl phthalate (DEHP), bisphenol A (BPA) and bisphenol S (BPS). Data is presented as the normalised weight (wt. %).

Table S3.1. The size distribution dynamics.

Size range	**Measurement**	**Mean (µm)**	**Standard deviation (µm)**	**Median (µm)**
Small (sieve range < 200 µm)	Maximum Feret diameter	136.2	144.8	91.0
	Minimum Feret diameter	88.0	87.9	62.2
	Major axis length	132.5	143.7	87.8
	Equivalent diameter of circle having the same area	100.9	99.0	71.6
Medium (sieve range 400 µm – 1 mm)	Maximum Feret diameter	593.2	513.7	622.4
	Minimum Feret diameter	394.5	336.2	459.8
	Major axis length	571.4	498.5	592.0
	Equivalent diameter of circle having the same area	448.2	372.5	516.0
Large (sieve range 1 – 2 mm)	Maximum Feret diameter	1447.1	381.0	1403.6
	Minimum Feret diameter	853.2	262.0	818.7
	Major axis length	1414.0	383.4	1317.5
	Equivalent diameter of circle having the same area	1026.3	205.3	965.4

Table S3.2. The method used for quantifying bisphenol S (BPS), bisphenol A (BPA), diethylhexyl phthalate (DEHP) and dioctyl terephthalate (DEHT) in the leachate solutions using High-Performance Liquid Chromatography.

	BPA	**BPS**	**DEHP and DEHT** [a]
Flow rate (mL/min)	1	0.7	2

Injection volume (µL)	10	100	
Mobile phase	0.1% formic acid: water and methanol	Water: acetonitrile	
Elution mode	Isocratic elution with 60% methanol	Gradient elution	
		Time (minutes)	Acetonitrile (%)
		0	65
		3.25	65
		3.75	83
		5.75	83
		6.25	95
		9	95
		11	65

[a] Due to the estimated concentration of DEHT and DEHP in the leachate solutions being below the limit of detection[230], samples were placed in a Savant SpeecVac™ (ThermoFisher) to evaporate excess water. Remaining plasticiser was then dissolved in 1.5 mL acetonitrile prior to analysis.

Table S3.3. Statistical output indicating differences in leaching behaviours among small, medium and large polystyrene microplastics incorporated with diehylhexyl phthalate (DEHP), dioctyl terephthalate (DEHT), bisphenol A (BPA) and bisphenol S (BPS) exposed to variations in solution salinity, pH, temperature and agitation using a p-value threshold of 0.05.

Sample comparison description; additive type (microplastic size, temperature (˚C), pH)	**p-value**	**Statistically different?**
Temperature Studies		
BPA (small, 5, 8.1) v BPA (small, 25, 8.1)	0.01	Yes
BPA (small, 5, 8.1) v BPA (small, 60, 8.1)	0.08	No
BPA (small, 25.5, 8.1) v BPA (small, 60, 8.1)	0.00004	Yes
BPS (small, 5, 8.1) v BPA (small, 25.5, 8.1)	0.11	No
BPS (small, 5, 8.1) v BPA (small, 60, 8.1)	0.01	Yes
BPS (small, 25.5, 8.1) v BPS (small 60, 8.1)	0.0006	Yes
DEHT (small, 5, 8.1) v DEHT (small, 25.5, 8.1) v DEHT (small, 60, 8.1)	0.75	No
DEHP (small, 5, 8.1) v DEHP (small, 25.5, 8.1) v DEHP (small, 60, 8.1)	0.91	No

Particle Size Studies		
BPA (small, 25.5, 8.1) v BPA (medium, 25.5, 8.1)	0.11	No
BPA (small, 25.5, 8.1) v BPA (large, 25.5, 8.1)	0.00003	Yes
BPA (medium, 25.5, 8.1) v BPA (large, 25.5, 8.1)	0.00003	Yes
DEHT (small, 25.5, 8.1) v DEHT (medium, 25.5, 8.1) v DEHT (large, 25.5, 8.1)	0.14	No
pH Studies		
BPA (small, 25.5, 8.1) v BPA (small, 25.5, 10)	0.57	No
BPA (small, 25.5, 8.1) v BPA (small, 25.5, 3)	0.24	No
BPA (small, 25.5, 3) v BPA (small, 25.5, 10)	0.031	Yes
BPS (small, 25.5, 8.1) v BPS (small, 25.5, 10)	0.001	Yes
BPS (small, 25.5, 8.1) v BPS (small, 25.5, 3)	0.043	Yes
BPS (small, 25.5, 3) v BPS (small, 25.5, 10)	0.36	No
DEHT (small, 25.5, 8.1) v DEHT (small, 25.5, 10) v DEHT (small, 25.5, 3)	0.28	No
DEHP (small, 25.5, 8.1) v DEHP (small, 25.5, 10) v DEHP (small, 25.5, 3)	0.18	No

Table 3.4. A comparison between the average bisphenol A (BPA), bisphenol S (BPS), dioctyl terephthalate (DEHT) and diethylhexyl phthalate (DEHT) concentrations remaining in the small microplastics (136 µm) exposed to seawater with adjusted pH of 1, 3, 10 and 11 after 24 hours at 5 and 25.5°C. Data is presented as the mean (*n* = 3 per plasticiser type and condition) rounded to the nearest whole number.

	25.5°C					**5°C**		
	pH 8.1 (ambient)	pH 1	pH 3	pH 10	pH 11	pH 8.1 (ambient)	pH 3	pH 10
DEHT	13	12	13	13	11	13	13	13

DEHP	13	12	13	13	13	13	13	13
BPA	9	9	10	9	8	12	12	7
BPS	11	8	10	8	8	13	13	9

Chapter 4. Impact of polymer molecular weight on plasticiser leaching from polystyrene microplastics

Figure S4.1. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of low molecular weight (35k g/mol) polystyrene-bisphenol A (PS-BPA) microplastics.

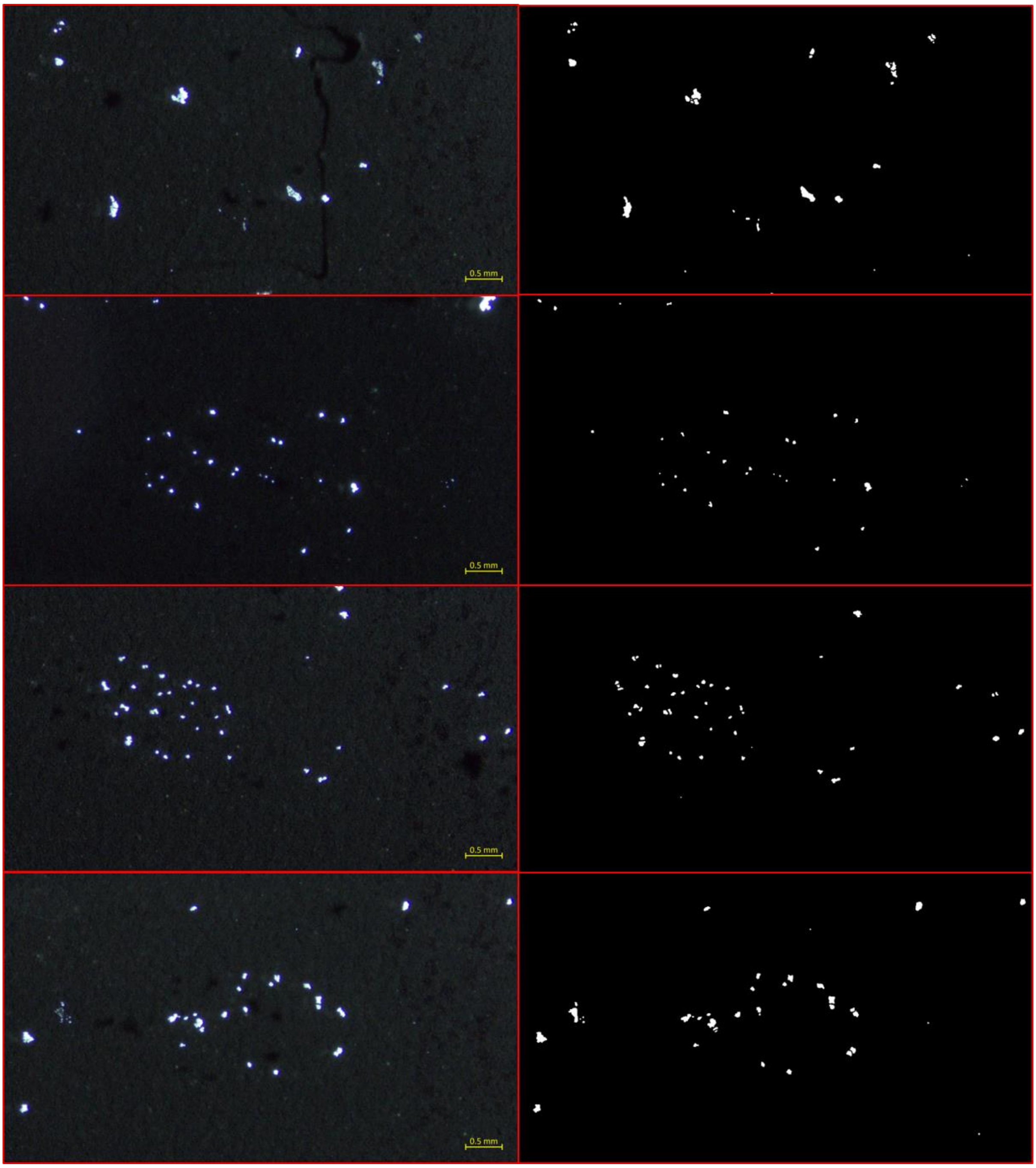


Figure S4.2. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of low molecular weight (35k g/mol) polystyrene-bisphenol S (PS-BPS) microplastics.

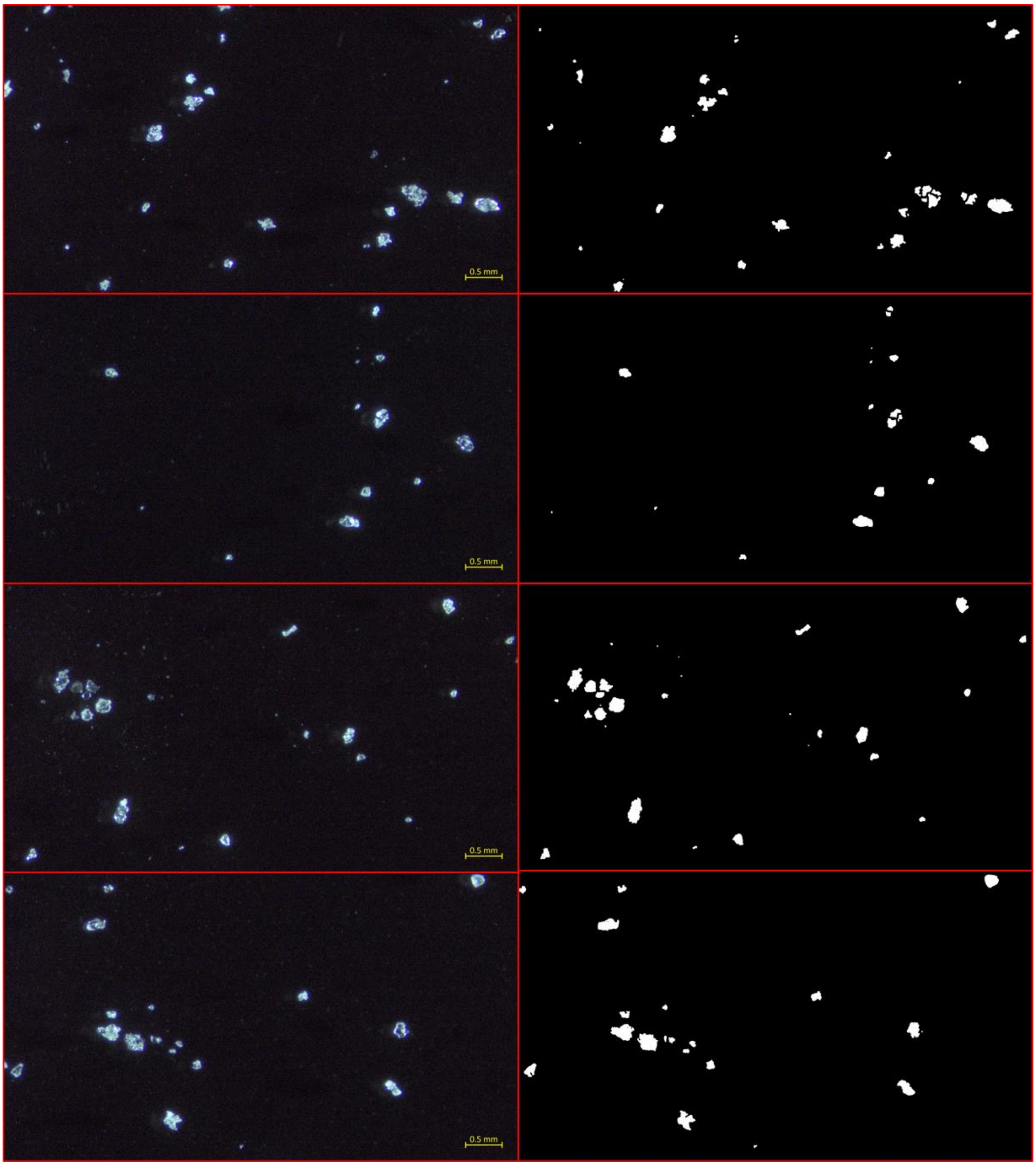


Figure S4.3. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of low molecular weight (35k g/mol) polystyrene-diethylhexyl phthalate (PS-DEHP) microplastics.

Figure S4.4. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of low molecular weight (35k g/mol) polystyrene-dioctyl terephthalate (PS-DEHT) microplastics.

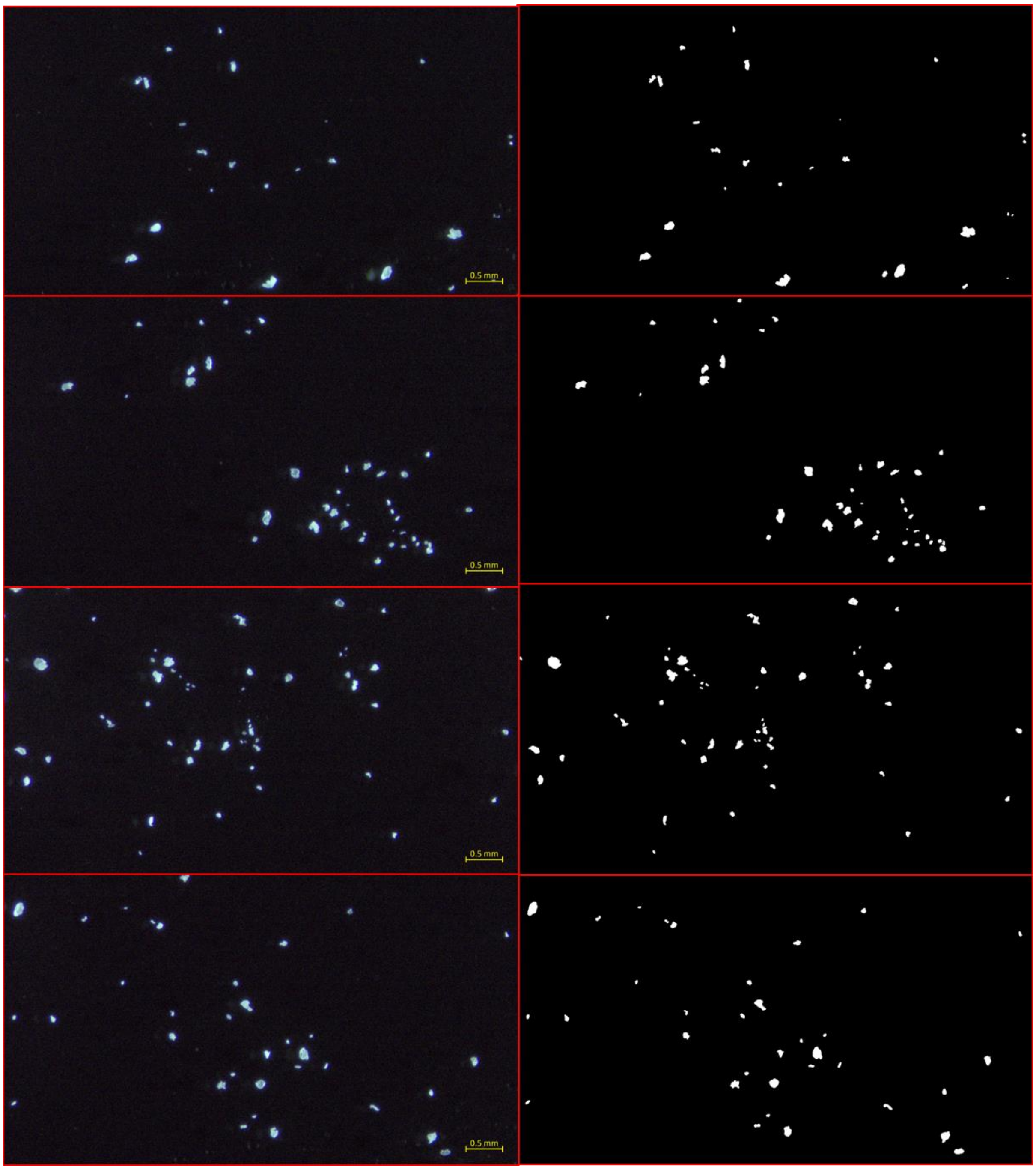


Figure S4.5. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of medium molecular weight (192k g/mol) polystyrene-bisphenol A (PS-BPA) microplastics.

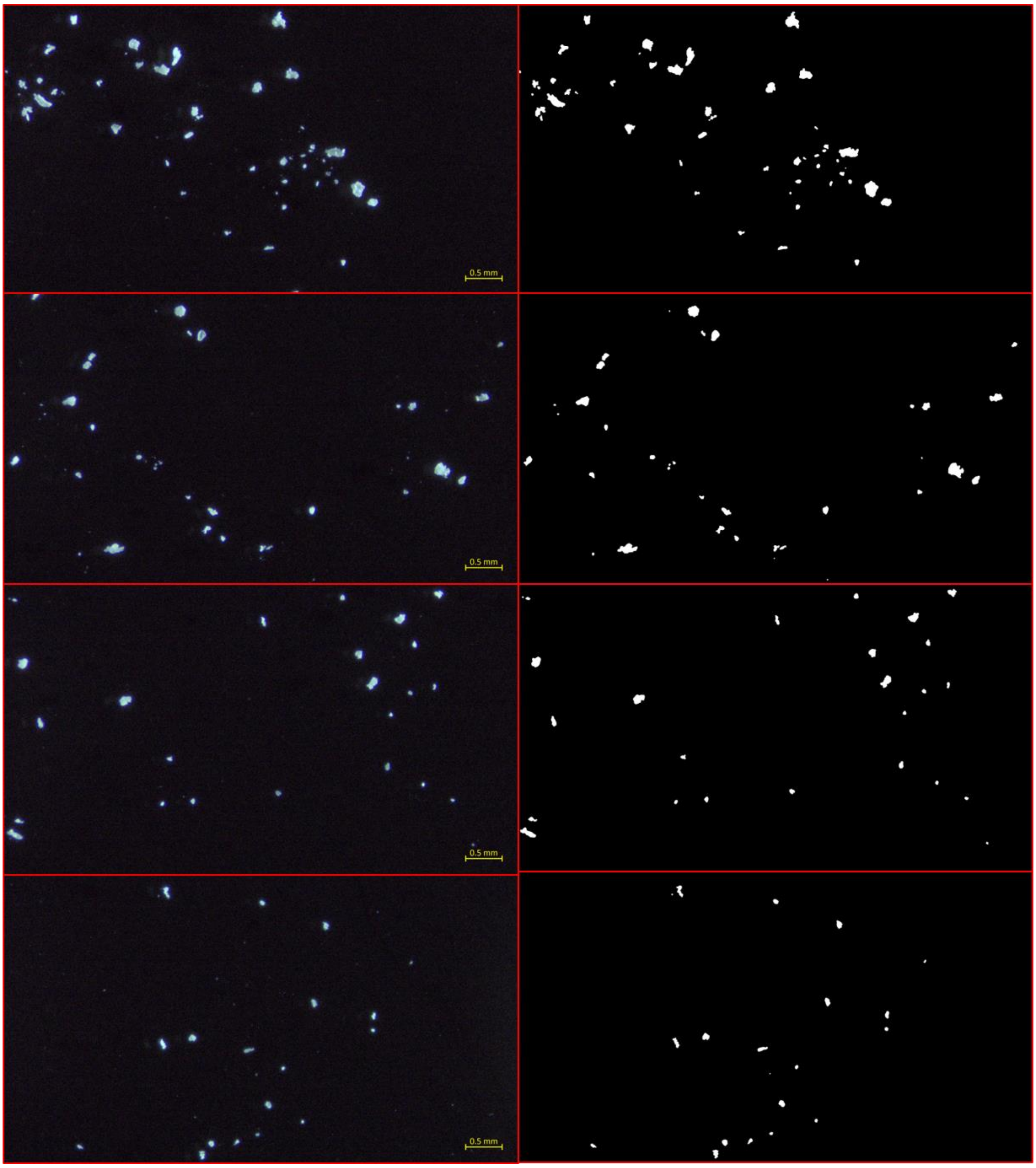


Figure S4.6. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of medium molecular weight (192k g/mol) polystyrene-bisphenol S (PS-BPS) microplastics.

Figure S4.7. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of medium molecular weight (192k g/mol) polystyrene-diethylhexyl phthalate (PS-DEHP) microplastics.

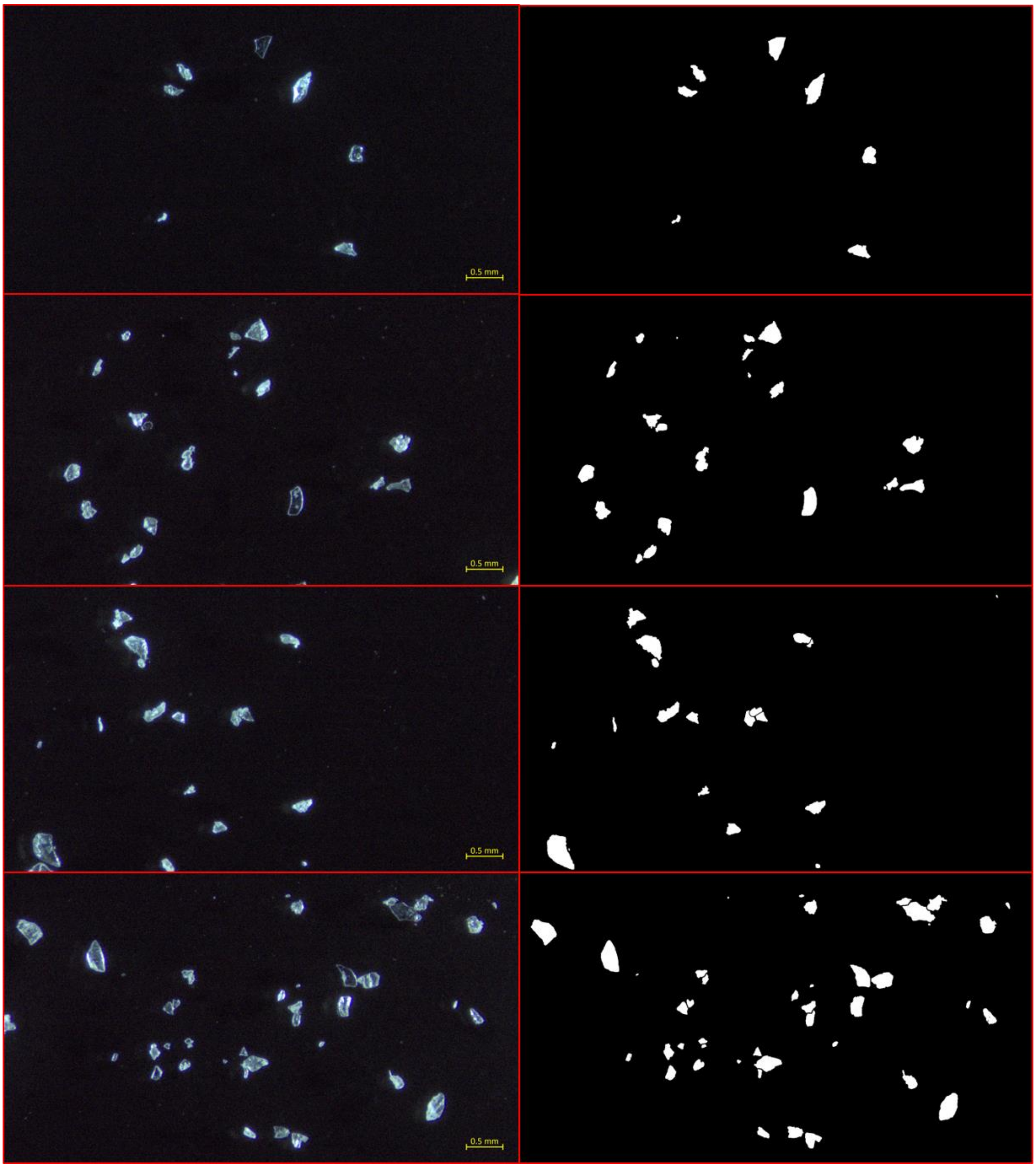


Figure S4.8. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of medium molecular weight (192k g/mol) polystyrene-dioctyl terephthalate (PS-DEHT) microplastics.

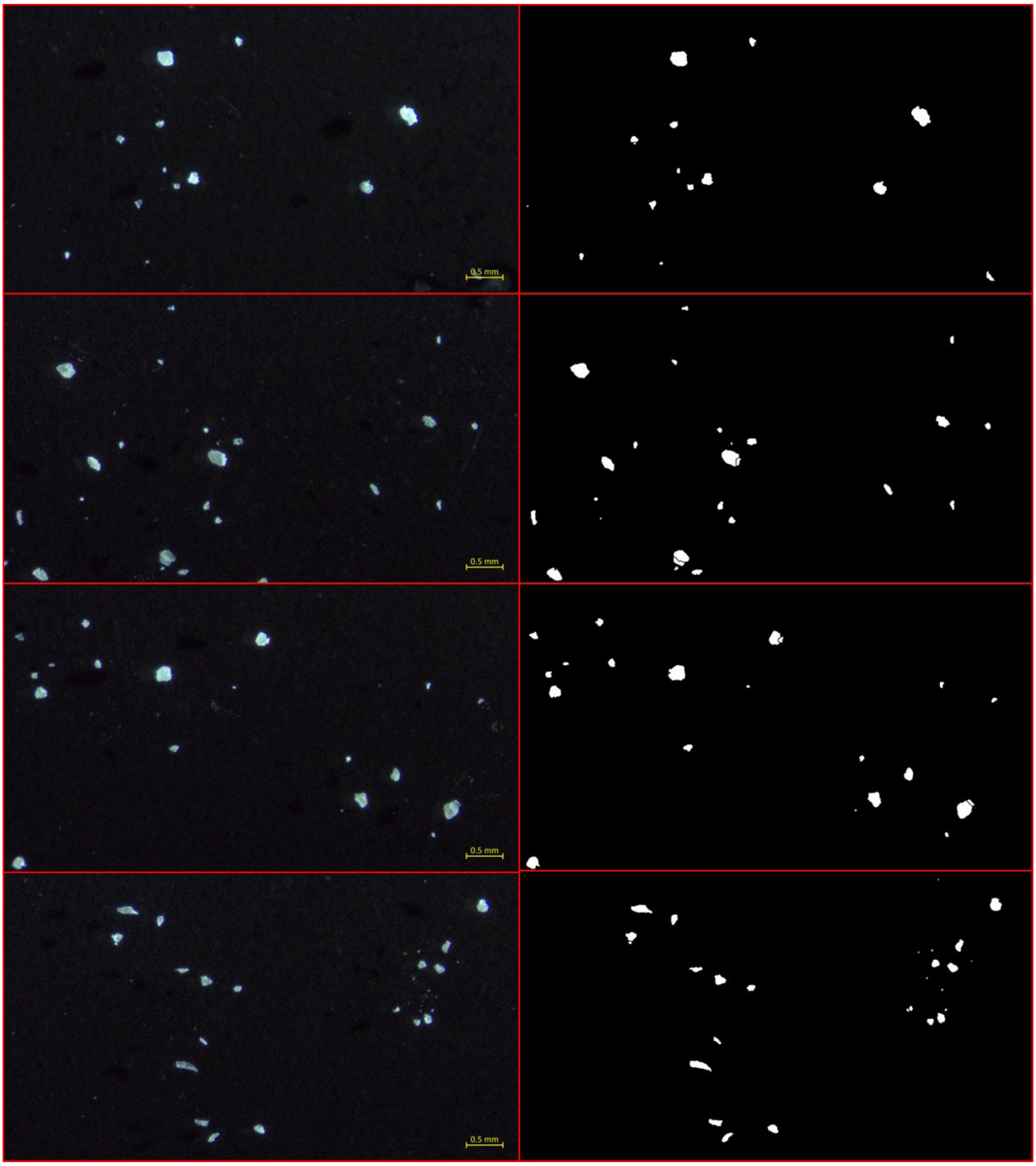


Figure S4.9. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of high molecular weight (350k g/mol) polystyrene-bisphenol A (PS-BPA) microplastics.

Figure S4.10. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of high molecular weight (350k g/mol) polystyrene-bisphenol S (PS-BPS) microplastics.

Figure S4.11. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of high molecular weight (350k g/mol) polystyrene-diethylhexyl phthalate (PS-DEHP) microplastics.

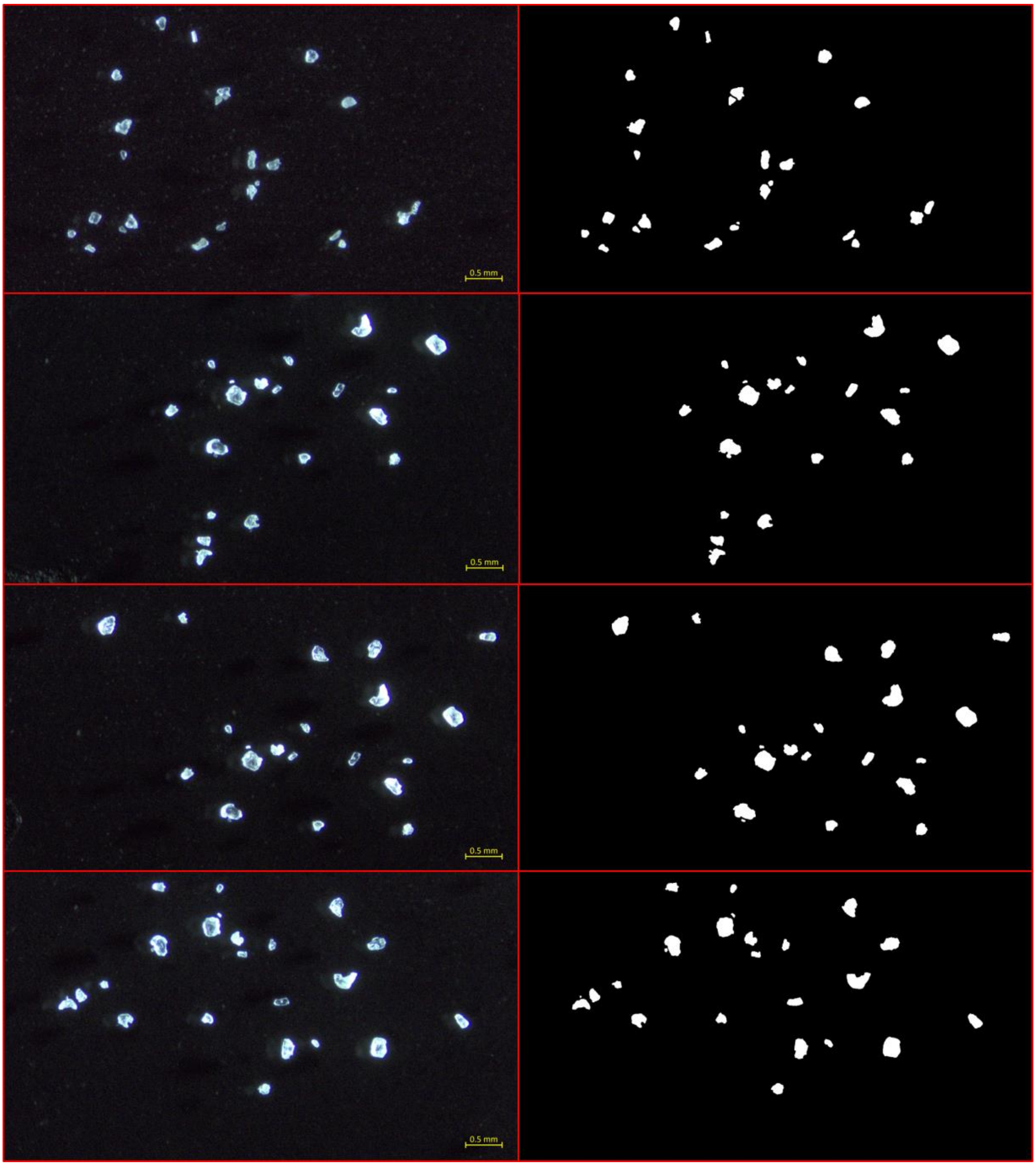


Figure S4.12. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of high molecular weight (350k g/mol) polystyrene-dioctyl terephthalate (PS-DEHT) microplastics.

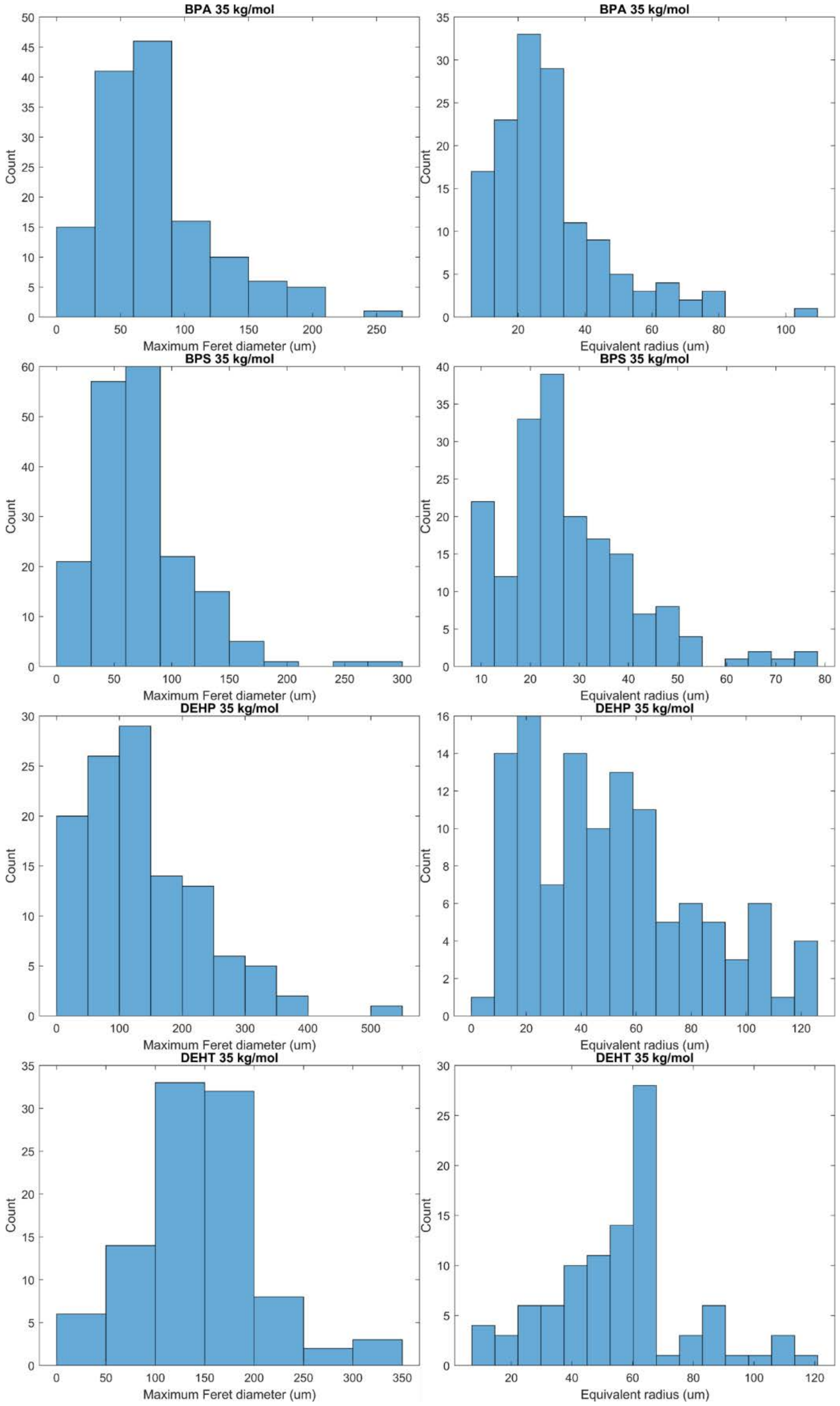


Figure S4.13. Histograms of particle sizes obtained from the images in Figures S4.1 – S4.4, plotted for low molecular weight polystyrene (35k g/mol) containing bisphenol A (BPA), bisphenol S (BPS), diethylhexyl phthalate (DEHP) and dioctyl terephthalate (DEHT). Sizes are measured using the maximum Feret diameter (left) and the equivalent radius (right). The maximum Feret diameter is the distance across the object in its longest direction. The equivalent radius is defined as the radius of a circle that has the same two-dimensional area as the microplastic particle in the image.

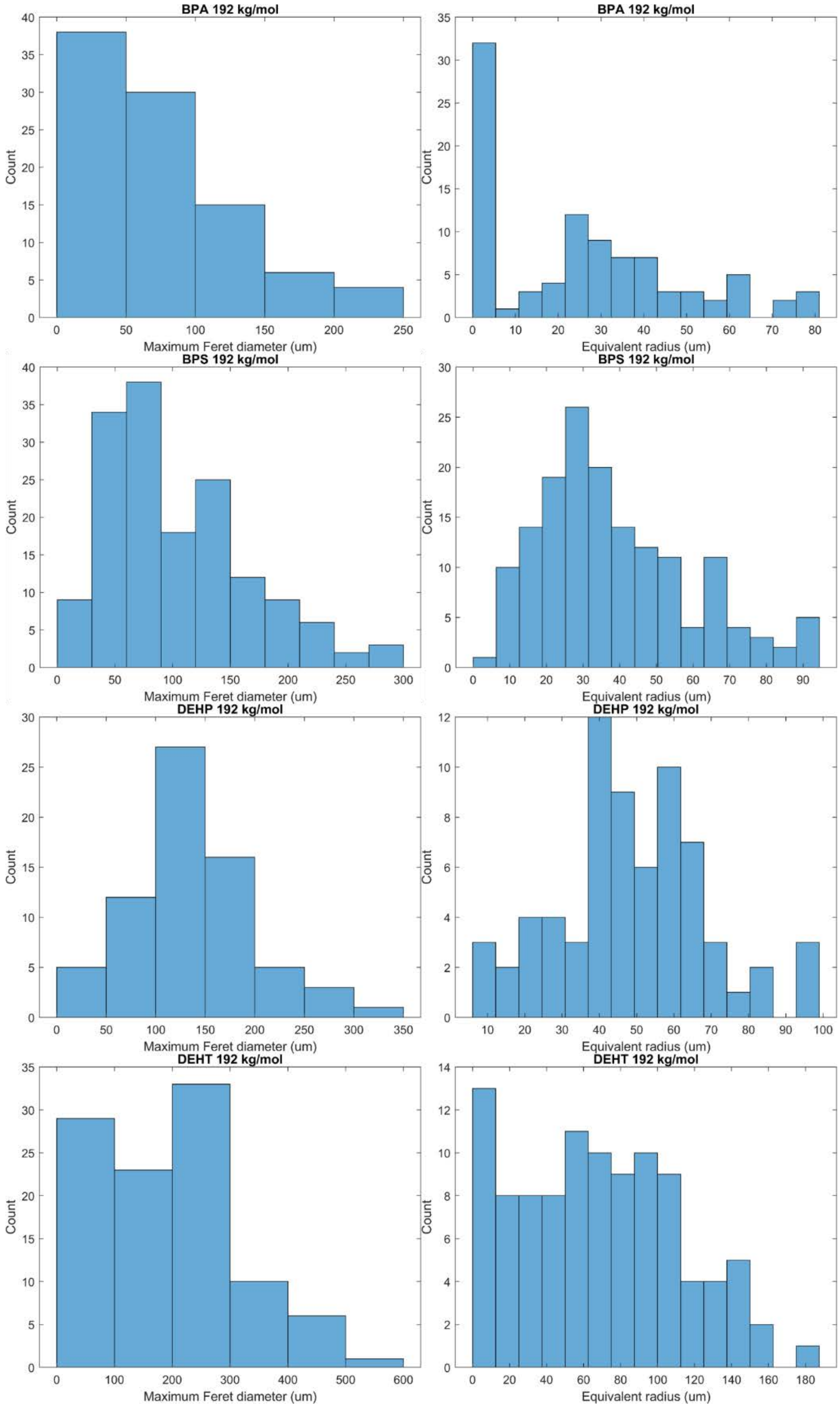


Figure S4.14. Histograms of particle sizes obtained from the images in Figures S4.5 – S4.8, plotted for medium molecular weight polystyrene (192k g/mol) containing bisphenol A (BPA), bisphenol S (BPS), diethylhexyl phthalate (DEHP) and dioctyl terephthalate (DEHT). Sizes are measured using the maximum Feret diameter (left) and the equivalent radius (right). The maximum Feret diameter is the distance across the object in its longest direction. The equivalent radius is defined as the radius of a circle that has the same two-dimensional area as the microplastic particle in the image.

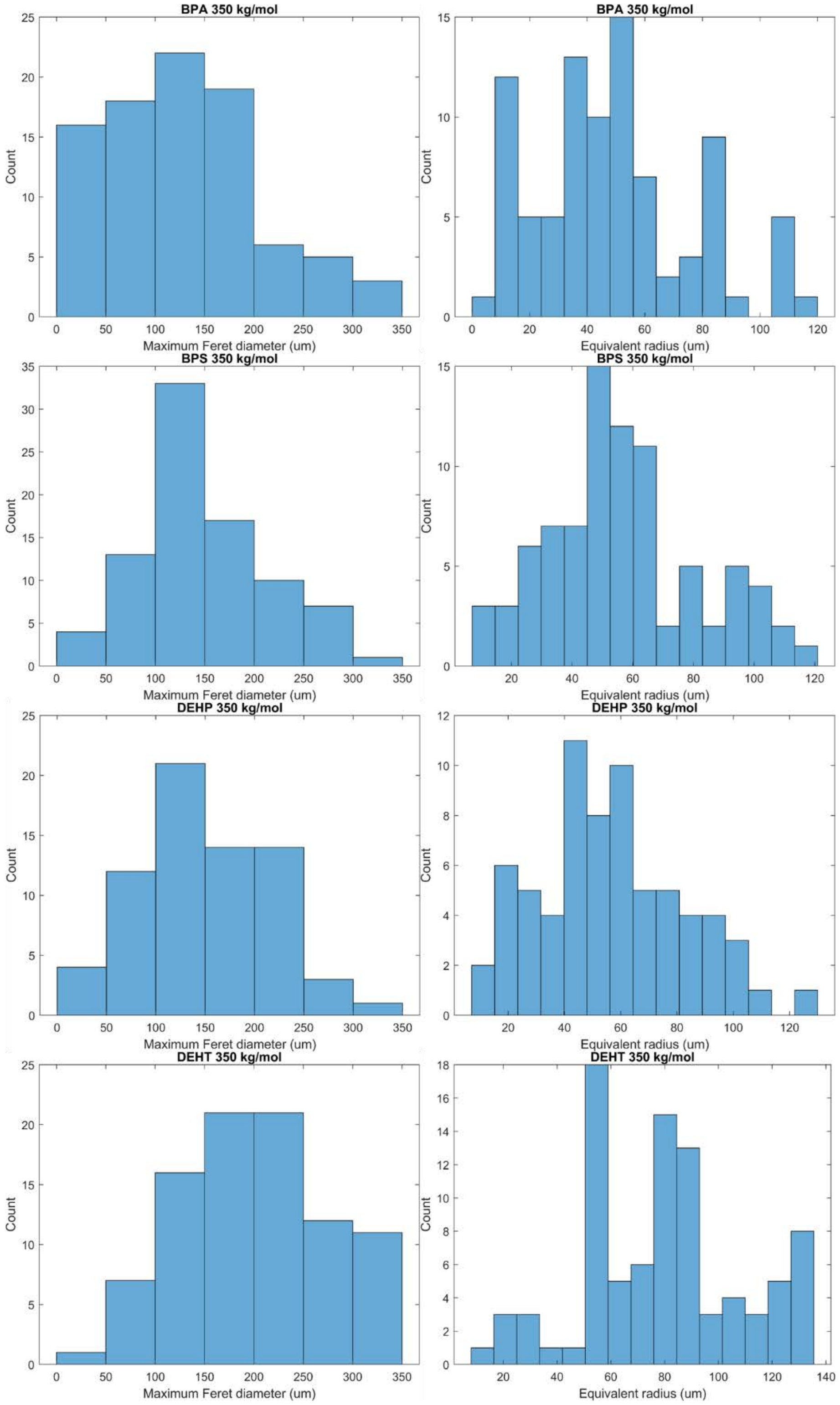


Figure S4.15. Histograms of particle sizes obtained from the images in Figures S4.9 – S4.12, plotted for high molecular weight polystyrene (350k g/mol) containing bisphenol A (BPA), bisphenol S (BPS), diethylhexyl phthalate (DEHP) and dioctyl terephthalate (DEHT). Sizes are measured using the maximum Feret diameter (left) and the equivalent radius (right). The maximum Feret diameter is the distance across the object in its longest direction. The equivalent radius is defined as the radius of a circle that has the same two-dimensional area as the microplastic particle in the image.

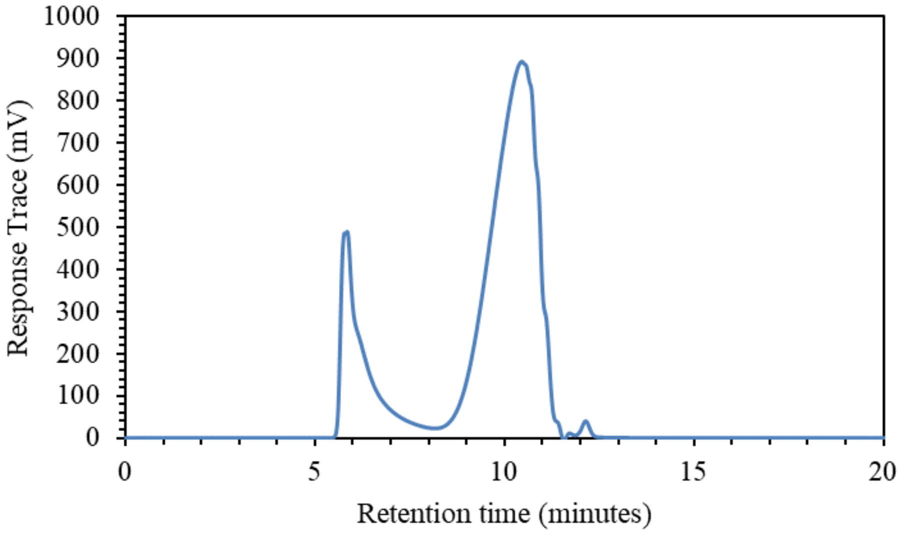


Figure S4.16. Gel permeation chromatography traces demonstrating the bimodal distribution of the low weight-average molecular weight polystyrene.

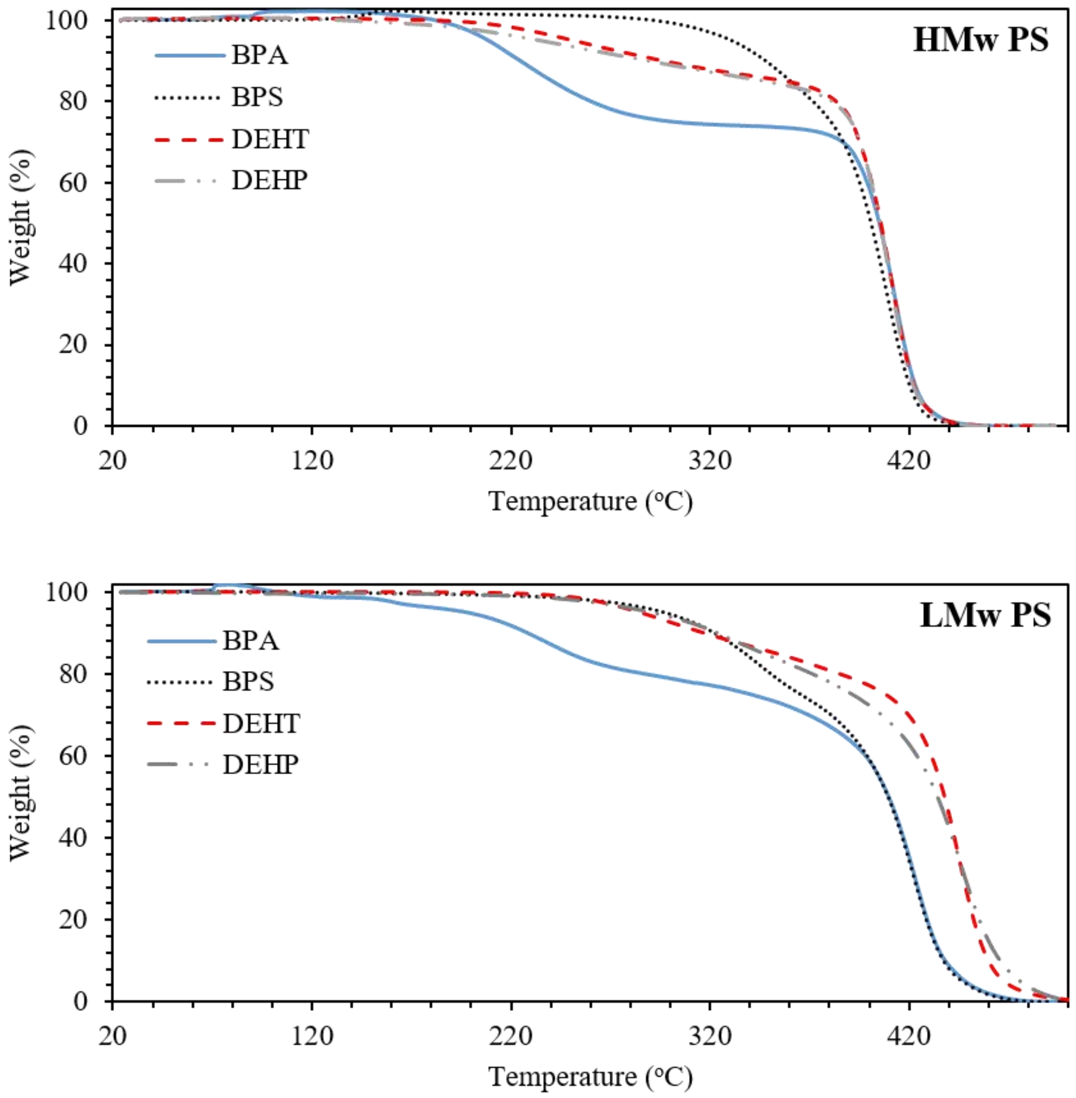


Figure S4.17. The thermograms of bisphenol A (BPA), bisphenol S (BPS), dioctyl terephthalate (DEHT) and diethylhexyl phthalate (DEHP) incorporated into the high (H*Mw*; 350k g/mol) and low (L*Mw*; 35k g/mol) molecular weight polystyrene (PS) microplastics. Thermograms were generated at ramp speed 10°C/min for all H*Mw*, L*Mw* BPA and BPS samples, and at 30°C/min for L*Mw* DEHT and DEHP samples.

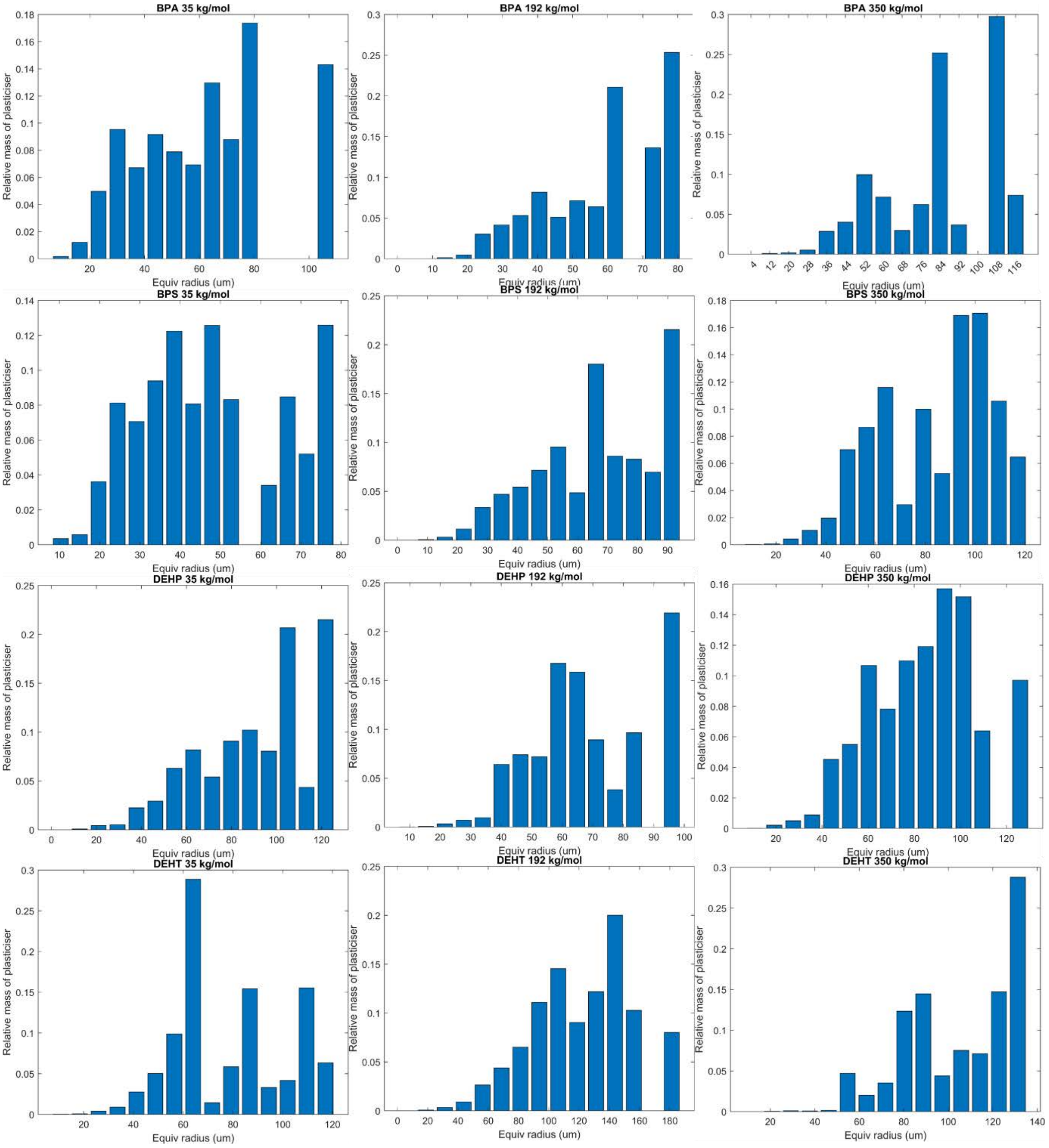


Figure S4.18. The leaching contribution of particles of different sizes (calculated by scaling the size distribution by$\frac{4}{3}\pi r^3$, where r is the equivalent radius from Figure S4.14 – S4.16). This estimates the amount of plasticiser that is available to leach from particles of different sizes. Results are shown for (a) bisphenol A (BPA), (b) bisphenol S (BPS), (c) diethylhexyl phthalate (DEHP) and (d) dioctyl terephthalate (DEHT) plasticisers leaching from low (35k g/mol), medium (192k g/mol) and high (350k g/mol) molecular weight polystyrene microplastics.

Table S4.1. Size distribution statistics of low (35k g/mol), medium (192k g/mol) and high (350k g/mol) molecular weight polystyrene microplastics incorporated with bisphenol A (BPA), bisphenol S (BPS), diethylhexyl phthalate (DEHP) and dioctyl terephthalate (DEHT). Since the particles were irregularly shaped, the predominant size definition was taken as the minimum/maximum Feret diameter, which is the shortest/longest distance across the object. The major axis length is defined as the major axis of the ellipse that has the same normalised central moments as the particle. The equivalent diameter is defined as the diameter of the circle that has the same area as the particle. These statistics were computed using the "regionprops" function in Matlab.

Plasticiser	**Molecular weight (g/mol)**	**Measurement**	**Mean (µm)**	**Standard deviation (µm)**	**Median (µm)**
BPA	35,000	Maximum Feret diameter	75.9	44.3	66.5
		Minimum Feret diameter	53.1	32.1	45.0
		Major axis length	73.4	42.8	64.8
		Equivalent diameter of a circle having the same area	73.4	42.8	64.8
	192,000	Maximum Feret diameter	70.9	59.1	63.7
		Minimum Feret diameter	43.4	38.5	40.0
		Major axis length	70.2	57.6	62.2
		Equivalent diameter of a circle having the same area	51.3	44.3	52.2
	350,000	Maximum Feret diameter	129.8	74.3	119.2
		Minimum Feret diameter	85.6	51.5	76.4
		Major axis length	125.5	71.7	115.5
		Equivalent diameter of a circle having the same area	98.0	55.2	92.3
BPS	35,000	Maximum Feret diameter	73.0	40.9	63.8
		Minimum Feret diameter	49.2	24.2	43.8
		Major axis length	70.6	40.6	60.8
		Equivalent diameter of a circle having the same area	55.1	26.3	49.7
	192,000	Maximum Feret diameter	104.4	60.5	88.0
		Minimum Feret diameter	67.2	37.4	59.7
		Major axis length	101.8	59.2	87.0
		Equivalent diameter of a circle having the same area	77.3	41.5	67.5

	350,000	Maximum Feret diameter	149.7	63.2	140.8
		Minimum Feret diameter	98.6	46.3	92.6
		Major axis length	145.8	60.6	134.7
		Equivalent diameter of a circle having the same area	113.7	49.3	109.8
DEHP	35,000	Maximum Feret diameter	139.4	91.4	126.9
		Minimum Feret diameter	93.2	56.2	85.9
		Major axis length	135.5	95.5	121.8
		Equivalent diameter of a circle having the same area	101.7	61.2	94.8
	192,000	Maximum Feret diameter	137.0	63.8	124.4
		Minimum Feret diameter	83.4	36.3	83.7
		Major axis length	133.5	64.4	122.5
		Equivalent diameter of a circle having the same area	97.2	40.1	97.4
	350,000	Maximum Feret diameter	149.3	67.5	139.7
		Minimum Feret diameter	99.0	48.5	96.2
		Major axis length	143.1	64.9	136.8
		Equivalent diameter of a circle having the same area	113.2	52.4	108.0
DEHT	35,000	Maximum Feret diameter	144.3	59.0	148.4
		Minimum Feret diameter	99.4	41.7	101.4
		Major axis length	140.1	58.1	144.7
		Equivalent diameter of a circle having the same area	111.3	45.1	114.9
	192,000	Maximum Feret diameter	190.6	131.1	191.1
		Minimum Feret diameter	114.6	76.9	110.6
		Major axis length	182.5	124.7	180.9
		Equivalent diameter of a circle having the same area	134.4	89.4	131.7
	350,000	Maximum Feret diameter	198.5	73.4	196.8
		Minimum Feret diameter	139.7	57.2	137.4
		Major axis length	193.9	72.5	188.4
		Equivalent diameter of a circle having the same area	157.2	59.7	155.2

Table S4.2. Average bisphenol A (BPA), bisphenol S (BPS), dioctyl terephthalate (DEHT) and diethylhexyl phthalate (DEHP) concentrations (wt. %) incorporated in the medium and high molecular weight (*Mw*) polystyrene microplastics calculated using gel permeation chromatography (GPC) and thermal gravimetric analysis (TGA). Error margin between GPC and TGA measurements is <6.7 wt. %.

Polystyrene molecular weight	Plasticiser	Average wt. %	
		GPC	TGA
Medium-*Mw* (192k gmol)	BPA	16.68	18.76
	BPS	17.03	14.37
	DEHT	15.17	16.35
	DEHP	14.24	15.45
High-*Mw* (350k g/mol)	BPA	20.06	22.43
	BPS	28.57	19.64
	DEHT	21.23	14.52
	DEHP	14.45	15.38

Chapter 5. Plasticiser leaching from polyvinyl chloride microplastics

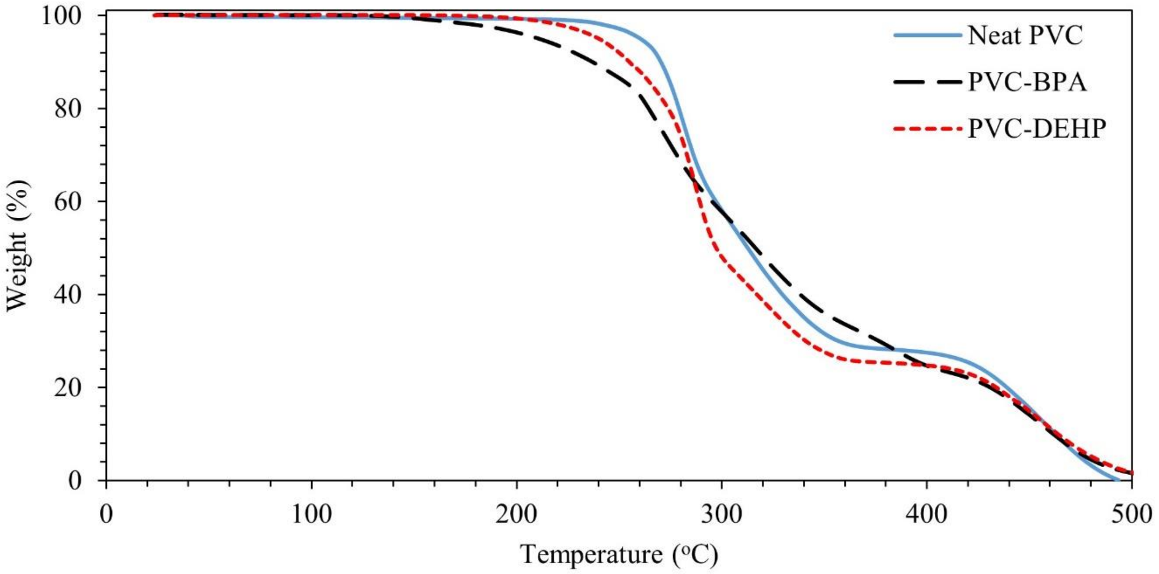


Figure S5.1. The thermal gravimetric analysis (TGA) thermograms of neat, virgin polyvinyl chloride (PVC; solid blue) incorporated with bisphenol A (BPA; black dashed) and diethylhexyl phthalate (DEHP; red dotted).

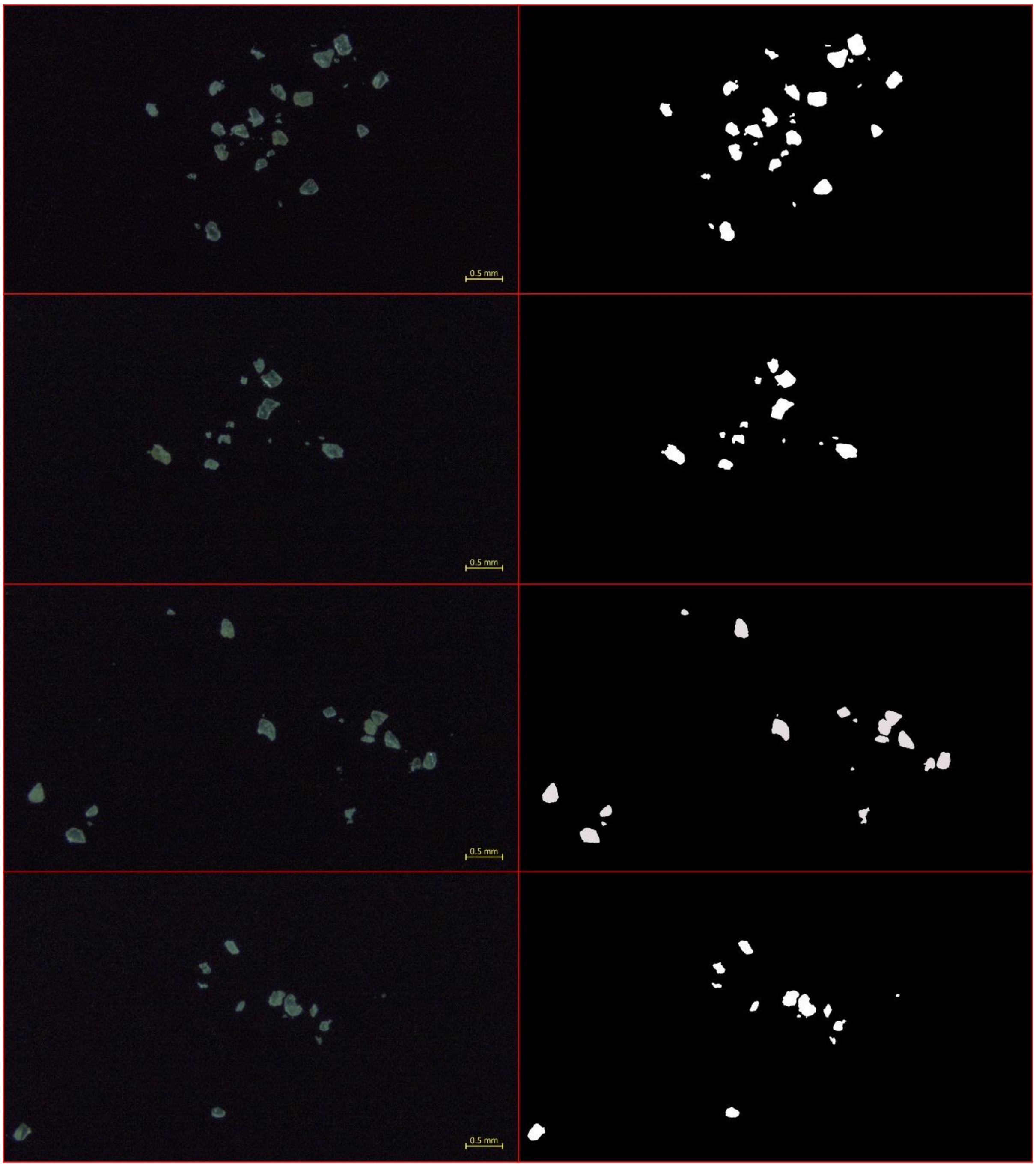


Figure S5.2. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of polyvinyl chloride-bisphenol A (PVC-BPA) microplastics.

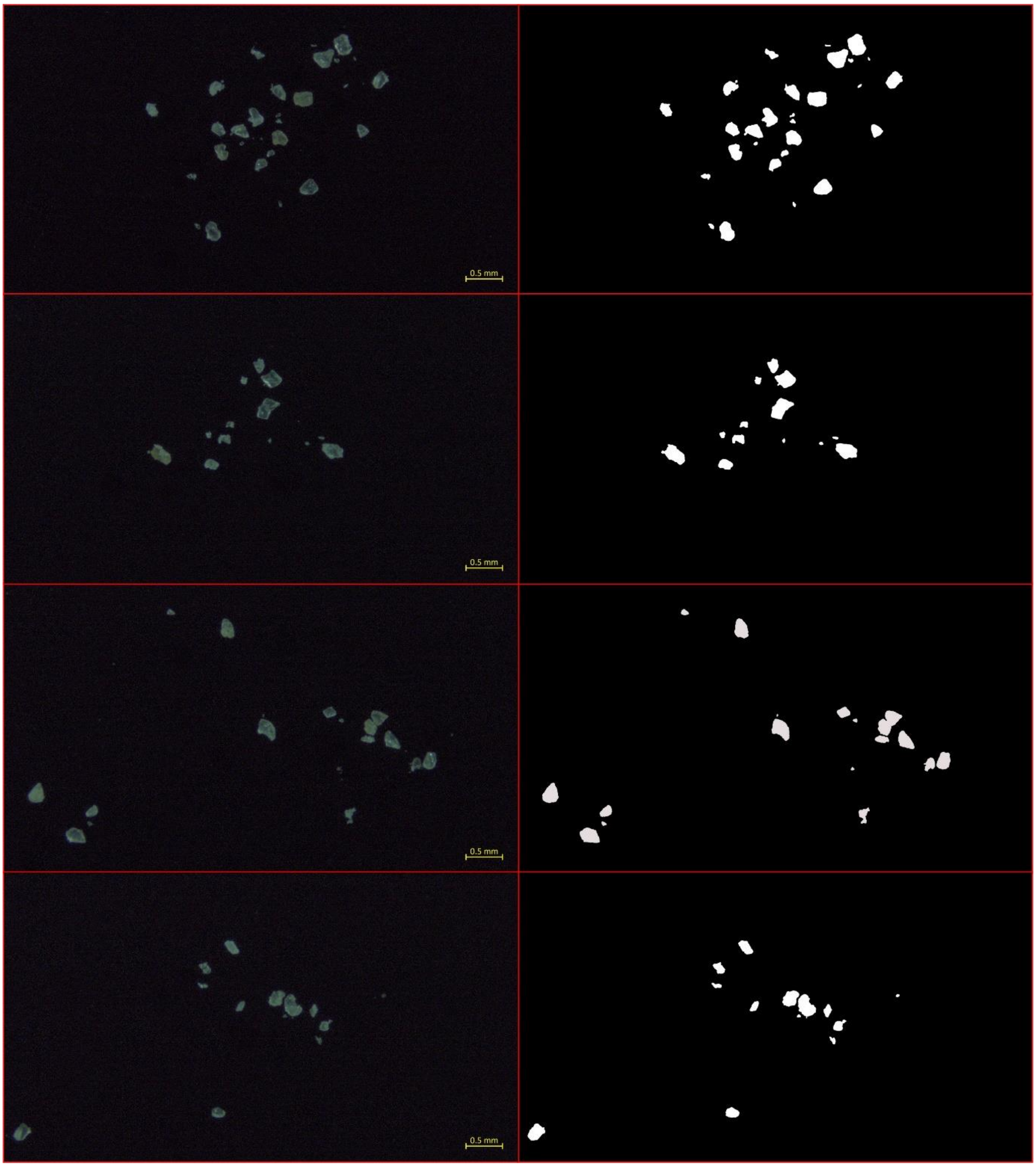


Figure S5.3. The microscope images (right) and corresponding binary masks (left) used for the particle size analysis of polyvinyl chloride-diethylhexyl phthalate (PVC-DEHP) microplastics.

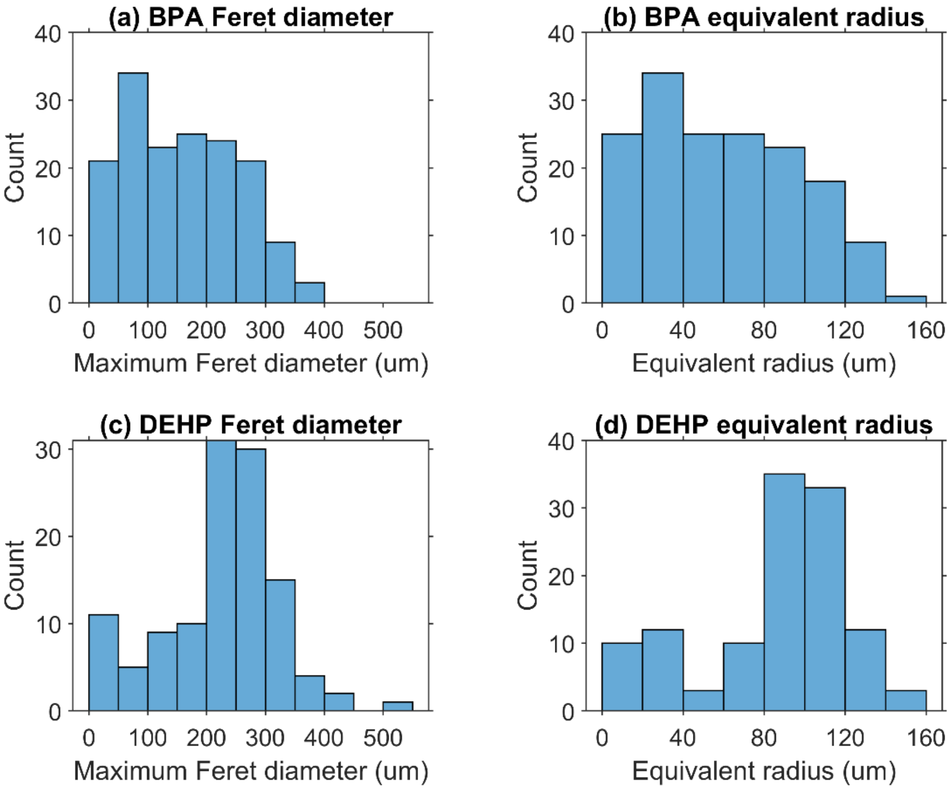


Figure S5.4. Histograms of particle sizes obtained from the images in Figures S5.2 and S5.3, plotted for (a-b) bisphenol A (BPA) and (c-d) diethylhexyl phthalate (DEHP). Sizes are measured using the maximum Feret diameter (left) and the equivalent radius (right). The maximum Feret diameter is the distance across the object in its longest direction. The equivalent radius is defined as the radius of a circle that has the same two-dimensional area as the microplastic particle in the image.

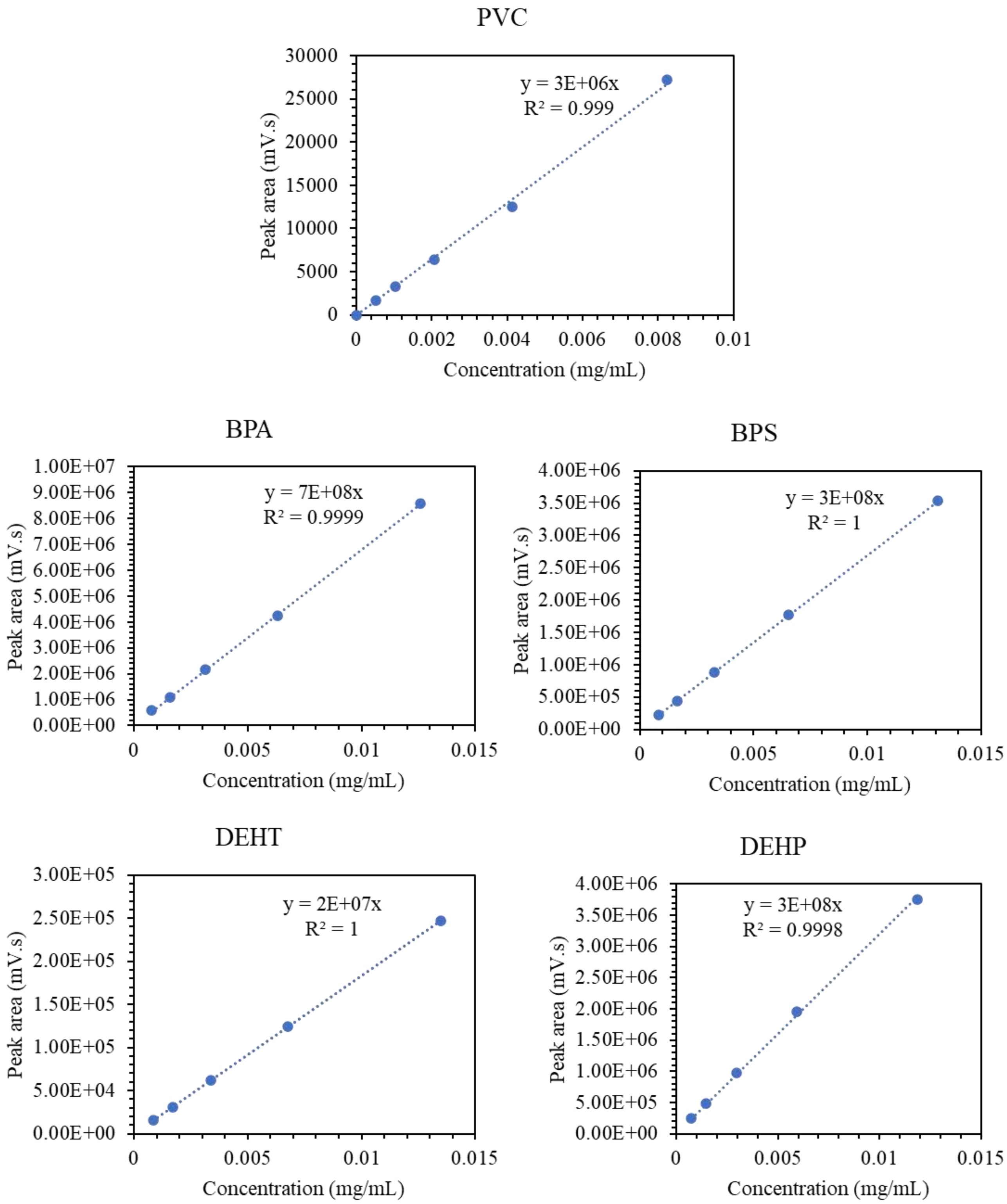


Figure S5.5. Gel permeation chromatography calibration curves of polyvinyl chloride (PVC), bisphenol A (BPA), bisphenol S (BPS), dioctyl terephthalate (DEHT) and diethylhexyl phthalate (DEHP) used for concentration determination.

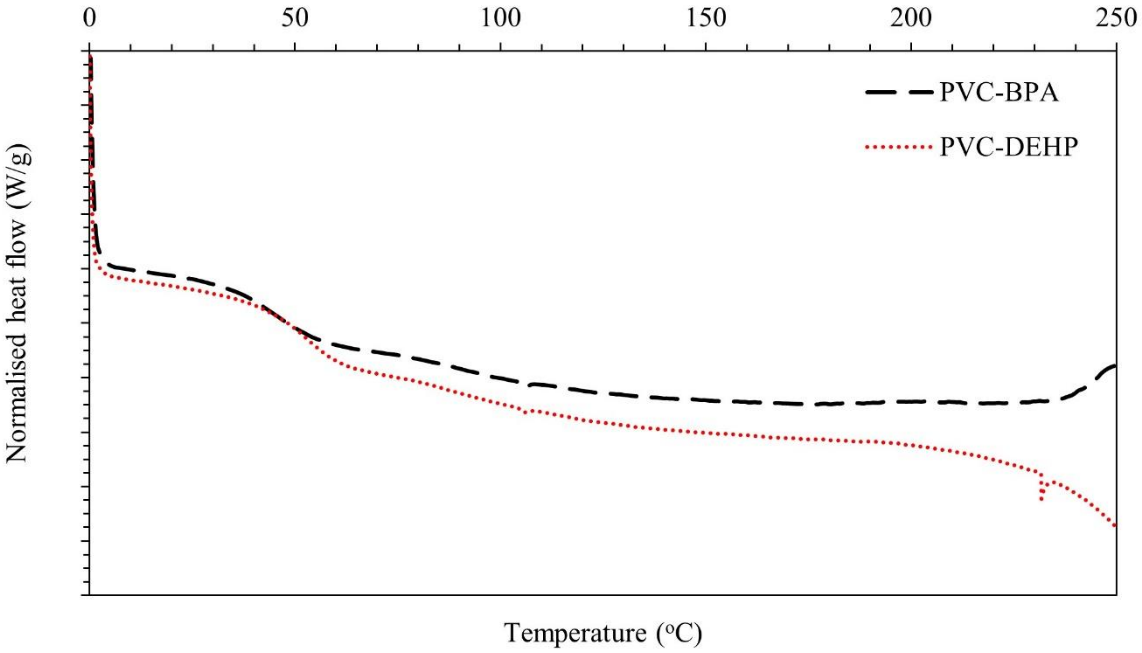


Figure S5.6. The differential scanning calorimetry (DSC) thermal profiles of polyvinyl chloride (PVC) incorporated with bisphenol A (BPA; black dashed) and diethylhexyl phthalate (DEHP; red dotted).

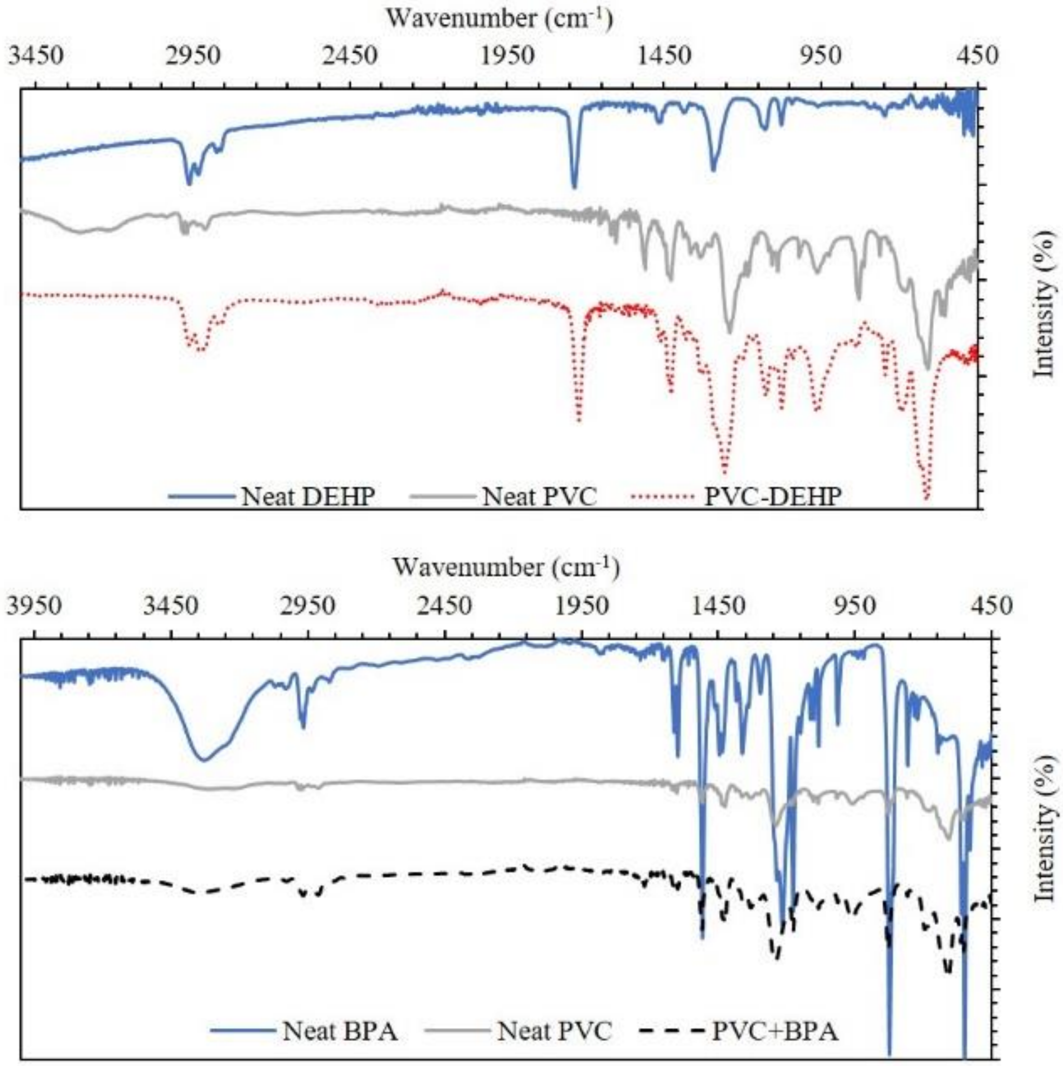


Figure S5.7. The infrared profiles of neat diethylhexyl phthalate (DEHP; blue solid) and bisphenol A (BPA; blue solid), virgin polyvinyl chloride (PVC; grey solid), as well as PVC incorporated with DEHP (PVC-DEHP; red dotted) and BPA (PVC-BPA; black dashed).

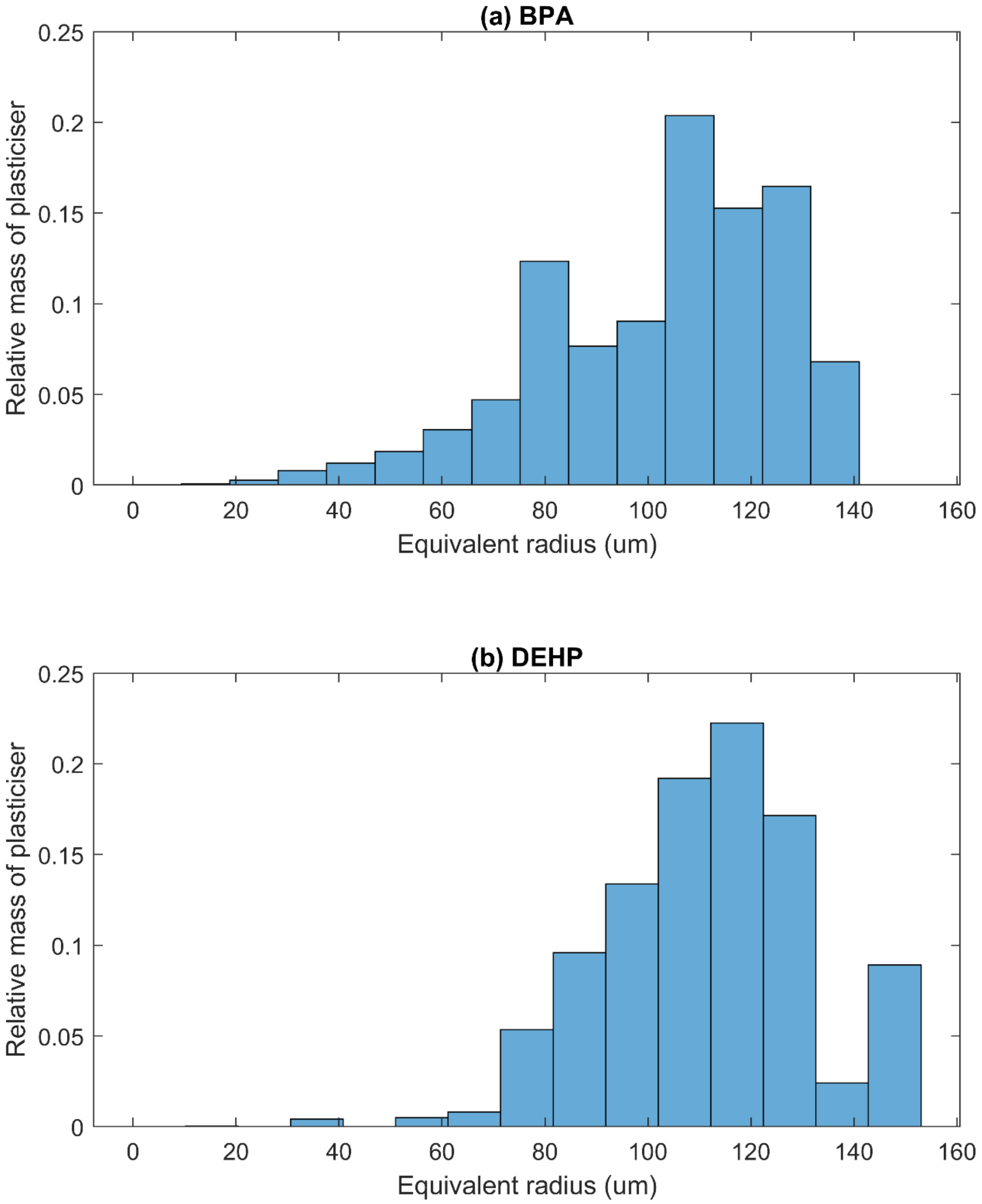


Figure S5.8. The leaching contribution of particles of different sizes (calculated by scaling the size distribution by: $\frac{4}{3}\pi r^3$, where r is the equivalent radius from Figure S5.4). This estimates the amount of plasticiser that is available to leach from particles of different sizes. Results are shown for (a) bisphenol A (BPA) and (b) diethylhexyl phthalate (DEHP) plasticisers leaching from polyvinyl chloride (PVC) microplastics.

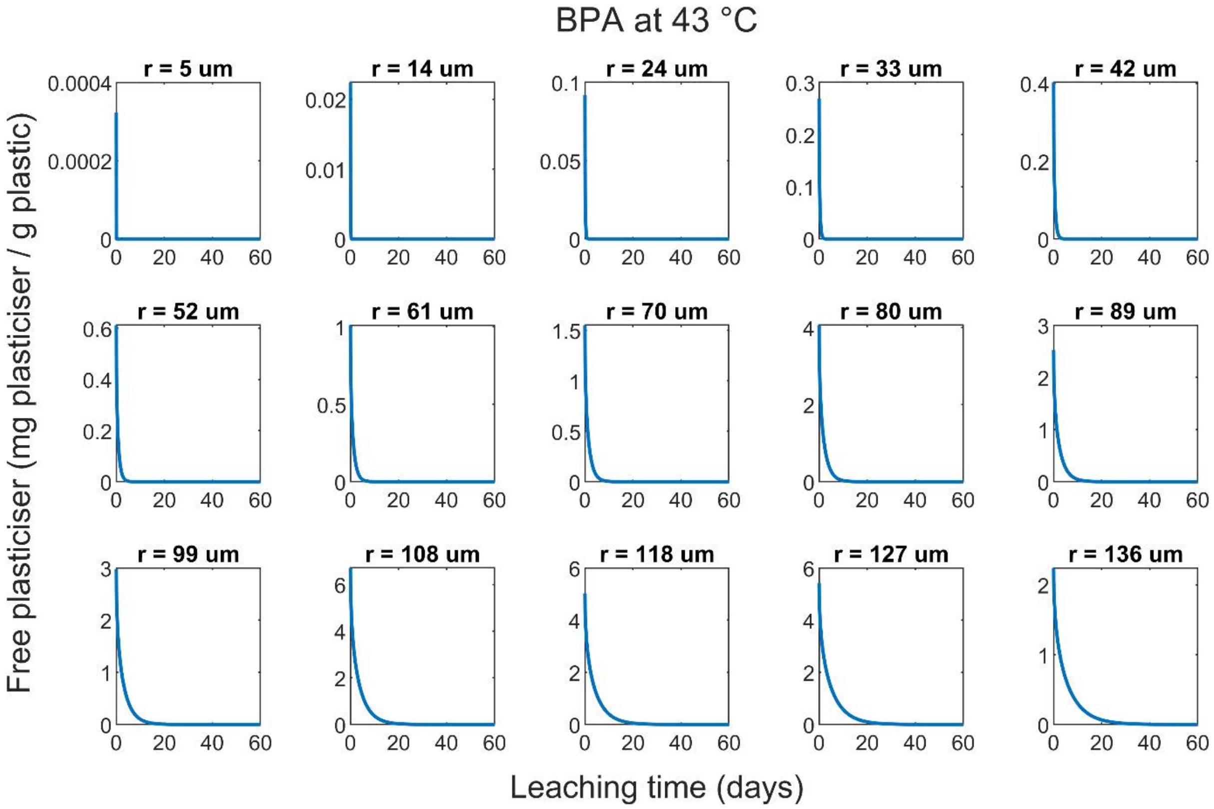


Figure S5.9. Size resolved leaching behaviour (based upon the model fit) for bisphenol A (BPA) from polyvinyl chloride microplastics at 43°C.

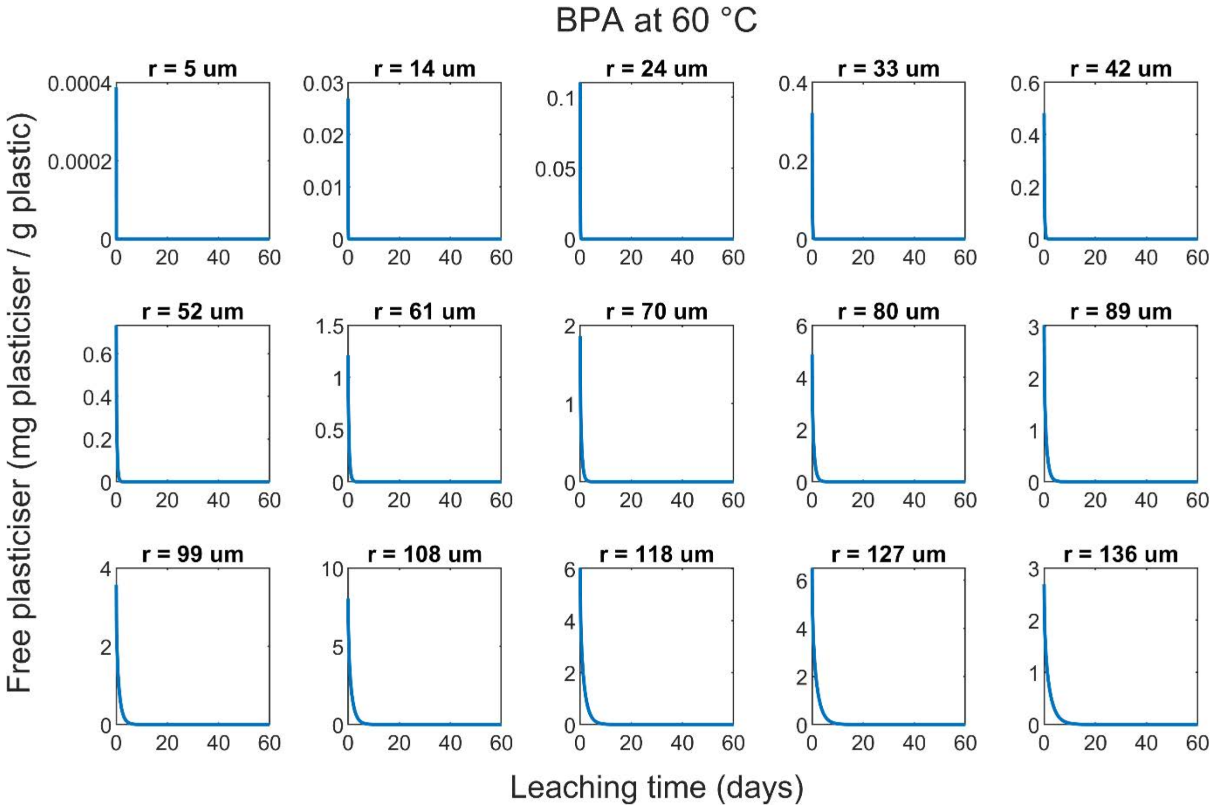


Figure S5.10. Size resolved leaching behaviour (based upon the model fit) for bisphenol A (BPA) from polyvinyl chloride microplastics at 60°C.

Table S5.1. The methods trialled for the dissolution of polyvinyl chloride (PVC), polypropylene (PP), high/low density polyethylene (HDPE/LDPE), nylon 6/6, polyethylene terephthalate (PET), polycaprolactone (PCL) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). Soluble polymers suitable for gel permeation chromatography (GPC) analysis at ambient temperatures were then casted onto a watch glass to allow for solvent evaporation and the mechanical properties of the plastic membrane assessed for processability (rigid/flexible).

Polymer*	Solubility Parameters				Mechanical properties of the casted and dried plastic membrane	Suitable for use in this study?
	Solvent(s)*	Time	Temperature (ºC)	Suitability for GPC analysis at ambient temperatures (Y/N)		
PVC	**THF (1:7 w/v)**	**Overnight**	**Ambient**	**Y**	**Rigid**	**Yes**
PP	THF, DCM, DMSO, toluene, xylenes	<4 days	<100ºC	N	-	No, insoluble at room temperature
HDPE	THF, DCM, DMSO, toluene, xylenes	<4 days	<100ºC	N	-	No, insoluble at room temperature
LDPE	Xylenes (1:9 w/v)	24 hours	100ºC	Y	Flexible	No, once dried is too malleable for processing
Nylon 6/6	THF, DCM, DMSO, toluene, xylenes	<4 days	<100ºC	N	-	No, insoluble at room temperature
PET	Phenol (1:5 w/w)	2 hours	90ºC	Y	Rigid	No, insoluble at room temperature

PCL	THF (1:4 w/v)	Overnight	Ambient	Y	Flexible	No, once dried is too malleable for processing
PHBV	THF, DCM, DMSO, toluene, xylenes	<4 days	<100°C	N	-	No, insoluble at room temperature

* Phenol, Tetrahydrofuran (THF), dichloromethane (DCM), PP, HDPE, LDPE, Nylon 6/6. PET and PCL were sourced from Sigma Aldrich, toluene, xylenes and dimethylsulfoxide (DMSO) was sourced from Unichrom and PHBV was obtained from a proprietary source.

Table S5.2. Size distribution statistics of prepared polyvinyl chloride (PVC) microplastics incorporated with bisphenol A (BPA) and diethylhexyl phthalate (DEHP). Since the particles were irregularly shaped, the predominant size definition was taken as the minimum/maximum Feret diameter, which is the shortest/longest distance across the object. The major axis length is defined as the major axis of the ellipse that has the same normalised central moments as the particle. The equivalent diameter is defined as the diameter of the circle that has the same area as the particle. These statistics were computed using the "regionprops" function in Matlab.

Plasticiser	Measurement	Mean (µm)	Standard deviation (µm)	Median (µm)
BPA	Maximum Feret diameter	159	94	157
	Minimum Feret diameter	105.5	65.1	95.8
	Major axis length	153.3	90.7	148.5
	Equivalent diameter of circle having the same area	120.1	72.3	113.4
DEHP	Maximum Feret diameter	223.7	95.8	240.8
	Minimum Feret diameter	152.5	64.2	167.1
	Major axis length	214.4	94.8	226.5
	Equivalent diameter of circle having the same area	172	70.6	188.4

Chapter 6. Biofilm development on plasticised marine microplastics

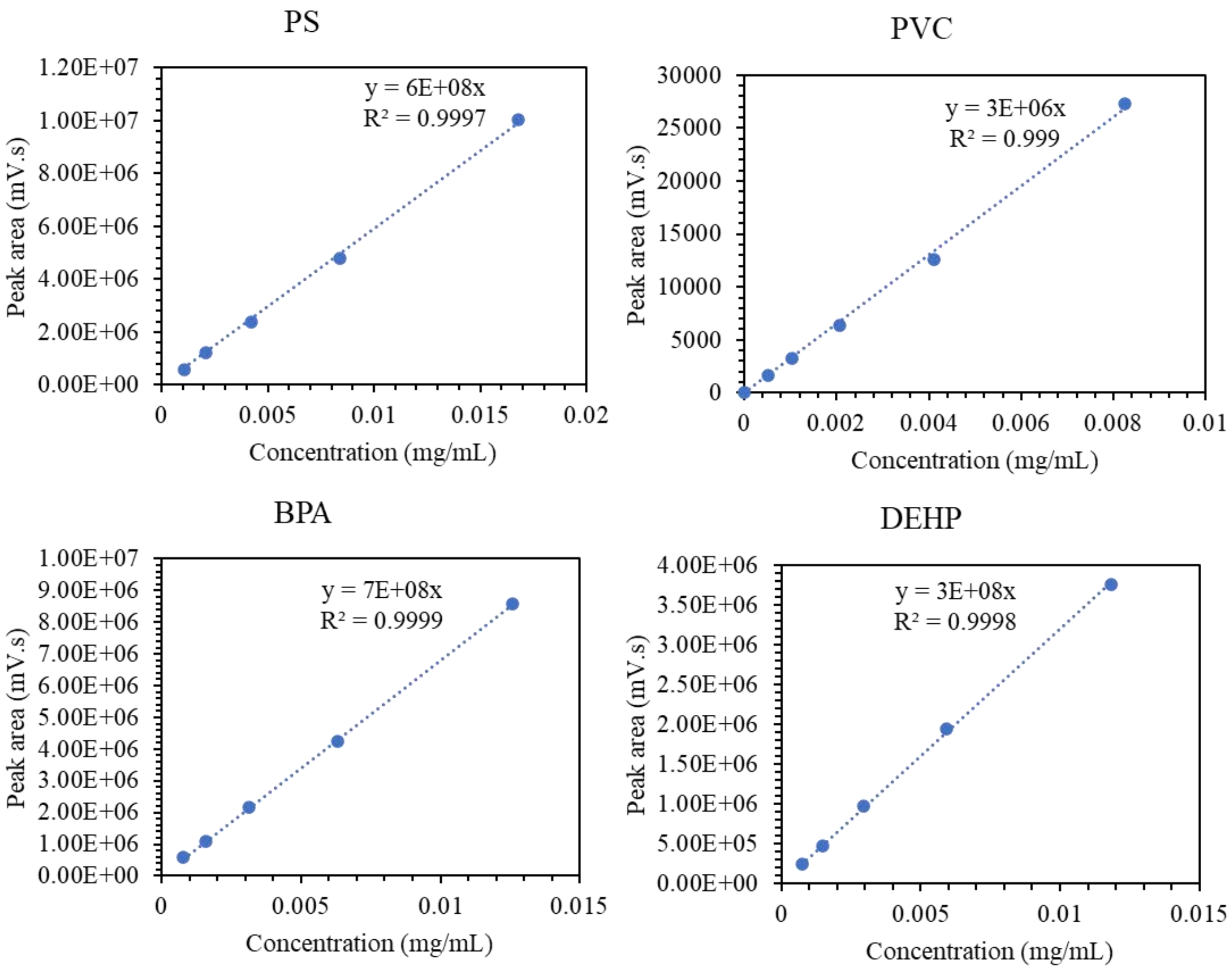


Figure S6.1. Gel permeation chromatography curves of polystyrene (PS), polyvinyl chloride (PVC), bisphenol A (BPA) and diethylhexyl phthalate (DEHP) for concentration determination.

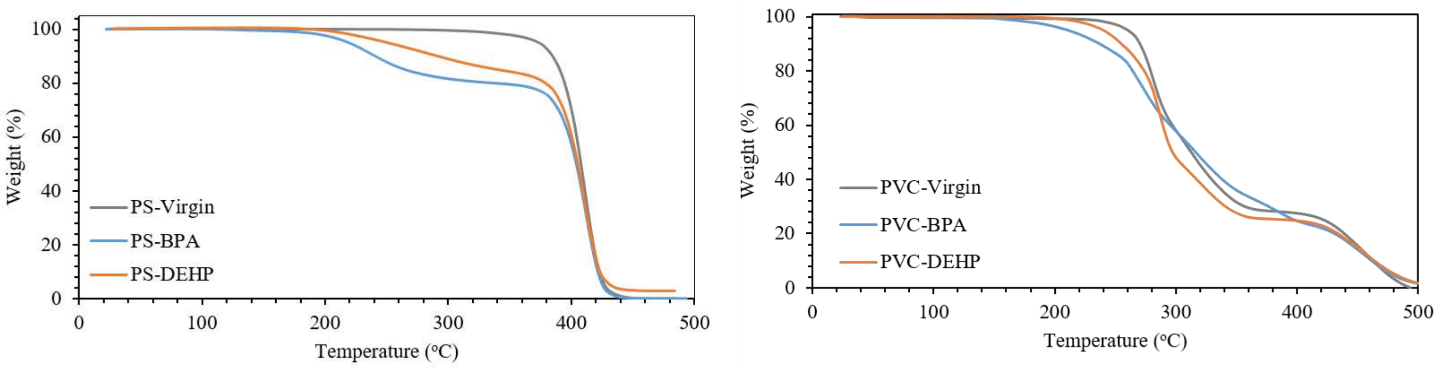


Figure S6.2. Thermal gravimetric analysis (TGA) thermograms of control (i.e., 0 days of exposure) polystyrene (PS) and polyvinyl chloride (PVC) microplastics; virgin (i.e., containing no plasticiser) and containing diethylhexyl phthalate (DEHP) and bisphenol A (BPA). Data was obtained in Chapter 3 (PS) and Chapter 5 (PVC).

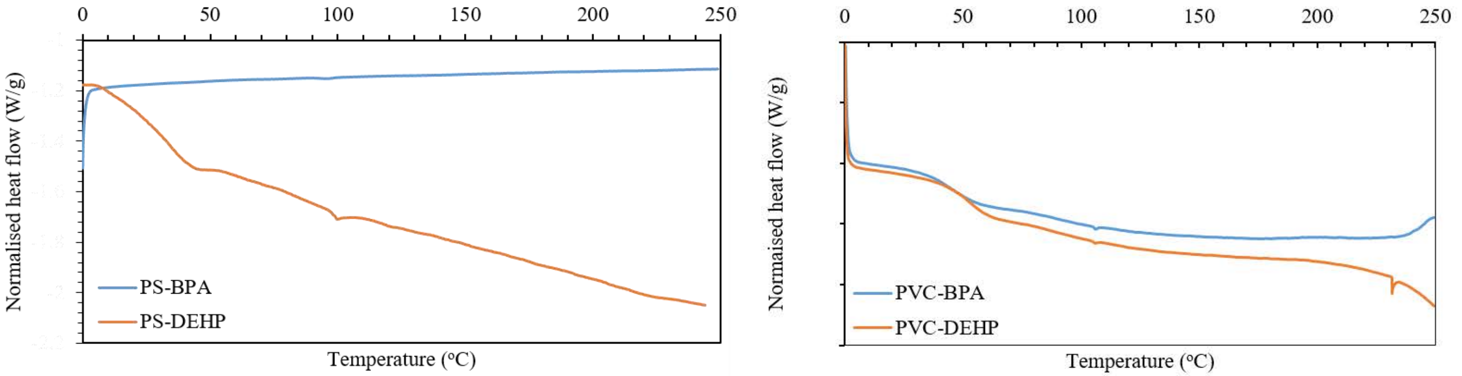


Figure S6.3. Differential scanning calorimetry (DSC) thermograms of control (i.e., 0 days of exposure) polystyrene (PS) and polyvinyl chloride (PVC) microplastics; virgin (i.e., containing no plasticiser) and containing diethylhexyl phthalate (DEHP) and bisphenol A (BPA). Data was obtained in Chapter 3 (PS) and Chapter 5 (PVC).

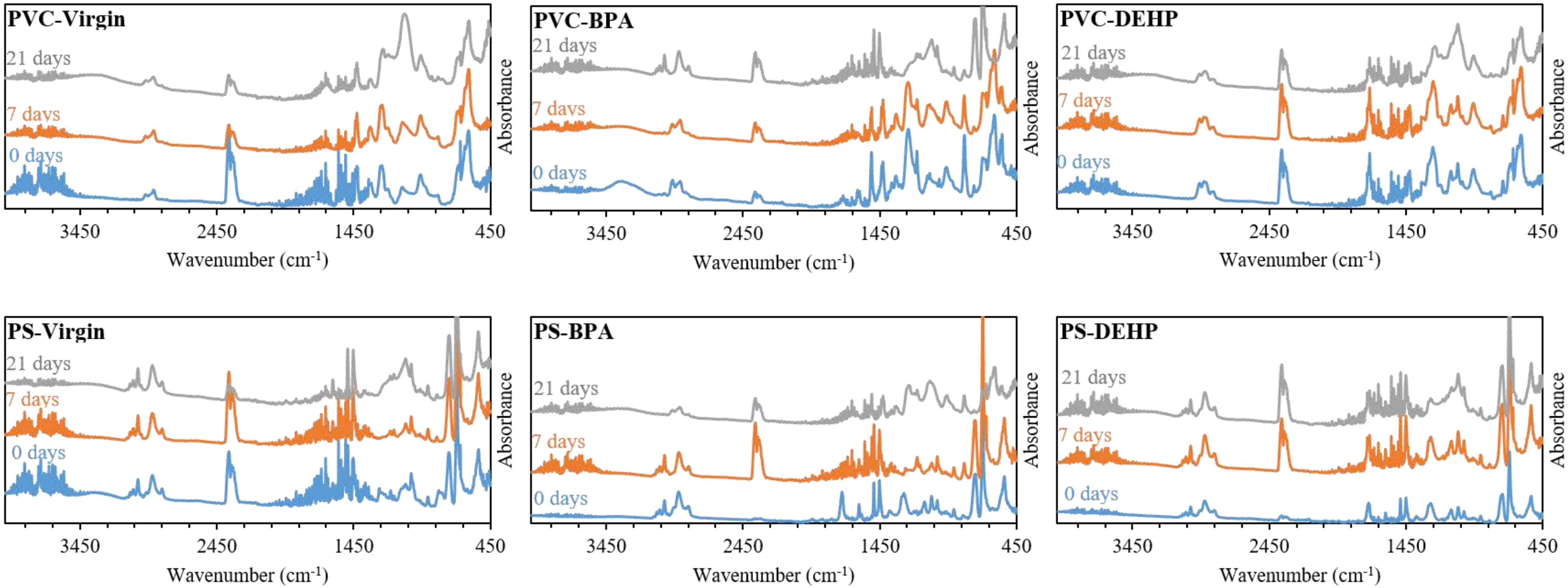


Figure S6.4. Infrared spectra of polystyrene (PS) and polyvinyl chloride (PVC) microplastics; virgin (i.e., no plasticiser) and containing diethylhexyl phthalate (DEHP) and bisphenol A (BPA) after 0 (blue), 7 (orange) and 21 days (grey) of experimental exposure.

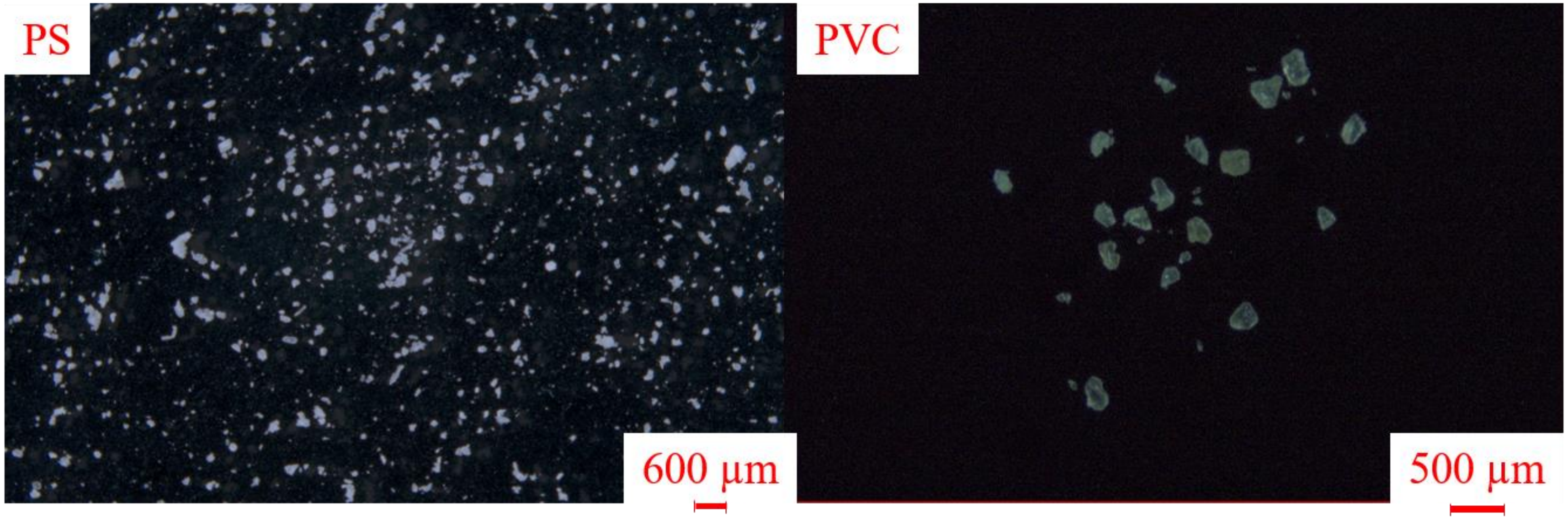


Figure S6.5. Representative microscope images of polystyrene (PS) and polyvinyl chloride (PVC) microplastics taken at 2.5X and 6.3X magnification, respectively. Data was obtained in Chapter 3 (PS) and Chapter 5 (PVC).

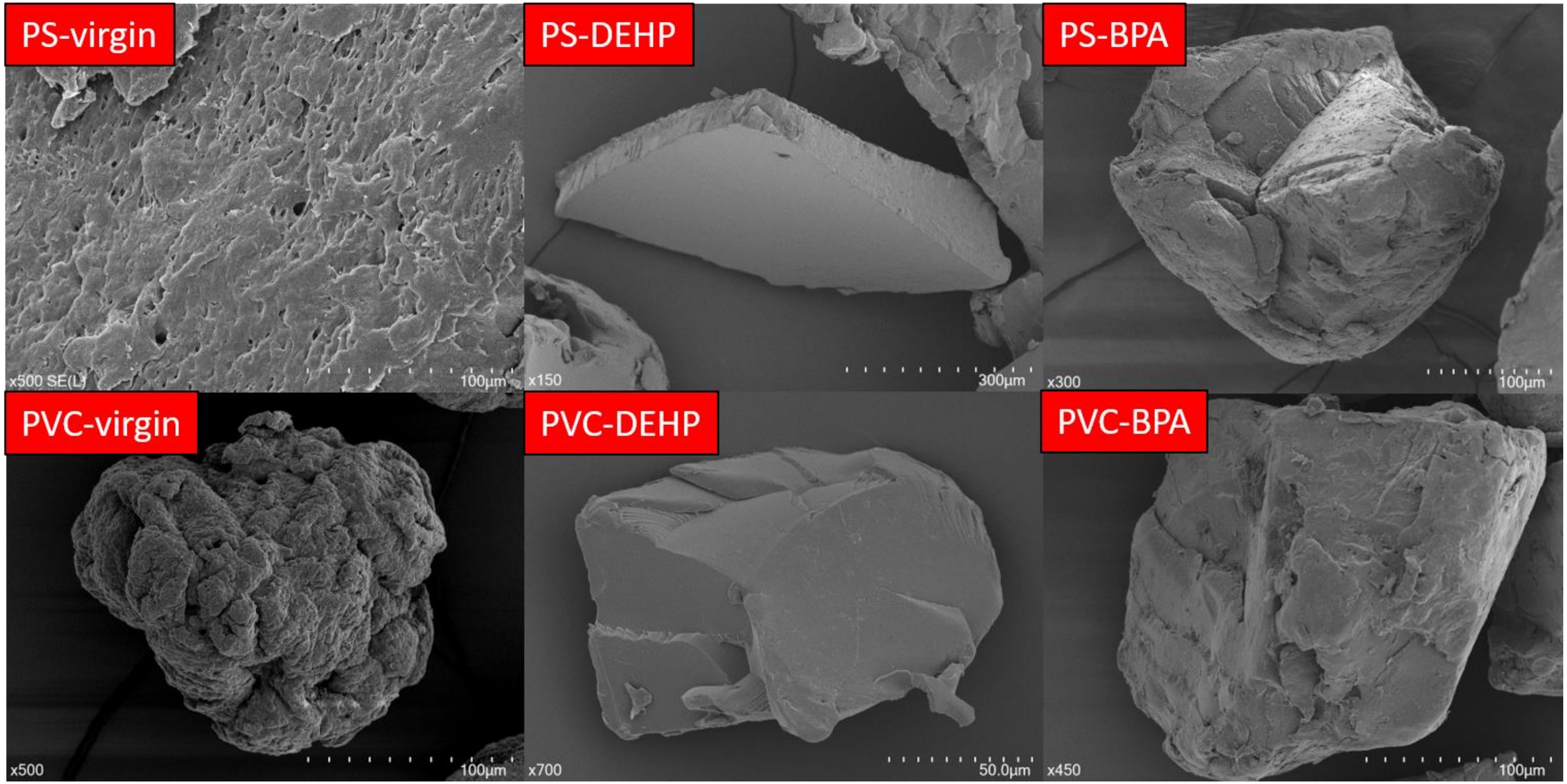
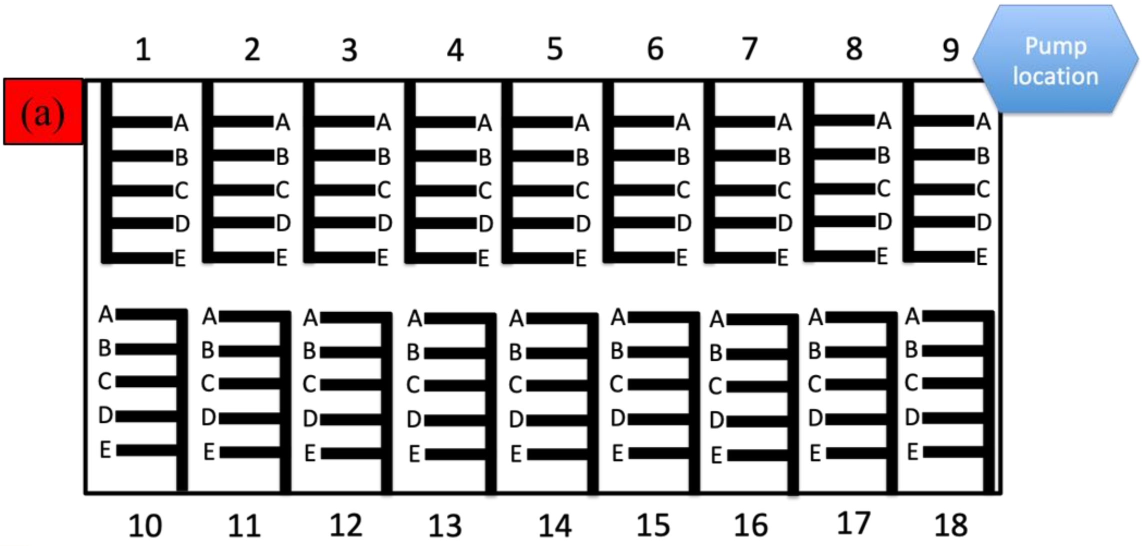


Figure S6.6. Representative images of the control (i.e., no exposure to seawater) polystyrene (PS) and polyvinyl chloride (PVC) microplastics; virgin (i.e., no plasticiser) and containing diethylhexyl phthalate (DEHP) and bisphenol A (BPA). Magnification and scale bars are indicated on each image.



(b)

Microplastic type	Additive Type	Location on the manifold system according to (a)	
		7-day exposure	21-day exposure
Polystyrene	Virgin	16	9
	BPA	5	18
	DEHP	15	7
Polyvinyl chloride	Virgin	14	1
	BPA	6	12
	DEHP	13	8
PHBV*	Virgin	4	11
	1.5% additives	2	10
	Composite	3	17

* A treatment using the biopolymer PHBV was run concurrently with the PS and PVC experiment, however, data analysis in this chapter was only undertaken for PS and PVC.

Figure S6.7. (a) Location of each polymer (polystyrene, polyvinyl chloride poly(3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) treatment group (*n* = 5 replicates; A – E) as described in (b). Polymers were incorporated without additive (virgin), or containing bisphenol A (BPA), diethylhexyl phthalate (DEHP), 1% boron nitride and 0.5% heatstabilisers (1.5% additives) and 40% calcium carbonate (composite), where stated. All treatment locations were randomized to minimize line effect and flow fluctuations due to their proximity to the pump.

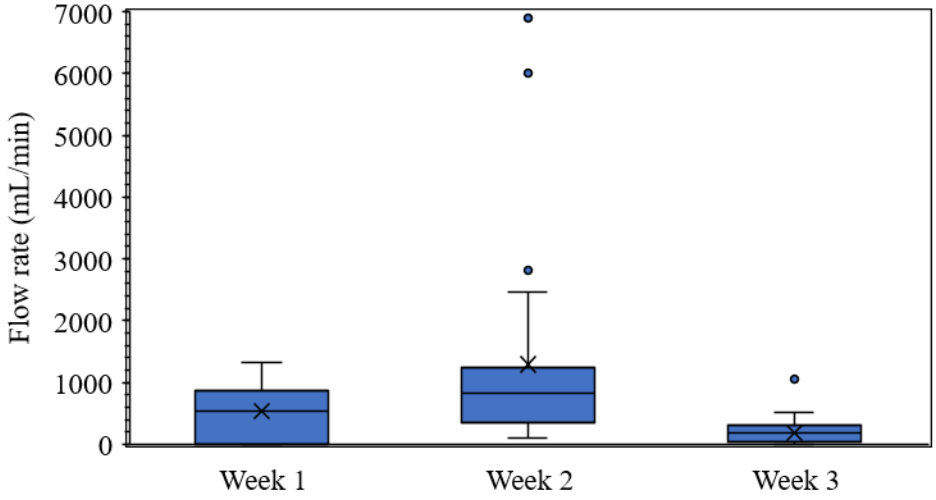


Figure S6.8. Flow rate (mL/min) seawater leaving each treatment cartridge weekly throughout the experiment.

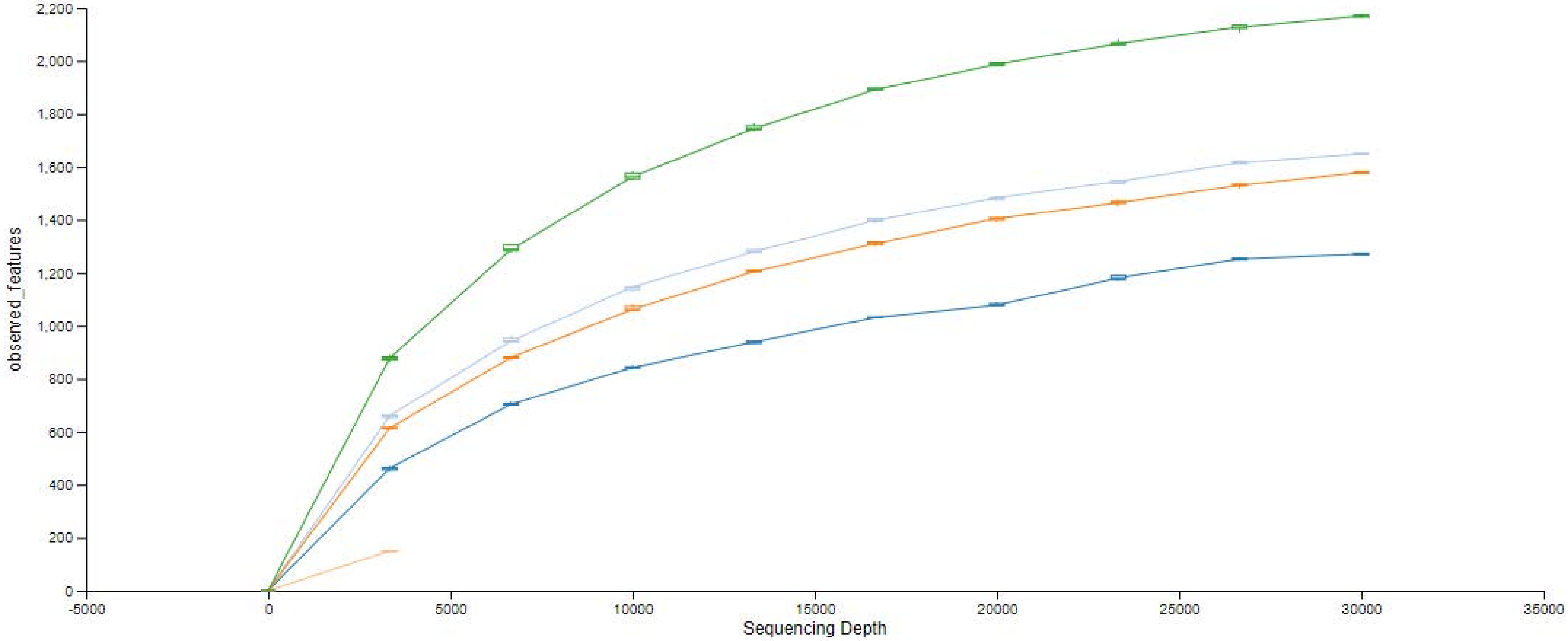


Figure S6.9. Sample-based rarefaction curve of background seawater (green), polystyrene (light blue), polyvinyl chloride (orange), poly(3-hydroxybutyrate-co-3-hydroxyvalerate (dark blue) and negative controls (i.e., kit blanks; yellow) number of operational taxonomic units (OTUs) in the rarefied dataset.

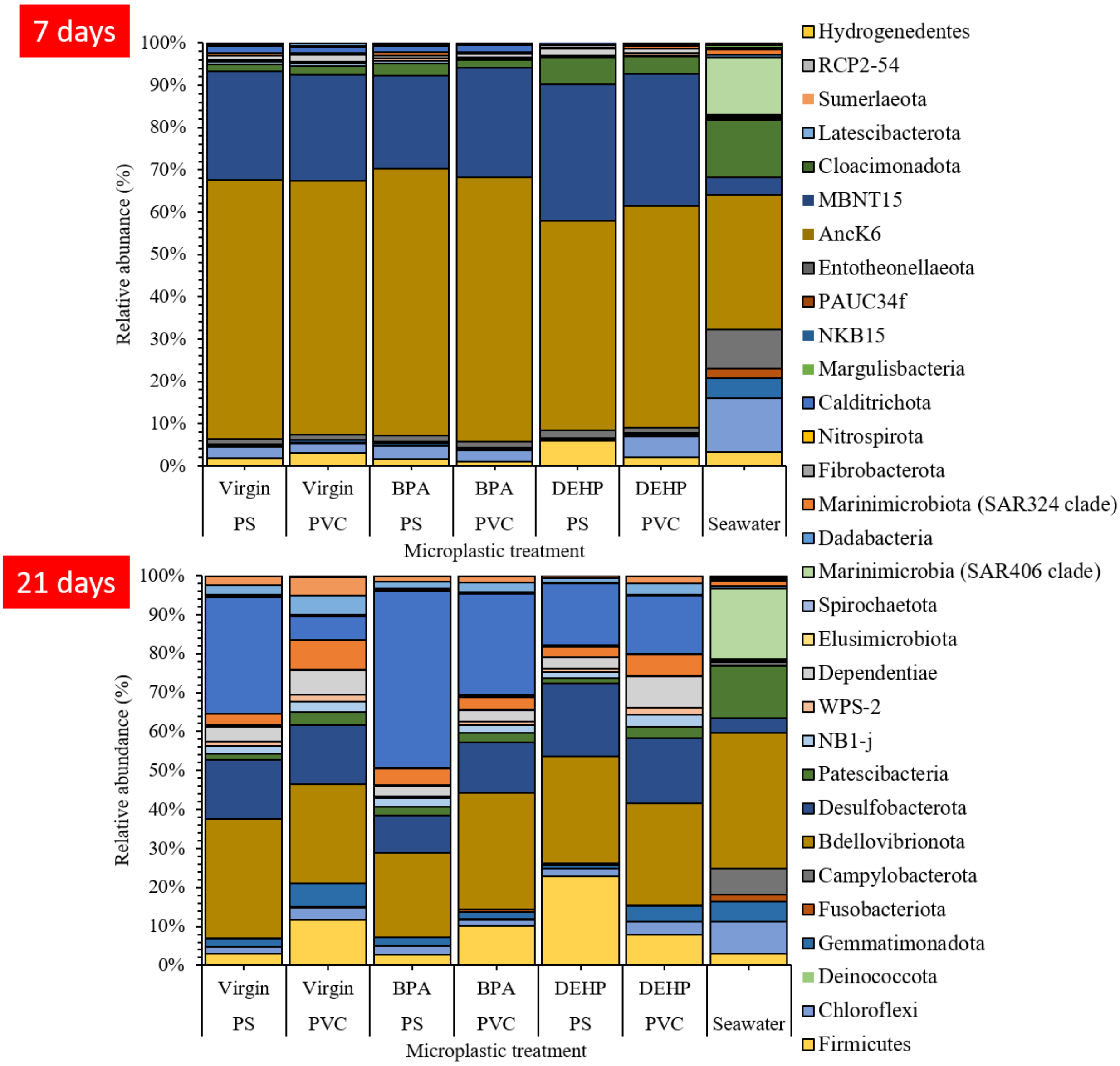


Figure S6.10. Mean relative abundance (%) of the low abundant phyla (present in less than 1% in more than 75% of samples) detected in the seawater and microplastic biofilms from polystyrene (PS) and polyvinyl chloride (PVC) microplastics; virgin (i.e., containing no plasticiser) and containing bisphenol A (BPA) and diethylhexyl phthalate (DEHP) after 7 and 21 days.

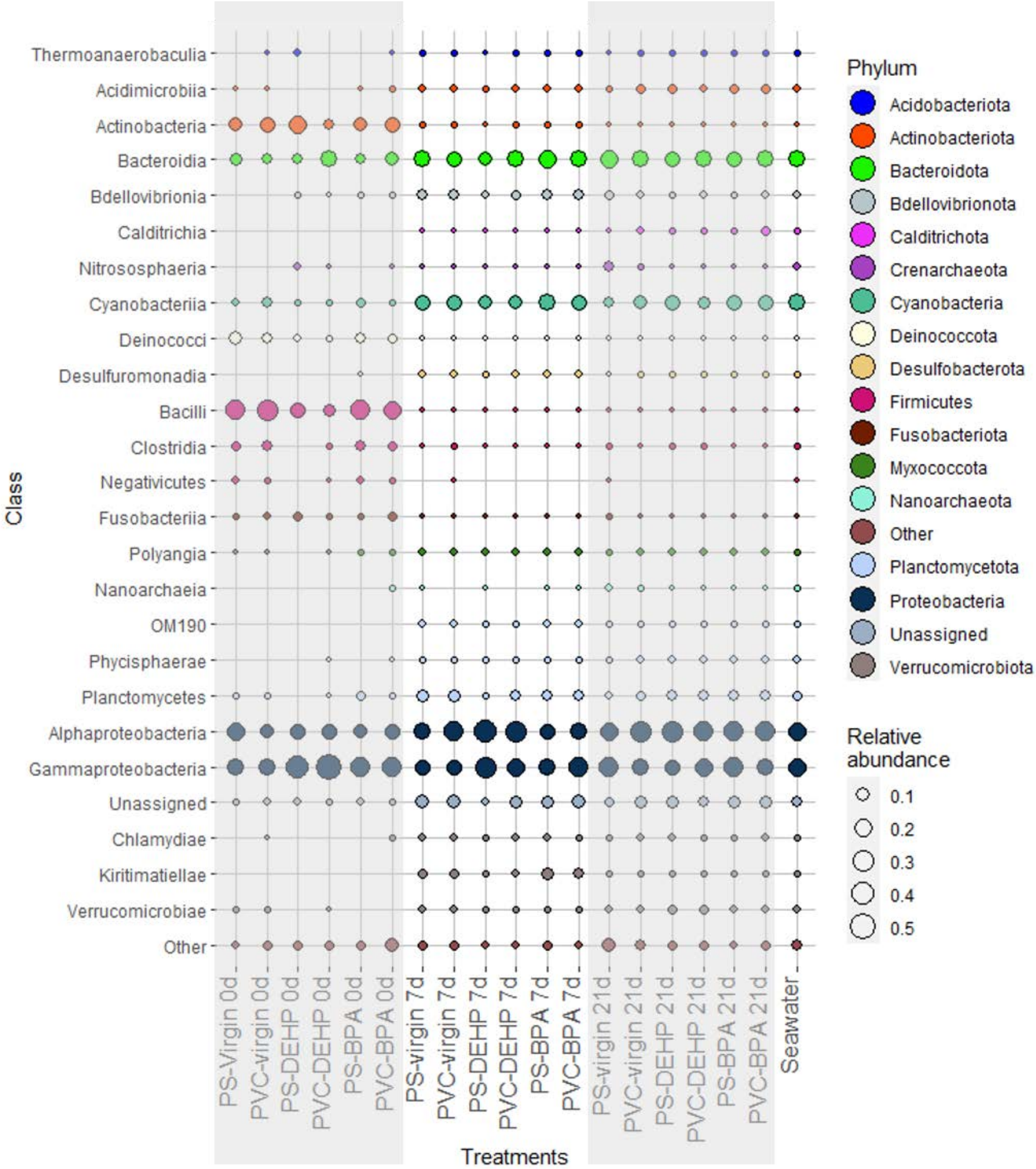


Figure S6.11. Mean relative abundance (%) of the top 25 most abundant bacterial taxa at the phylum and class level (present in greater than 1% abundance in more than 75% of samples) detected in the seawater and microplastic biofilms from polystyrene (PS) and polyvinyl chloride (PVC) microplastics; virgin and containing plasticiser diethylhexyl phthalate (DEHP) or bisphenol A (BPA) after 0, 7 and 21 days. "Other" refers to bacterial taxa with less than 1% relative abundance, and "unassigned" refers to amplicon sequences that could not be classified.

Table S6.1. The weather and water conditions throughout the duration of the experiment

		Week 1			Week 2			Week 3			Average conditions ± standard deviation
		23/06/2021 - 29/06/2021			30/06/2021 - 06/07/2021			07/07/2021 - 14/07/2021			
		Min.	Mean	Max.	Min.	Mean	Max.	Min.	Mean	Max.	
Ambient weather [a]	Temperature (°C)	13.1	21.1	27.6	10.7	18.6	25.8	7.9	18.3	24	19.33 ± 6.8
	Number of rainfall events (dd/mm); **total rainfall (mm)**	0			4 (02/07, 03/07, 06/07); **49.8**			2 (07/07; 08/07); **24.2**			-
Water conditions (umol/L) [b]	Ammonium	0.25			0.34			0.18			0.26 ± 0.08
	Phosphate	0.13			0.23			0.12			0.16 ± 0.06
	Nitrogen dioxide	0.06			0.11			0.05			0.073 ± 0.03
	Nitrate	0.51			2.00			0.37			0.96 ± 0.90
	Silicon oxide	3.24			5.65			2.30			3.73 ± 1.72

[a] Weather data was obtained from the Australian Government Bureau of Meteorology, Queensland website (http://www.bom.gov.au/qld)

[b] Incoming water quality data was obtained through onsite testing by the National Sea Simulator (Sea Sim) through the Australian Institute of Marine Science (AIMS)

Table S6.2. Mean DNA concentrations (µg/µL) of biofilm material extracted from polystyrene and polyvinyl chloride blends (virgin, containing bisphenol A (BPA) and diethylhexyl phthalate (DEHP)) after 0, 7 and 21 days using the NanoDrop UV spectrometer. Background seawater and negative controls are also reported. Replicates are indicated for each sample type.

Polymer type (*n* = 5 per polymer)	**Polymer blend (*n* = 5 per additive type)**	**Mean [DNA] (µg/µL)**		
		Biofilm formation time (days) (*n* = 5 per time point)		
		0	**7**	**21**
Polystyrene microplastics	Virgin	16	9	19
	BPA	46	20	105
	DEHP	6	12	13
Polyvinyl chloride microplastics	Virgin	16	8	25
	BPA	16	20	29
	DEHP	6	6	21
Background seawater (*n* = 2 per time point)		-	59	63
Kit blank (*n* = 3)		4.2		

Table S6.3. Mean DNA concentrations (ng/µL) of biofilm material extracted from polystyrene and polyvinyl chloride blends (virgin, containing bisphenol A (BPA) and diethylhexyl pthahate (DEHP) after 0, 7 and 21 days using the Qubit flurophotometer. Background seawater and negative controls are also reported. All samples were normalised through dilution to ~5 ng/uL for 16S-V4 analysis.

Sample description **(polymer type; additive type; morphology; exposure time (days))**	**[DNA] (ng/uL)**	**Dilution factor for 96-well plate**
Positive control (polystyrene; virgin; microplastic; 0)	0	1:1
Experimental (polystyrene; virgin; microplastic; 7)	-	1:1
Experimental (polystyrene; virgin; microplastic; 21)	13	1:2
Positive control (polystyrene; BPA; microplastic; 0)	-	1:1
Experimental (polystyrene; BPA; microplastic; 7)	3	1:1
Experimental (polystyrene; BPA; microplastic; 21)	-	1:2
Positive control (polystyrene; DEHP; microplastic; 0)	0	1:1
Experimental (polystyrene; DEHP; microplastic; 7)	-	1:1
Experimental (polystyrene; DEHP; microplastic; 21)	14	1:2
Positive control (polyvinyl chloride; virgin; microplastic; 0)	-	1:1
Experimental (polyvinyl chloride; virgin; microplastic; 7)	9	1:1
Experimental (polyvinyl chloride; virgin; microplastic; 21)	-	1:2
Positive control (polyvinyl chloride; BPA; microplastic; 0)	0	1:1
Experimental (polyvinyl chloride; BPA; microplastic; 7)	-	1:1
Experimental (polyvinyl chloride; BPA; microplastic; 21)	13	1:2
Positive control (polyvinyl chloride; DEHP; microplastic; 0)	-	1:1
Experimental (polyvinyl chloride; DEHP; microplastic; 7)	0.7	1:1
Experimental (polyvinyl chloride; DEHP; microplastic; 21)	-	1:2
Background seawater	14	1:2
Negative control (background seawater, 7)	-	1:2
Negative control (background seawater; 21)	0	-

Table S6.4. Pair-wise analysis of variance using a Tukey's posthoc test comparing the relative abundance (%) of dominant bacterial phyla and classes present on all microplastic polymer (polystyrene (PS) and polyvinyl chloride (PVC)) and plasticiser (no plasticiser (virgin) diethylhexyl phthalate (DEHP) and bisphenol A (BPA)) treatments after immersion in flowing, filtered seawater for 7 and 21 days. Significant differences are indicated by * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Polymer-plasticiser composition	PS-virgin*PVC-Virgin		PS-DEHP*PVC-DEHP		PS-BPA*PVC-BPA		PS-virgin*PS-DEHP		PS-virgin*PS-BPA		PS-DEHP*PS-BPA		PVC-virgin*PVC-DEHP		PVC-virgin*PVC-BPA		PVC-DEHP*PVC-BPA	
Exposure time (days)	7	21	7	21	7	21	7	21	7	21	7	21	7	21	7	21	7	21
Phylum																		
Cyanobacteria	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bacteroidota	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Proteobacteria	*	-	*	-	-	-	***	-	-	-	***	-	-	-	-	-	-	-
Acidobacteriota	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Actinobacteroita	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Myxococcota	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Plantomycetota	*	*	*	*	*	*	-	-	-	-	-	-	-	-	-	-	-	-
Verrumicrobiota	-	-	-	-	-	-	-	-	**	-	***	-	-	-	**	-	***	-
Class																		
Acidimicrobiia	*	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Alphaproteobacteria	\	\	\	\	\	\	***	\	\	\	***	\	***	\	\	\	***	\
Bacteroidia	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Chlamydiae	*	*	\	*	*	*	\	\	\	\	\	\	\	\	\	\	\	\
Cyanobacteriia	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Gammaproteobacteria	\	\	\	\	\	\	***	***	\	\	**	**	\	\	\	\	\	\
Kiritimatiellae	\	\	\	\	\	\	\	\	***	\	***	\	\	\	***	\	\	\

Myxococcia	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
OM190	***	\	\	\	\	\	***	\	\	\	\	\	\	\	\	\	*	\
Phycisphaerae	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Planctomycetes	*	\	***	\	\	\	***	\	*	\	***	\	**	\	**	\	\	\
Polyangia	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Rhodothermia	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Thermoanaerobaculia	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\
Verrucomicrobiae	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\	\

Table S6.5. Bray-Curtis intra-individual dissimilarity indices for seawater and polystyrene and polyvinyl chloride microplastics; virgin and containing the plasticisers diethylhexyl phthalate (DEHP) and bisphenol A (BPA) after 0, 7 and 21 days exposure.

Polymer type	**Plasticiser presence**	**Mean Bray-Curtis Index**		
		Exposure time (days)		
		0	**7**	**21**
Polystyrene	Virgin	0.8504	0.7629	0.8298
	DEHP	0.7314	0.7864	0.7967
	BPA	0.8893	0.7612	0.7961
Polyvinyl chloride	Virgin	0.8524	0.7925	0.8089
	DEHP	0.8778	0.8018	0.8035
	BPA	0.8877	0.7689	0.8121
Seawater	-	-	0.7988	0.7891

Table S6.6. Mean weight-average molecular weight (*Mw*) and polydispersity index (PDI) values (± standard deviation) for polystyrene (PS) and polyvinyl chloride (PVC) microplastics; virgin (i.e., no plasticiser) and containing diethylhexyl phthalate (DEHP) and bisphenol A (BPA) after 0 (control), 7 and 21 days of biofouling. Values in **bold** are significantly different from the control (p-value < 0.05).

Polymer Type	**Plasticiser**	**Exposure time (days)**	**Mean *Mw***	**Mean PDI**
PS	Virgin	0	171, 000 ± 600	2.54 ± 0.08
		7	154, 000± 900	2.37 ± 0.06
		21	155, 000 ± 1500	2.39 ± 0.05
	DEHP	0	171, 000 ± 6000	2.54 ± 0.08
		7	163, 000 ± 3500	**2.13 ± 0.04**
		21	166, 000 ± 1500	**2.23 ± 0.02**
	BPA	0	171, 000 ± 6000	2.54 ± 0.08
		7	168, 000 ± 1000	2.36 ± 0.01
		21	**167, 000 ± 1500**	2.43 ± 0.005
PVC	Virgin	0	58, 000 ± 1000	1.97 ± 0.21
		7	59, 000 ± 1500	2.19 ± 0.21
		21	60, 000 ± 500	1.99 ± 0.04
	DEHP	0	58, 000 ± 1000	1.97 ± 0.21
		7	57, 000 ± 1000	1.97 ± 0.05
		21	58, 000 ± 400	2.03 ± 0.05
	BPA	0	58, 000 ± 1000	1.97 ± 0.21
		7	60, 000 ± 2000	1.97 ± 0.04
		21	60, 000 ± 1000	1.97 ± 0.04

Table S6.7. Total relative frequency (%) of bacterial genera with putative degradative potential (as identified in literature[73,171,175,254,312,322]) detected in the seawater and microplastic microbiomes; polystyrene (PS) and polyvinyl chloride (PVC), virgin (i.e., no plasticiser) and containing bisphenol A (BPA) and diethyhexyl phthalate (DEHP) after 0, 7 and 21 days of exposure to marine bio-fouling conditions.

Treatment condition	**Pseudomonas**			**Deslufovibrio**			**Cohaesibacter**			**Bacillus**			**Brevundimonas**			**Erysipelothrix**			**Corynebacterium**		
Time (days)	**0**	**7**	**21**	**0**	**7**	**21**	**0**	**7**	**21**	**0**	**7**	**21**	**0**	**7**	**21**	**0**	**7**	**21**	**0**	**7**	**21**
PS-virgin	470	49	18	0	75	59	0	3	0	228	4	8	131	0	0	0	0	0	2125	0	2
PS-BPA	32	0	5	123	45	34	0	9	3	1023	4	0	0	0	0	0	0	2	908	0	0
PS-DEHP	0	55	2	0	17	15	0	21	270	13	2	0	148	0	0	0	0	0	609	0	0
PVC-Virgin	753	65	11	0	72	51	0	0	35	2739	15	19	0	0	0	0	0	4	1154	0	0
PVC-BPA	134	39	8	0	41	28	0	8	80	1295	4	0	103	0	0	0	0	2	1837	0	0
PVC-DEHP	1095	187	27	0	55	35	0	3	0	722	5	9	69	0	3	0	0	5	1981	0	2
Seawater	-	21	28	-	133	95	-	27	22	-	0	0	-	15	98	-	2	2	-	4	0