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Challenges in optimising methods for extraction of microplastics from complex environmental matrices

by

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Abstract

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Microplastics are a widespread contaminant of concern globally. Society, researchers and policy makers are interested in the quantities of microplastics present in the environment and the harm they pose to habitats, organisms and humans. Due to the rapid rise of research published finding microplastics in new complex environments, there has also been an increase in the development of complicated, multi-staged microplastic extraction methods. Successful and impactful results often rely on efficient and reliable methods. Researchers in this field face challenges when developing methods for these new, complex environments, such as standardisation, validation and producing accessible methods that consider monetary cost and environmental impact.

Validation of methods is rarely undertaken, but when it is, it is achieved by performing a recovery rate study. These studies involve ‘spiking’ the environmental matrix with known amounts of microplastics, running the method and counting the recovered microplastics. To investigate published validation studies, a meta-analysis of 71 recovery rate studies, was carried out. Recovery rates were found to vary dependant on the environment studied, but were highest from plant material, whole organisms and excrement (>88% recovery). The average recovery rates provided an indication that microplastics in the environment could be underestimated by approximately 14% depending on the methods used. Methods were then replicated and developed to extract microplastics from fishmeal, which is a complex environmental medium found to contain microplastics. Fishmeal is a foodstuff that is of high importance for global food security and the microplastic contaminants have potential to enter food chains. Fishmeal properties were measured to investigate their influence on recovery of microplastics. An environmentally benign, generally safe and affordable method using calcium chloride, a dispersant and potassium hydroxide was found to produce the highest recovery rate (66.3%). Positive correlations were found between recovery rate and protein content of fishmeal, whereas negative correlations were found between recovery rate and organic content. To fully optimise methods for assessment of microplastics it is important to consider emerging analytical methods in the field and to test these with environmentally relevant material. The use of solvent extractions and thermal desorption GC-MS were investigated for their effectiveness at identifying chemical indicator markers of virgin microplastics, artificially weathered microplastics and naturally weathered microplastics. Chemical indicator markers were more commonly present in direct thermal desorption samples than the solvent extraction samples, and in some cases the most appropriate markers were present for weathered microplastics, but not for the virgin microplastics.

The findings of this thesis highlight the importance of validating methods when trying to achieve standardisation, and the implications of underestimating true microplastic abundance due to low recovery rates. Development of affordable, safe and environmentally conscious methods is possible, however there may be a trade-off between this and effectiveness of the method. Moreover, this thesis has demonstrated how the recovery of microplastics can vary dependant on environmental and microplastic properties. Thus microplastics should not be considered a single contaminant, but more like a mixture of chemicals and additives with different histories in various matrices. With the prospect of standardised, validated, affordable, safe, environmentally benign and realistic methods, research in this field would become more accessible to many people worldwide, which is needed when studying such a ubiquitous group of pollutants.

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Research Thesis: Declaration of Authorship

Print name: CHLOE JANE WAY

Title of thesis: Challenges in optimising methods for extraction of microplastics from complex environmental matrices

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University.
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
3. Where I have consulted the published work of others, this is always clearly attributed.
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
5. I have acknowledged all main sources of help.
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.
7. Parts of this work have been published as:

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Abbreviations

ABS	Acrylonitrile Butadiene Styrene
ATR-FTIR	Attenuated Total Reflection Fourier Transform Infrared
AW	Artificially Weathered
BEPP	Basic Enzymatic Purification Protocol
BHT	Butylated Hydroxytoluene
BPA	Bisphenol A
CA	Cellulose Acetate
$CaCl_2$	Calcium Chloride
CIMs	Chemical Indicator Markers
CLP	Classification, Labelling and Packaging of substances
DCM	Dichloromethane
EPS	Expanded Polystyrene
FAO	Food and Agriculture Organization
Fe	Iron
FTIR	Fourier Transform Infrared
GC	Gas Chromatography
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HDPE	High-Density Polyethylene
HNO_3	Nitric Acid
IFFO	International Fishmeal and Fish Oil Organisation
IQR	Interquartile Range
KI	Potassium Iodide
KOH	Potassium Hydroxide
KWS	Korona-Walzen-Scheider
LDPE	Low-Density Polyethylene

Li_2WO_4	Lithium Metatungstate
LMP	Large Microplastic
LOI	Loss on Ignition
MAC	Macroplastic
MP	Microplastic
MPSS	Munich Plastic Sediment Separator
MS	Mass Spectrometry
MSFD	Marine Strategy Framework Directive
MSP	Monosodium Phosphate
$Na_2WO_42H_2O$	Sodium Tungstate Dihydrate
NaBr	Sodium Bromide
NaCl	Sodium Chloride
NaClO	Sodium Hypochlorite
NaI	Sodium Iodide
NaOH	Sodium Hydroxide
NCC	Norwegian Coastal Current
NOAA	National Oceanic and Atmospheric Administration
NW	Naturally Weathered
OEP	Oil Extraction Protocol
PA	Polyamide
PAH	Polycyclic Aromatic Hydrocarbon
PBAT	Polybutylene Adipate Terephthalate
PBDE	Polybrominated Diphenyl Ethers
PBS	Polybutylene Succinate
PBT	Persistent, Bioaccumulative and Toxic
PC	Polycarbonate
PE	Polyethylene
PET	Polyethylene terephthalate

PLA	Polylactic Acid
PMMA	Polymethyl Methacrylate
POM	Polyoxymethylene
POPs	Persistent Organic Pollutants
PP	Polypropylene
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PS	Polystyrene
PTFE	Polytetrafluoroethylene
PU	Polyurethane
PVC	Polyvinyl Chloride
PVDC	Polyvinylidene Chloride
Pyr	Pyrolysis
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
RR	Recovery Rate
SBR	Styrene-Butadiene Rubber
SDS	Safety Data Sheet
SEM	Scanning Electron Microscope
SMI	Sediment-Microplastic Isolation Unit
SPT	Sodium Polytungstate
TD	Thermal Desorption
TICC	Total Ion Current Chromatogram
TWP	Tyre Wear Particles
UNEP	United Nation Environment Program
UV	Ultraviolet
WWTP	Wastewater Treatment Plant
$ZnBr_2$	Zinc Bromide

$ZnCl_2$	Zinc Chloride

Chapter 1 Introduction

1.1 Defining plastic and microplastic

Plastic production begins with the distillation of crude oil into fractions of hydrocarbon chains. During polymerisation, polymer chains are formed from monomers such as ethylene and catalysts (Plastics Europe, 2022). With the addition of additives, thermosets and thermoplastics are produced. Thermoplastics are those plastics which can be heated and cooled to change their properties, whilst thermosets change chemically when heated producing a plastic with irreversible properties (Plastics Europe, 2020). Additives are used for functional aspects of plastics (*e.g.* flame retardants and plasticisers), colouring the plastics (pigments), as a filler (calcium carbonate) and as reinforcements to the plastics (glass fibres) (Hahladakis *et al.*, 2018). The majority of these additives are not polymerised with the plastic monomers and thus are not chemically bound to the polymer, meaning they have the potential to disassociate and enter the surrounding environment (Hahladakis *et al.*, 2018).

Wide production of the plastic products we know today began in the 1950s, however the first synthetic plastic called Bakelite was produced in the early 1900s (Geyer *et al.*, 2017). With plastic now generally being manufactured with a single use lifecycle, production has increased exponentially. In 2019, 368 million tonnes of plastic was produced, with China accountable for 31% of this total (Plastics Europe, 2020). Globally, polypropylene (PP) and polyethylene (PE) are the most-used types of plastic due to their low production cost and wide transformability into a range of products. These plastics are used in many types of food packaging, household goods, carrier bags, cling film, door frames and building supplies (Andrady and Neal, 2009). With such a large increase in production over time, there comes a large increase in the creation of plastic waste. Currently, only 14% of the plastic used for packaging globally has been recycled (Stiftung, 2020), meaning a large proportion is left over as waste and often deposited into landfills or incinerated. Of this plastic waste destined for disposal, it is estimated that between 15-40% is mismanaged and then lost to the environment (Jambeck *et al.*, 2015). Due to the way most plastics are made, with structures of long carbon chains, it makes the majority extremely resistant to degrading (Zheng *et al.*, 2005). Therefore, if plastic material is mismanaged and is lost in the environment, it will become exposed to forces which allow it to weather and be broken into smaller pieces as microplastics or nanoplastics.

Plastic may be weathered in a variety of ways including by ultraviolet (UV) radiation, microorganisms, physical abrasion and changes in temperature and salinity (Jahnke *et al.*, 2017), ultimately reducing the plastic into smaller sizes and also causing some chemical changes. Not only do the smaller sized plastics continue to be weathered, but they may also release potentially harmful additives which are not chemically bonded to the plastic, such as phthalates, metals, polybrominated diphenyl ethers (PBDEs) and bisphenol-A (BPA) into the surrounding environment (Sheng *et al.*, 2021). Moreover, plastic can also be a vector for harmful substances such as polycyclic aromatic hydrocarbons (PAHs), BPAs, PBDEs and metals through adsorption (Sheng *et al.*, 2021). Smaller size plastics are often categorised into either primary or secondary microplastics. Primary microplastics are those which are manufactured to be a small size (usually <5 mm), such as nurdles (pre-production pellets), whereas secondary microplastics are those which have been fragmented by environmental weathering described previously, or are produced when plastic becomes brittle and breaks down due to loss of plasticisers (Horton and Dixon, 2018). Examples of secondary microplastics could include fibres released from washing clothing or tyre wear particles (Hartmann *et al.*, 2019).

Although the term 'microplastics' has been used in the literature since the early 2000s, there is still no clear consensus between researchers in this field about what size categories these plastics should fall in to (Table 1.1). A definition was proposed by a meeting of experts in the field in 2008 which defined microplastics as those below 5 mm (Arthur *et al.*, 2008), which was then adopted by many going forward. However, no lower limit was set by the same group as it was seemingly assumed that as 333 μm neuston nets were used to collect the plastics in marine environments, thus there would be no need to consider plastics of a smaller size, but this view has since been much challenged. A unified definition of these size categories is essential to allow for accurate comparison across studies, and for meaningful results to be portrayed to others. Therefore a definition encompassing the physiochemical aspects of plastics would be more suitable. Frias and Nash (2019) attempted to unify researchers by proposing a definition which includes the properties of microplastics as well as the size. This definition is: *"Microplastics are any synthetic solid particle or polymeric matrix with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water"*. Since this has been published, large organisations have also offered definitions. International Organization for Standardization (2020) suggested that nanoplastics are those below 1 μm and microplastics are divided into small microplastics (1 μm-1 mm) and large microplastics (1-5 mm). As this has been developed by an internationally recognised organisation committed to standardising, this definition is used and followed in this thesis.

Table 1.1 **Examples of plastic sizes used by previous studies**

Different sizes used to classify nanoplastics, microplastics, mesoplastics and macroplastics in previously published studies, including the size categories to be used in this thesis. Adapted from Hartmann *et al.* (2019).

Authors	**Nano plastic**	**Micro plastic**		**Meso plastic**	**Macro plastic**
Andrady (2003)		67-500 μm		5-10 mm	1-15 cm
Browne *et al.* (2007)	<1 μm	1-1000 μm			>5 mm
Moore (2008)		<5000 μm			>5 mm
Ryan *et al.* (2009)		<2000 μm		2-20 mm	>2 cm
Costa *et al.* (2010)		<1000 μm			
Desforges *et al.* (2014)		1-5000 μm			
Wagner *et al.* (2014)	<20 μm	20-5000 μm		5-25 mm	>2.5 cm
Koelmans *et al.* (2015)	1-100 nm	100 nm-5000 μm			>5 mm
Andrady (2015b)	<1 μm	1-1000 μm		1-25 mm	2.5-100 cm
Koelmans *et al.* (2017)	<335 μm	335-5000 μm			>5 mm
Alimi *et al.* (2018)	<100 nm	0.1-5 mm		5-25 mm	>25 mm
Alimba and Faggio (2019)	1 nm-20 μm	20 μm-5 mm		5-25 mm	>25 mm
Bucci *et al.* (2020)		<5 mm			>5 mm
Wang *et al.* (2021b)	<100 nm	<5 mm			
International Organization for Standardization (2020)	<1 μm	Small	Large		
		1 μm-1 mm	1-5 mm		
This Thesis	**<1 μm**	**Small**	**Large**		
		1 μm-1 mm	**1-5 mm**		

1.2 Microplastics in the environment

1.2.1 Distribution of microplastics

Global literature reporting the presence of microplastics is increasing, and current research has highlighted the widespread abundance of microplastics in the environment. For example, Yu *et al.* (2020) analysed 1138 publications which showed that microplastics were present on seven continents and in four oceans. Researchers are now investigating quantities of microplastics in various environmental media. Some of this media includes seawater, freshwater, sediments, agricultural soils, wastewater treatment effluent, sludge and air. However, in a recent meeting by the UN Environment Assembly, where the widespread nature of plastic pollution was acknowledged, a resolution to create a legally binding global agreement was created to end plastic pollution, including moves to make analytical methods more widely accessible (UNEP, 2022).

Microplastics are present in many seawater bodies, but the quantities (and the units reported[1]) vary considerably. For example, in the mid-west Pacific Ocean, an average of 34,039 microplastics/ km^2 were found in the sea surface (Wang *et al.*, 2020), and 990-6,999 microplastics/ m^3 were found in near-surface samples of the Atlantic Ocean, (Pabortsava and Lampitt, 2020). Microplastics are also present in smaller seawater bodies. In Algerian waters of the Mediterranean Sea, 0.86 microplastics/ m^3 were found (Setiti *et al.*, 2021); in a study spanning 6 years, 91,128 microplastics/ km^2 were found in the surface waters around Scotland (Russell and Webster, 2021); and microplastics have now been found in seawater and associated coral reef polyps (Lei *et al.*, 2021). The distribution of macroplastic quantities are extremely uneven across the ocean basins. Eriksen *et al.* (2014) created a model that estimates that there are approximately 5.25 trillion plastic particles floating in the ocean, and that more of these plastics are situated in the Northern Pacific and Northern Atlantic (55%) compared to the Southern Pacific and Southern Atlantic (15%). However these authors explain how this is a minimum value and could be higher as other aspects of plastic pollution such as beach plastics and ingested plastics are not included in the estimate.

Microplastics are present in bodies of freshwater. In the largest freshwater lake system of China, microplastics have been found in quantities of 1,064 microplastics/ m^3 (Jian *et al.*, 2020). Researchers have discovered the presence of microplastics in pristine environments such as the Antarctic. For example, Gonzalez-Pleiter *et al.* (2020) found 0.95 microplastics/ 1000 m^3 in Antarctic freshwater. The abundance of microplastics has been researched in areas in which fresh and seawater mix. For instance, 545.8 microplastics/ m^3 were discovered in the Terengganu estuary in Malaysia (Taha *et al.*, 2021) and 10-168 microplastics/ m^3 in an estuary in Morocco (Haddout *et al.*, 2022).

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[1] The units used to report the presence/amounts of microplastics vary from study to study. For example, the units could be based on number of particles, weight of particles per mass, volume, area or length of a given environmental matrix (Hanvey *et al.*, 2017). The differences in units used make for difficult conversions and subsequent difficult comparisons.

Microplastics within water bodies often settle into the sediment below. In the Western Pacific, researchers found 240 microplastics/ kg of sediment taken from the deep sea (Zhang *et al.*, 2020). As well as deep sea sediment, microplastics have been found in beach sediments of India (40.7 particles/ m^2) (Robin *et al.*, 2020), and in the marine sediment of North East Spain (32.4 microplastics/ kg) (Exposito *et al.*, 2021).

Researchers have detected microplastics in environmental matrices inland, such as within agricultural soil. Researchers from China have found up to 3,410 microplastics/ kg of soil in Shaanxi province (Ding *et al.*, 2020) and 161 microplastics/ 100 g of soil in Xinjiang (Hu *et al.*, 2021). Similarly, in Spanish agricultural soil, up to 1,100 microplastics/ kg were found in soil before being treated with sewage sludge (van den Berg *et al.*, 2020).

Researchers have recently been focusing on wastewater treatment plants (WWTP) as a source of microplastics into the environment. Microplastics may enter the WWTPs from cosmetics that are flushed down household drains, or from washing machines (Carr *et al.*, 2016). The effluent is often discharged into water systems, and sludge can be applied to agricultural soils, furthering the spread of microplastics. Akarsu *et al.* (2020) found that 180×10^6 microplastics are released per day from a WWTP effluent into Mersin Bay in Turkey. Similarly, 2.4×10^5 microplastics are released from a WWTP in New Zealand into surrounding coasts (Ruffell *et al.*, 2021). With regard to the sludge within WWTP, researchers have found 6.74×10^3 microplastics per kg of dry sludge at a WWTP in Nanjing China (Yuan *et al.*, 2022).

As microplastics, most commonly microfibres, are lightweight and small in size, they are often found to be airborne. For example, up to 4.18 microplastics/ m^3 was found in the air in Shanghai (Liu *et al.*, 2019b). However, airborne microplastics have been found in abundance indoors. Liao *et al.* (2021) found more microplastics indoors (1,583 microplastics/ m^3) than outdoors (189 microplastics/ m^3) in China. More recently, it has been found that air conditioner units increase the amount of microplastics within indoor spaces. When an air conditioner unit is turned on microfibres were found to increase from 0.49/ m^2/ day to 1.19/ m^2/ day inside a room (Chen *et al.*, 2022). Furthermore, this is often a misconception that there are more fibres in air samples than fragments. However, many studies have found more fragments when sampling the air (Klein and Fischer, 2019; Dehghani *et al.*, 2017; Allen *et al.*, 2019). The reason for this may be due to the different sampling locations or different sampling techniques (Gaston *et al.*, 2020).

This evidently shows the ubiquitous nature of microplastics, now present in multiple different environmental matrices. These particles can be found gathered in “hotspots” in certain environments. Although there is now hope to “end plastic pollution” via international policy initiatives, this will take large efforts and still relies on scientists and researchers to continue monitoring microplastics in new complex environments.

1.2.2 Transportation of microplastics

Microplastics can be transported around the environment in many ways such as through the air (with support of rain, wind and snow), through river systems, through processes within the ocean (e.g. Stokes drift, Ekman currents, thermohaline current, melting of sea ice and interacting with organisms), through farming processes, by agricultural run-off, from WWTP effluent and from road dust (Figure 1.1). However, the way in which these small particles move can be affected by factors such as their size, density and by how bio-fouled/weathered they may be (YanfangLi *et al.*, 2020).

Research on microplastics in the air is beginning to emerge, with evidence that the weather, including rain and snow has an effect on their deposition and how far they travel. For example, microplastics found in the remote Pyrenees Mountains were discovered to be from a source located 95 km away, and that the long distance travelled was due to the precipitation and air movements in the area (Allen *et al.*, 2019). Similarly, Purwiyanto *et al.* (2022) found that more microplastics were deposited in Jakarta, India, during the rainy season (23 microplastics/ m^2/ d^1) than during the dry season (5 microplastics/ m^2/ d^1), and in the Western Italian Alps, up to 49 microplastic/ L were deposited in snow (Parolini *et al.*, 2021).

Microplastics have the potential to travel within river systems. For example, researchers in South Korea found more microplastics in the downstream area of a river (4,760 microplastics/ m^3) than the upstream area of a river (293 microplastics/ m^3), and more microplastics at the surface, than mid-water in the Nakdong River (Eo *et al.*, 2019). The microplastics made from denser polymers or those that are biofouled may sink to the bottom of the rivers and become integrated within the sediment (He *et al.*, 2021). Microplastics are often released into rivers in effluent from WWTPs. A study in Spain estimated that 350 microplastics/ m^3 were released from treated effluent into the Henares River each day, which at typical flow rates equates to 300 million microplastic particles per day (Edo *et al.*, 2020). A review has found that between 0.5-50 microplastics/ L are found in WWTP effluent (Gkatzioura *et al.*, 2021). River systems are a key transport pathways of microplastics into the ocean. Lebreton *et al.* (2017) created a model that estimated that between 1.15 and 2.4 million tonnes of plastic enters oceans worldwide from rivers every year. However, this estimate must be interpreted with caution as the model only considers estimates of floating riverine plastic, and there can be more plastics present in the water column, potentially increasing this estimation.

Estuarine process can affect the movement of plastics into the oceans. Biltcliff-Ward *et al.* (2022) examined transport pathways of microplastics in estuaries and found that microplastic concentrations were higher in the sediments than in the water column, meaning that estuaries act as a trap for microplastics in these systems. Furthermore, Stead *et al.* (2020) found that microplastics can become trapped within sediments of salt marshes in the UK. They also found that microplastic fibres decreased in abundance during the ebb tide compared to the flood tide.

Once microplastics enter the ocean, oceanographic and biological processes facilitate the transport of the particles further away from the original source. The process of Stokes drift (waves) is often responsible for the transport of microplastics to Arctic regions, whereas Ekman currents (wind-driven ocean currents) are responsible for the transport of microplastics to accumulation zones in ocean gyres, particularly in subtropical areas (Onink *et al.*, 2019). Currents can meet the seafloor and have the potential to transport microplastics, or deposit them into sediments (Huang *et al.*, 2021). Thermohaline currents create areas of suspended sediment in the deep sea and are known to be responsible for resuspension and transport of microplastics (Kane and Clare, 2019). In more recent years, with global temperatures increasing, melting sea ice has been found to be a source of microplastics into the ocean (von Friesen *et al.*, 2020). In areas where algal concentrations are high, microplastics may become biofouled, making them more likely to sink faster into the deep ocean (Lobelle *et al.*, 2021). One final way microplastics may be transported around the ocean is when they are ingested and egested by fish and other marine organisms. However, this could take between 7 and 49 days (Ory *et al.*, 2018).

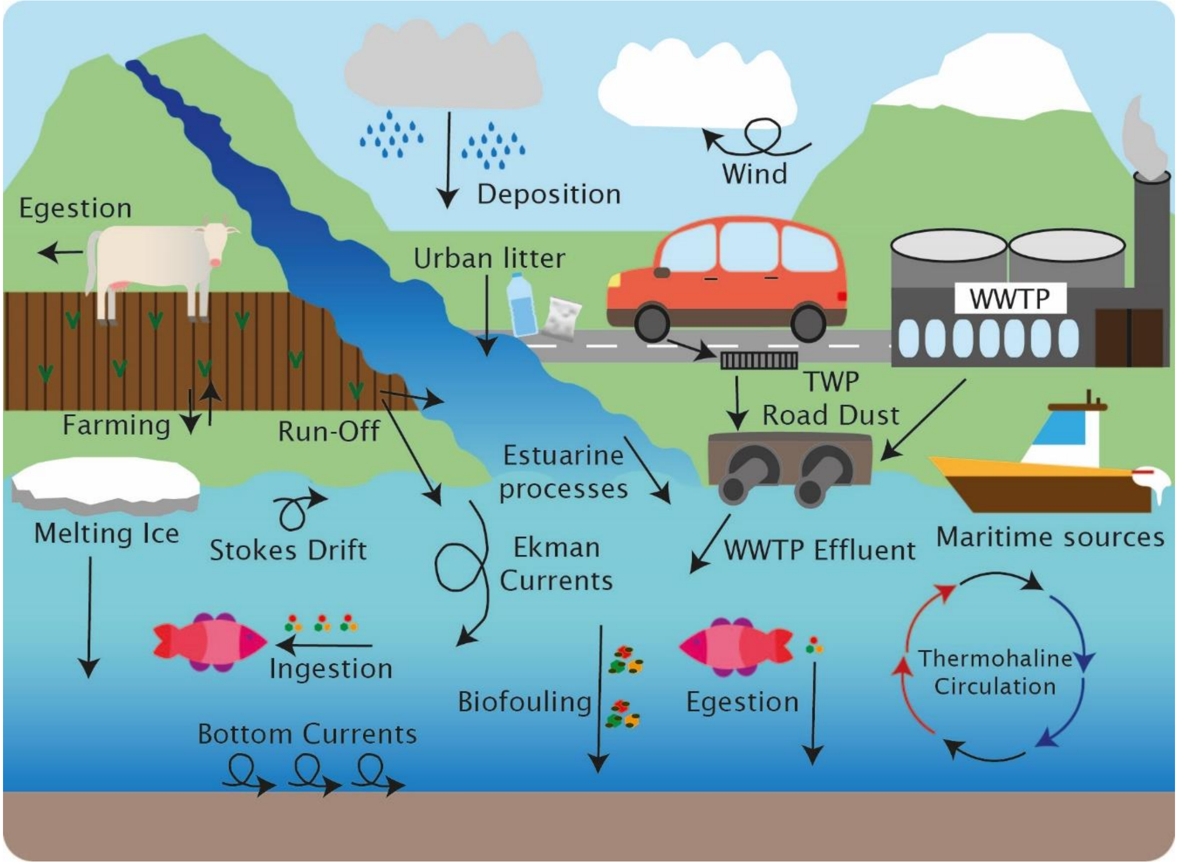

Figure 1.1 **Transport of microplastics in the environment**

Potential transport pathways of microplastics around the environment. Adapted from Huang *et al.* (2021).

Microplastics can be transported when on land. Sludge from WWTP is often deposited on agricultural land as soil improver, and other farming processes such as harvesting can contribute to the disturbance and transport of microplastics elsewhere (Huang *et al.*, 2021). Similarly, mulching in agricultural soils can transport the plastics further into the soils, where they may be broken down further by physical and chemical processes (Wong *et al.*, 2020). During heavy rainfall, microplastics may also be swept into ocean and rivers from agricultural run-off.

Even though tyre wear particles (TWPs) were considered a pollutant as early as the 1970's (Cadle and Williams, 1979), they have been largely overlooked until recently. They contain a mixture of styrene butadiene rubbers, natural rubbers, carbon black and other chemicals, ultimately classing them as a microplastic (Knight *et al.*, 2020). The inclusion of TWPs as a type of microplastic has been under much debate. The international standards organisation (ISO) do not class rubber as a plastic, and due to the many types of TWPs (recycled tyre crumb (RTC) and tyre repair-polished debris (TRD)) (Luo *et al.*, 2021), the decision of inclusion is complicated. However TWPs have been discovered in large amounts and are classed as a source of microplastics in the environment by many researchers. These have been found on roads in areas associated with high levels of acceleration and breaking. Average amounts of TWPs were 0.6-65 particles in 5 mL of material tested (O'Brien *et al.*, 2021). It has been estimated that the average emission of tyre wear dust per person is 0.81 kg/ year (Kole *et al.*, 2017). These particles can be swept down stormwater drains which then have the potential to be transported to aquatic environments during heavy rainfall. In Perth, Australia, an average of 664 microplastics/ kg of sediments were found in the stormwater drains (Lutz *et al.*, 2021).

As a result of microplastics being small in size thus being easily transported, spreading wide across the globe and interacting with many organisms; microplastics are often being discovered in new, often complex environmental matrices.

1.3 Complex environmental matrices

Due to the ubiquitous nature of microplastics, it is no surprise they are now being found in many complex environmental matrices. Environmental matrices can be complex due to the amounts of rich organic and carbonate components. Examples of some complex matrices include soils and biosolids found in wastewater treatment plants. These matrices are often challenging for researchers when it comes to extracting plastics, as organic rich components are often difficult to fully digest (Hurley *et al.*, 2018). The organic material in soil varies, with many different functional groups, including residues of decomposing plants and organisms, often making it difficult to distinguish and identify the chemical properties of microplastics within (Bläsing and Amelung, 2018). Another environmental matrix which is emerging to be contaminated with microplastics is fishmeal. Fishmeal is made from fish that are cooked, strained and broken down into a foodstuff. It has a high organic content and differing levels of proteins, fats and vitamins. It is essential in poultry, pig and aquaculture farming as feed, and is of great economic value, meaning the monitoring of microplastic contaminants is necessary. Fishmeal will be the complex environmental matrix of focus in this thesis.

Fishmeal has a high nutritional content including proteins, omega-3 fatty acids, amino acids and vitamins, supporting the diet of farmed animals (IFFO The Marine Ingredients Organisation, 2020). Small, oily, bony fish are most commonly used in the production of fishmeal as they are often not used for human consumption (Food and Agriculture Organization of the United Nations, 1986). Some are produced from the trimmings of fish caught for human consumption, however only a small amount. For example, 98% of landings of Peruvian anchovies (*Engraulis ringens*) are used to produce fishmeal, leaving 2% to be sold for consumption (Wijkstrom, 2010). To produce fishmeal, captured fish are broken down, cooked and strained before being pressed into a presscake. The presscakes are dried and debris is removed before being milled, ready for packaging (Food and Agriculture Organization of the United Nations, 1986). The steps in this process are shown in Figure 1.2.

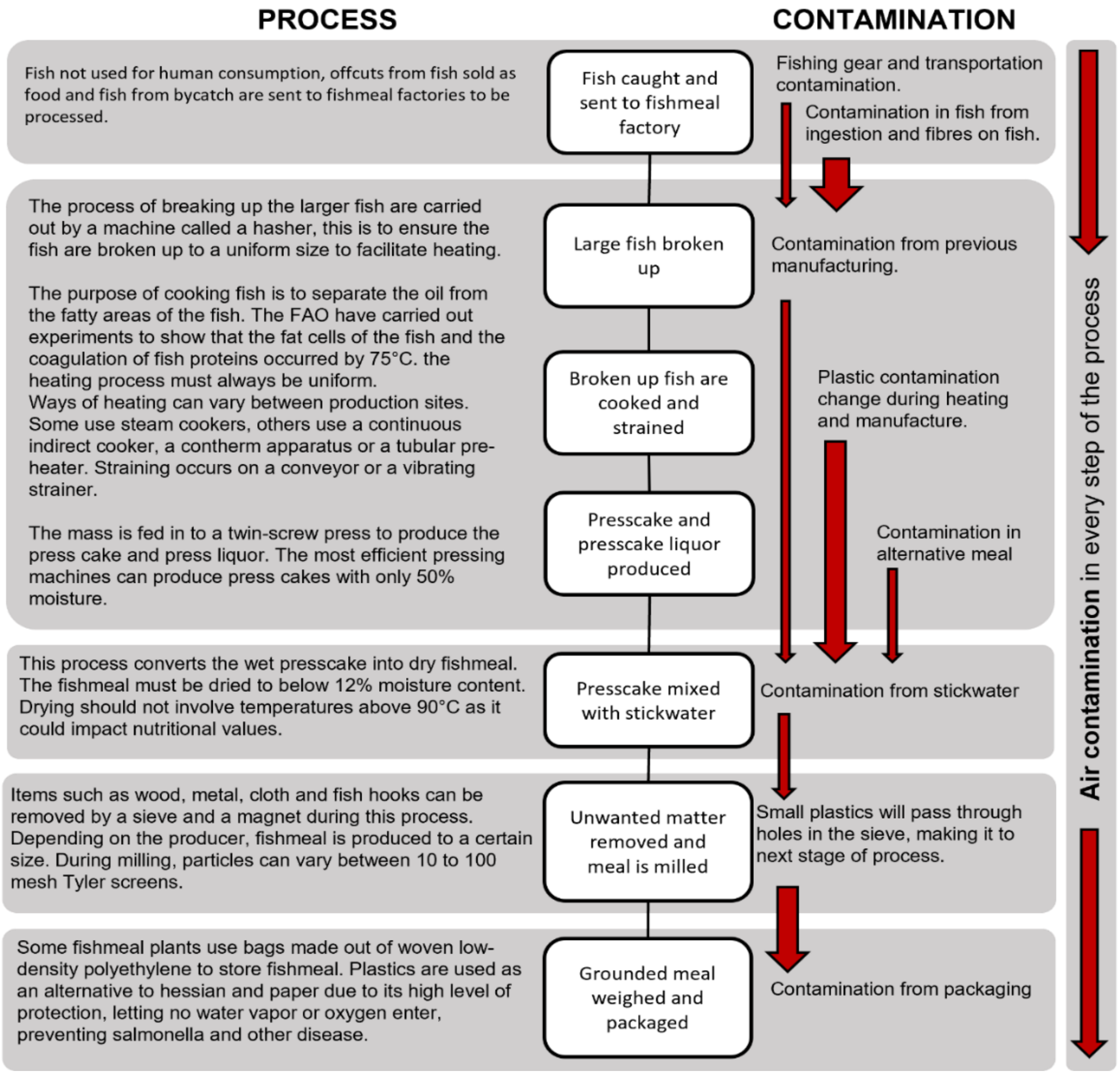

Figure 1.2 **Fishmeal production process**

Process of the production of fishmeal according to Food and Agriculture Organization of the United Nations (1986), adapted to included potential sources and pathways of microplastic contamination, with thickness of arrows representing the potential for the contamination to move to the next step of the process.

Over time, the main use and consumption of fishmeal has drastically shifted. In the 1960s, 50% of fishmeal was used as pig feed and 48% as poultry feed, with only 2% used as feed for aquaculture (Figure 1.3). The 1980s showed an increase in aquaculture salmon farming, thus pig feed dropped to 36%, poultry feed increased to 50% and aquaculture increased to 10% (Figure 1.3). With alternative feeds now available, by 2010, fishmeal used for poultry and pig feed dropped to only 25% combined. However, fishmeal used for feed in aquaculture is now the most dominant, using 75% (Figure 1.3) (Shepherd and Jackson, 2013). In 2016, aquaculture provided 171 million tonnes of fish, with the majority (88%) to be used as food for humans (FAO, 2018). This highlights the importance of the aquaculture industry with respect to food security globally.

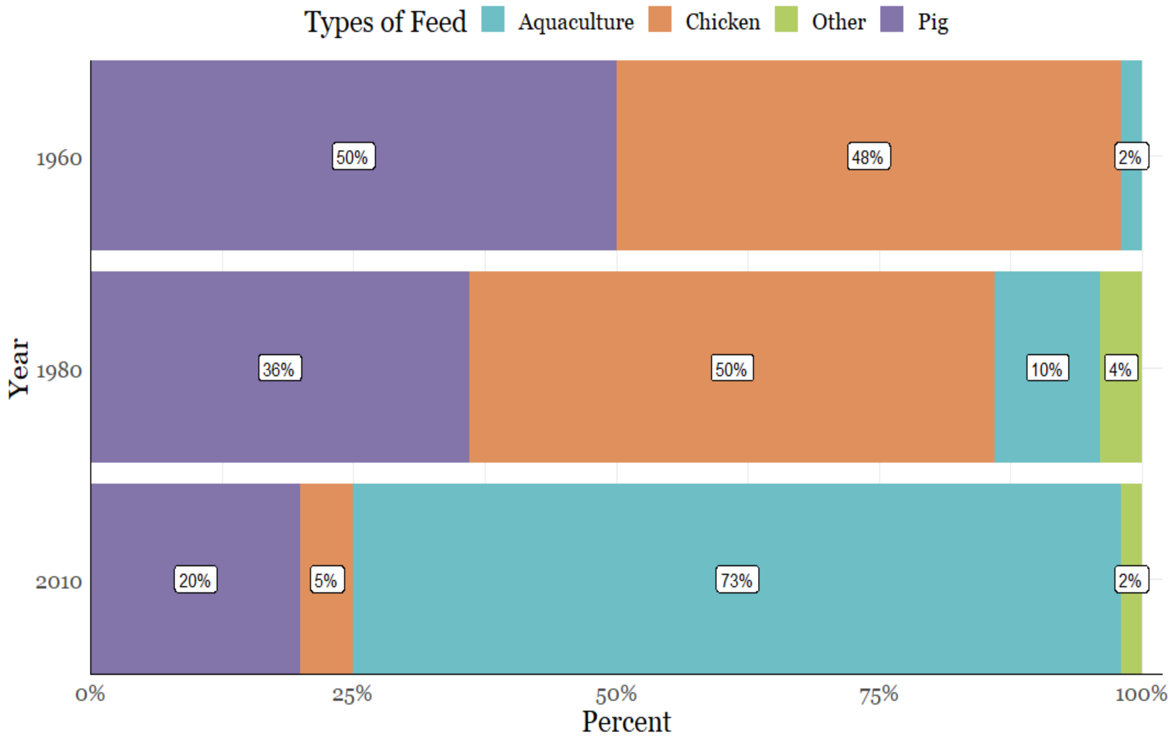

Figure 1.3 **Fishmeal consumption**

Percentage of consumption of fishmeal worldwide for each industry sector from 1960, 1980 and 2010. Adapted from Shepherd and Jackson (2013).

Research on the amount of microplastics in fishmeal is still in its infancy, with the first research being published in 2019. Hanachi *et al.* (2019) began looking at the amount of microplastics in sardine, salmon and kilka fishmeal. An average of 565 microplastics/ kg was found (Table 1.2), with fragments being the most common shape of plastic, and polypropylene and polystyrene being the most common polymers found. Next, Karbalaei *et al.* (2020) studied Malaysian fishmeals, including fish waste, and found on average 5,000 microplastics/ kg, with fragments also the most dominant shape of microplastic found. In 2021, Gündoğdu *et al.* (2021) examined 26 different types of fishmeal and found on average 176 microplastics/ kg, with the highest levels in fishmeal sourced from China (337 microplastics/ kg) and Morocco (253 microplastics/ kg). Thiele *et al.* (2021) assessed microplastics in commercial fishmeal samples and found on average 123 microplastics/ kg. The most common fragment was polyethylene, and the most common fibre was the semi-synthetic polymer rayon. Yao *et al.* (2021) studied the microplastics within fish and shrimp meals and found on average 45 microplastics/ kg. This research observed eight different colours of microplastics and the most dominant shape of microplastics were films, mainly made from polyethylene and paraffin. Although paraffin is a wax, it is classed as a plastic by the International Organisation for Standardisation (International Organization for Standardization, 2020). Wang *et al.* (2021a) studied microplastics from ten different source countries and found on average 5,460 microplastics/ kg. Unlike the studies mentioned previously, this study found fibres to be the most common shape and cellophane, polypropylene and polyethylene terephthalate (PET) to be the most dominant polymer type.

Table 1.2 **Average microplastics found in fishmeal from existing literature**

Average amount of microplastics found per kg of fishmeal studied by existing authors, including the type of fish used for fishmeal, fishmeal source location, method detail, average amount of microplastics found, the most common polymer and shape of microplastic found and the quality assurance/quality control (QA/QC) undertaken, for each study.

Author	**Fish used in fishmeal**	**Fishmeal source location**	**Method detail**	**MPs found (items/kg)**	**Shape and polymer found**	**QA/QC**
Hanachi *et al.* (2019)	Sardine (*Clupeidae)*, Salmon (*Salmonidae)*, Kilka (*Clupeonella cultriventris)*	Persian Gulf & Caspian Sea	KOH digestion, heated at 40 °C for 72 hrs, NaI sonication and centrifugation	565	Fragment PP and PS	Laminar flow, glassware cleaned with ethanol, solutions filtered, cotton lab coats and gloves, procedural blanks.
Karbalaei *et al.* (2020)	Mackerel (*Scombridae)* & fish waste	South China, Malaysia	KOH digestion, heated at 40 °C for 72 hrs, NaI sonication and centrifugation	5,000	Fragment PE and PP	Laminar flow, procedural banks, cotton lab coats and gloves, glassware cleaned with ethanol, equipment covered with foil, solutions filtered.
Gündoğdu *et al.* (2021)	Krill (*Euphausiacea)*, Anchovy (*Engraulidae*), Sardine, Sandeel (*Ammodytes*), Herring (*Clupeidae*), Blue Whiting (Micromesistiu	Antarctica, Chile, China, Denmark, India, Morocco, Mauritania, Norway, Peru, South Africa, Turkey	30% KOH: NaClO digestion and NaI density separation	176	Fragment PE and PP	Jars covered with foil, glassware cleaned and placed in acetone bath, solutions filtered, laminar flow, surfaces cleaned with acetone, five replicates of negative control blanks, petri dish left open for environmental contamination.

Author	**Fish used in fishmeal**	**Fishmeal source location**	**Method detail**	**MPs found (items/kg)**	**Shape and polymer found**	**QA/QC**
	s poutassou), Pilchard (*Sardina pilchardus*), Sprat (*Sprattus sprattus*), Ammodytes, Triggerfish (*Balistidae*), Ribbon Perch (*Percidae*), Carangids (*Carangidae*), Mackerel					
Thiele *et al.* (2021)	Whitefish	N/A	NaCl density separation	123	Fibres PE and Rayon	Cotton clothing and lab coats, clothing lint rolled, surface cleaned with ethanol, clean air cabinet, glass and metal equipment used, filter placed in furnace at 500°C, solutions filtered, damp filter left for contamination, two procedural blanks.
Yao *et al.* (2021)	Fishmeal and shrimp meal	China, Russia, Denmark, Peru, Thailand	NaCl density separation, settle for 24 hrs, filtered, filtered washed with H_2O_2 for 72 hrs at 65 °C with shaking at 80 rpm	45	Films PE and paraffin	Tools and containers cleaned with filtered Milli Q water, samples kept in enclosed space, three procedural blanks.
Wang *et al.* (2021a)	Anchovy, Dace (*Leuciscus leuciscus*), Trash fish, Carp (*Cyprinidae*), Sandeel, Brevoortia, Sardine, Mackerel, Pollock (*Pollachius*), Flounder (*Paralichthys*), Cod (*Gadus morhua*), Herring	USA, Russia, Peru, Panama, Myanmar, Mexico, Mauritania, Denmark, China, Chile	H_2O_2 and KOH heated at 75 °C for 6 hours	5,460	Fibres Cellophane, PP and PET	Solutions filtered, glass petri dishes rinsed with filtered water, cotton lab coats and gloves, laminar flow, three procedural blanks.

The numbers of microplastic items found in a kg of fishmeal can be as high as 5460. Whether these amounts have impact on global food security is dependent on the effect microplastics will have on human health, which is an area that is yet to be fully understood (Vethaak and Legler, 2021). However, large amounts of fishmeal is used as feed for aquaculture, for example Alltech (2013) estimates that 45 million tonnes was used as aquaculture feed in

2012. These aquaculture facilities will ultimately produce fish which is used as food for humans worldwide. Therefore it is important that microplastic contamination in fishmeal is monitored regularly and impacts of the pollution on the fish and humans is communicated between researchers, fishmeal producers and regulatory bodies.

Fishmeal is an extremely varied and diverse medium. Table 1.2 shows the large variety of fish that fishmeal can be made from. Each different species will have a different chemical makeup, meaning the oil, protein and mineral content will differ, therefore producing largely different fishmeal. Having a medium that can vary significantly from sample to sample, increases the complexity when it comes to isolating and extracting microplastics from within. Fish contain very high levels of organic material, and many inorganic components such as bones and scales, which will need to be digested or removed to isolate the microplastics. Methods to remove this material have been developed over the years in the field of microplastics and include techniques such as density separations and chemical and enzymatic digestions, examples of these are reviewed in Section 1.4. Furthermore, each of the researcher teams behind these methods have deployed varying levels of quality assurance and quality control to ensure levels of contamination are monitored and kept to a minimum (Table 1.2). For example, all of the researchers in Table 1.2 ran procedural blanks, all researchers but Yao *et al.* (2021) used a laminar flow cabinet and filtered solutions before use, and two research teams (Thiele *et al.*, 2021; Gündoğdu *et al.*, 2021) placed dampened filter papers in the laboratory to check for environmental contamination. The results from the studies with the more stringent QA/QC can be considered more reliable, as the risk of mistakenly assuming plastics are from the fishmeal rather than from the environment is lower.

1.4 Microplastic extraction methods

The study of microplastics has expanded enormously over the last 10 years. A search on Google Scholar (12/07/2022) using the term "microplastics" for the year of 2010 produced 195 results, whereas as the same search term for the year of 2020 produced 10,600 results. New methodologies are frequently being developed to increase the recovery rates of microplastics from environmental media, thus increasing the effectiveness of the method. However, having multiple new methods brings issues when trying to create quantitative comparisons, therefore the need for standardisation is of growing importance in the study of microplastics (Underwood *et al.*, 2017). At present, the methods used to extract plastics from environmental media include density separations with saline solutions and oils, digestions with acids, bases, oxidising agents and enzymes, physical separations with magnets, sieves and funnels, each with differing effectiveness, costs and safety issues (Table 1.4). Each of these methods are valued differently by different researchers and may be used individually or combined.

1.4.1 Density separation

1.4.1.1 Saline solutions

The use of density separation as a way to extract microplastics was first reported in 2004 by Thompson *et al.* (2004). This form of extraction uses high density saline solutions to allow floatation of less dense plastics. Some microplastic types such as low-density polyethylene (LDPE), high-density polyethylene (HDPE) and polypropylene (PP) already have a lower density than water (Table 1.3), allowing them to float with ease (Rodrigues *et al.*, 2018). Some commonly used saline solutions with varying densities include sodium chloride (NaCl) (1.19 g/cm^3) (Nuelle *et al.*, 2014), calcium chloride ($CaCl_2$) (1.39 g/cm^3) (Thomas *et al.*, 2020), sodium bromide (NaBr) (1.41 g/cm^3) (Liu *et al.*, 2019c), zinc chloride ($ZnCl_2$) (1.7 g/cm^3) (Wang *et al.*, 2018), sodium iodide (NaI) (1.8 g/cm^3) (Nuelle *et al.*, 2014), zinc bromide (1.71 g/cm^3) ($ZnBr_2$) (Quinn *et al.*, 2017), sodium polytungstate (SPT)(1.4-1.65 g/cm^3) (Zhang *et al.*, 2020), sodium tungstate dihydrate (Na_2WO_4 $2H_2O$) (1.4 g/cm^3) (Frias *et al.*, 2018), lithium metatungstate (Li_2WO_4) (1.62 g/cm^3) (Masura *et al.*, 2015), potassium iodide (KI) (1.7 g/cm^3) (Santana *et al.*, 2022) and monosodium phosphate (MSP) (1.45 g/cm^3) (Cutroneo *et al.*, 2021). The densities of these saline solutions will change dependent on concentration of salt added and the temperature of the solutions. The concentrations of these solutions are rarely reported per concentrations and temperature and is something that needs to be reported as standard practice in the literature. These solutions are more often used independently, but occasionally researchers may combine more than one solution to lower costs or increase effectiveness of a method (Constant *et al.*, 2021).

1.4.1.2 Sodium chloride

Sodium chloride is the most frequently used saline solution in microplastic research due to its low cost and wide availability (Cutroneo *et al.*, 2021; Tirkey and Upadhyay, 2021). Using this solution was recommended by the NOAA Marine Debris program (Masura *et al.*, 2015). However, with a density of only 1.19 g/cm^3, sodium chloride does not have the ability to float the denser microplastic polymers such as PET and PVC (Table 1.3). With this being said, dependent on the spiking plastic used, some studies have achieved recovery rates up to 95% using NaCl as a saline solution to extract microplastics from sediment (Quinn *et al.*, 2017). NaCl is not seen as a hazardous substance. It may only cause organoleptic (smell/taste) issues in humans if directly consumed in amounts higher than 250 mg/L (Siegel, 2007).

1.4.1.3 Sodium iodide

Sodium iodide (NaI) is used less than sodium chloride but is nonetheless incorporated into many methods due to its potential of reaching a higher density. With a higher density (1.8 g/cm^3) it is more likely to recover the higher-density plastics. Using NaI in a microplastic separator unit with freshwater and marine sediment, Nakajima *et al.* (2019b) achieved recovery rates of 94-98%. More recently this saline solution has been used to successfully extract tyre wear particles (Jarlskog *et al.*, 2021; Jarlskog *et al.*, 2020). The clearest argument against using this solution is due to the cost of making large amounts. For example Kedzierski *et al.* (2017) estimated that it would cost ~£780 to prepare the 2 L NaI (1.8 g/cm^3) solution needed to extract microplastics from 1 kg of sediment. Researchers have attempted to solve this issue by using NaI as a second, smaller, density separation stage after using a large NaCl density separation (Nuelle *et al.*, 2014), or by recycling the same NaI solution after each use (Kedzierski *et al.*, 2017). However, recycling comes with disadvantages such as lowered density, thus lowered recoveries over time, and the risk of exposure to contamination (Kedzierski *et al.*, 2017). Another reason against the use of this solution is its hazardous properties. According to REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) and CLP (Classification, Labelling and Packaging of substances) regulations, NaI can have long-lasting hazardous effects on aquatic environments and may cause respiratory issues within humans (Cutroneo *et al.*, 2021).

Table 1.3 **Densities of common plastic polymers**

Densities gathered from Polymer Processing (2008) and (British Plastics Federation, 2020).

Plastic polymer	**Density (g/cm^3)**
Expanded polystyrene (EPS)	0.03-0.05
Polypropylene (PP)	0.91
Low-density polyethylene (LDPE)	0.92-0.93
High-density polyethylene (HDPE)	0.94-0.97
Polyamide (Nylon 6) (PA)	1.13-1.41
Polyamide (Nylon 6,6) (PA)	1.13-1.41
Polymethyl methacrylate (PMMA)	1.17
Polycarbonate (PC)	1.20
Polyethylene terephthalate (PET)	1.37
Polyvinyl chloride (PVC)	1.38
Rayon	1.50
Polytetrafluoroethylene (PTFE)	2.30

1.4.1.4 Zinc chloride

Another widely used saline solution in microplastics extraction research is zinc chloride ($ZnCl_2$). This solution has been used in extracting microplastics from freshwater (Rodrigues *et al.*, 2018), soils (Radford *et al.*, 2021), marine sediment (Coppock *et al.*, 2017), compost (Prosenc *et al.*, 2021), aquatic sediment (Imhof *et al.*, 2012) and gastrointestinal tracts (Jaafar *et al.*, 2020). $ZnCl_2$ has a high density of up to 1.7 g/cm^3. High recovery rates of between 93-100% have been found using this saline solution to extract microplastics from soil (Prosenc *et al.*, 2021), and recoveries of 96% from sediments (Coppock *et al.*, 2017). Similarly to NaI, $ZnCl_2$ is also expensive when producing such high densities in large quantity. To make 1 L of $ZnCl_2$ at a density of 1.8 g/cm^3 it would cost approximately £65 (Coppock *et al.*, 2017). To reduce costs, researchers have developed methods which reuse the $ZnCl_2$ solutions. Rodrigues *et al.* (2020) found that using $ZnCl_2$ can still retain recovery rates above 95% after being re-used five times. This saline solution is known to be toxic to aquatic life and harmful to humans (Crichton *et al.*, 2017), and is often not used for this reason.

1.4.1.5 Zinc bromide

Another high-density salt solution that is not as commonly used as the three previously mentioned is zinc bromide. This saline solution has been shown to produce similar recovery rates to $ZnCl_2$. For example, in the experiments by Quinn *et al.* (2017), $ZnBr_2$ provided the highest recovery rates for twelve spiking plastics used. However, this solution can be hazardous to humans (causing skin burns, eye damage and respiratory issues) and can cause long-lasting toxicity to aquatic life (Cutroneo *et al.*, 2021). Moreover, this solution is expensive and has needed to be re-used to make experiments cost effective (Quinn *et al.*, 2016).

1.4.1.6 Calcium chloride

Another solution that is used for density separation is calcium chloride, this saline solution has a lower density of approximately 1.39 g/cm^3. However, Adams *et al.* (2021) found high recovery rates of between 80 and 100% using calcium chloride to extract microplastics from lake sediment. Moreover, calcium chloride is of much lower cost than NaI and $ZnCl_2$ to produce, and is not toxic to aquatic life (Bellasi *et al.*, 2021), so may be favoured to be used in density separations for these reasons.

1.4.1.7 Oil separations

A form of density separation used to extract microplastics from an environmental medium is the use of an oil. Plastics have strong oleophilic properties, so when low density oil is added to a matrix containing plastics, the oil combined with the plastics will rise, allowing for an easy extraction (Radford *et al.*, 2021). This method has been used for a variety of matrices such as suspended solids (Mani *et al.*, 2019), agricultural soils (Radford *et al.*, 2021) and sediment (Crew *et al.*, 2020). Existing studies have used castor oil and canola oil to extract microplastics and have often found high recovery rates. Mani *et al.* (2019) used castor oil to extract PP, PS, PMMA and PET from suspended solids and sediment and found an average recovery rate of 99%. Similarly, Crichton *et al.* (2017) found a high recovery of 96% using the oil extraction protocol (OEP) to extract microplastics from sediment; recovery was slightly less for fibres than fragments.

Researchers using this method have suggested the advantages of ease of use and low cost per sample (Crew *et al.*, 2020). However, a large drawback is the interference oil can cause when following up density separation with spectroscopic methods such as FTIR. Many studies have counteracted this disadvantage by using a clean-up step post density separation, using ethyl alcohol (Crichton *et al.*, 2017) or hexane (Crew *et al.*, 2020). However, it has been noted that adding this extra step, will increase cost, hazard and laboratory/environmental risk of these methods (Bellasi *et al.*, 2021), which was originally suggested as an advantage of using this method.

As well as plastics floating during density separation, other debris and organic material may float, hindering the extraction and isolation of the plastic. For this reason, many researchers follow density separations with a digestion to breakdown this material (Bellasi *et al.*, 2021).

1.4.1.8 Other density separation techniques

Over time, researchers have developed equipment that can assist with already established methods. For example, Imhof *et al.* (2012) developed the Munich Plastic Sediment Separator (MPSS) to improve the efficiency of density separation methods with saline solutions. The MPSS consists of a sediment container at the base, a standpipe in the middle and a dividing chamber at the top that contains a ball valve and a filter holder. Once turned upside down the ball valve opens and the sediment can be filtered (Figure 1.4 B). Recovery rates of between 95.5-100% were found when using this equipment. A similar piece of equipment was developed by Coppock *et al.* (2017) called the Sediment-Microplastic-Isolation (SMI) unit. This allows for risen microplastics in a saline solution to be separated from sunken sediment by closing a valve (Figure 1.4 A). Recovery rates using this equipment were 95.8%. The SMI is smaller and more portable than the MPSS. However, the SMI is made of plastic, compared to the stainless steel MPSS, possibly leading to contamination from the apparatus or reduced recoveries due to the microplastics sticking to the plastic apparatus.

Other equipment that can assist in separating microplastics is sieves. Nakajima *et al.* (2019a) created a stainless-steel sieve that can be placed into laboratory beakers and transferred easily between treatments, saving time and reducing the amount of microplastics lost. However, the sieve has a mesh size of 32 µm, meaning smaller microplastics would not be counted.

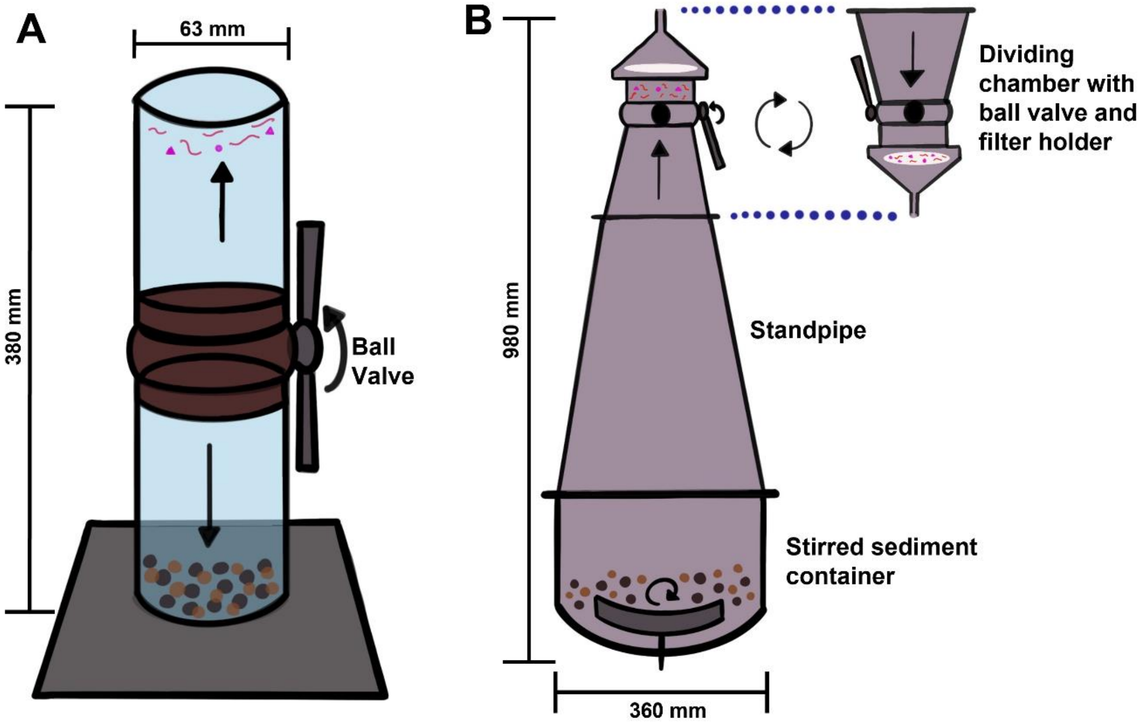

Figure 1.4 **Schematics of the SMI (A) and the MPSS (B)**

Schematics of the sediment-microplastic-isolation unit (SMI) (A) developed by Coppock *et al.* (2017) and the Munich plastic sediment separator (MPSS) (B) developed by Imhof *et al.* (2012), designed to separate microplastics from sediment using saline solutions and density separation.

1.4.2 Chemical digestion

Many types of environmental media may require a form of chemical digestion to breakdown high levels of organic matter in order to make the microplastics available to be extracted and identified. Commonly used acids, bases and oxidising agents that are used for chemical digestion include hydrogen peroxide (H_2O_2), Fenton's reagent (H_2O_2 and Fe), nitric acid (HNO_3), hydrochloric acid (HCl), potassium hydroxide (KOH) and sodium hydroxide (NaOH). Each of these regents have advantages and disadvantages of use when it comes to digesting efficiency, polymer degradation and user/environmental hazards (Table 1.4).

1.4.2.1 Hydrogen peroxide

Hydrogen peroxide is one of the most frequently used reagents when digesting highly organic environmental matrices. However it is more efficient at digesting certain matrices than others. Karami *et al.* (2017a) found that H_2O_2 was unable to digest fish tissues at low temperatures (40 °C) but had better digestion efficiency at 50 °C and 60 °C. However, others have found high recovery rates (>95%) of microplastics when extracting microplastic from freshwater with the same reagent (Rodrigues *et al.*, 2018). Often hydrogen peroxide is used after a density separation step, also giving high recovery rates (Phuong *et al.*, 2021). Hydrogen peroxide can undergo exothermic reactions, which increases the temperature of the solution. These high temperatures have the potential to degrade polymers (Schrank *et al.*, 2022). Moreover, H_2O_2 has been found to change the intensity of Raman peaks, which is also a sign of plastic degradation (Karami *et al.*, 2017a). Hazards associated with H_2O_2 include DNA damage in aquatic life and this reagent can be classed as PBT (persistent, bioaccumulative and toxic) (Picó and Barceló, 2021).

1.4.2.2 Fenton's reagent

A catalyst (Fe) can be added to hydrogen peroxide to make Fenton's reagent. Fenton's reagent is more effective at organic material breakdown in sludge and soil than H_2O_2 alone (Hurley *et al.*, 2018). However, an optimum pH must be kept to prevent a foam forming. The exothermic reaction could reach temperatures up to 89 °C, potentially degrading polymers, and creating a safety issue for users (Munno *et al.*, 2018). Munno *et al.* (2018), recommended that temperatures of chemical reactions should be kept below 60 °C to prevent plastic degradation. However, this study was carried out on fish tissue, so it is difficult to compare results to other media. Fenton's reagent will have the same hazardous properties as mentioned for H_2O_2, however it will also have added monetary costs for the addition of the iron catalyst, making the use of this reagent just as harmful and more expensive than H_2O_2 alone.

1.4.2.3 Nitric acid

Naidoo *et al.* (2017) used nitric acid (HNO_3) to digest whole juvenile fish and found that increasing the temperature can shorten the digestion time drastically. This would benefit studies with a large sample size. Nitric acid can be combined with other reagents. Yan *et al.* (2020) used Fenton's reagent followed by nitric acid to extract microplastics from human and chicken faeces and found an average recovery rate of 97.78%. However, it has been shown that nitric acid can degrade some of the polymers. In a study by Dehaut *et al.* (2016), 15 different plastics were tested for signs of degradation by different methods used. It was found that nitric acid degraded and reduced the weight of polyamide. According to the safety data sheets of Fisher Scientific (2022) nitric acid can cause skin corrosion/irritation and serious eye damage (Table 1.4), making this a hazardous reagent to use in a method.

1.4.2.4 Hydrochloric acid

Hydrochloric acid has been used to digest environmental matrices. Very high recovery rates (100%) can be found when using high concentrations (37%) of this acid (Karami *et al.*, 2017a), but it was found to melt PET fragments and made polymers stick to one another. Other studies have found HCl to be the least effective digesting method, with digestion efficacies of 82.6% (Cole *et al.*, 2014). HCl has been known to slightly degrade plastic polymers (Zhang *et al.*, 2022). Safety data sheets have highlighted the hazardous properties of this reagent, including the potential to cause skin corrosion, serious eye damage and organ toxicity (Fisher Scientific, 2022) (Table 1.4).

In many cases, chemical digestion has been combined with density separation to increase microplastic extraction efficiencies. Rodrigues *et al.* (2018) combined a wet peroxide method with $ZnCl_2$ density separation to produce the most effective extraction for removing microplastics from artificial freshwater including organic matter and sediment. This was the most expensive method tested, so it is not likely to be useful for studies with a large number of samples. Similarly, Nuelle *et al.* (2014) combined density separations of NaCl, NaI and a digestion of H_2O_2 and found recovery rates as high as 99%.

1.4.2.5 Basic reagents

Basic reagents such as KOH and NaOH have been reported to not break down organic material, such as sludge and soil sufficiently (Hurley *et al.*, 2018). However, Thiele *et al.* (2019) found KOH was the most effective at digesting bivalve tissues and was filterable to <25 µm, showing that effectiveness of a chemical depends on the medium being tested. Roch and Brinker (2017) heated NaOH to 50 °C for 15 mins before neutralising and further digesting with HNO_3. They found organic material (gastrointestinal tracts of fish) completely digested in a sample within 1 hour. By combining this further with a NaI density separation, recovery rates of spiking microplastics were reported as >95%. However, by combining two reagents it will increase costs and time needed for the method. There are mixed results on whether KOH would damage polymers when using it during microplastic extraction methods. Some researchers have found no damage when using KOH at temperatures of 40 ˚C (Karami *et al.*, 2017a) or 50 ˚C (Prata *et al.*, 2019). However some researchers have found that it can damage polystyrene (López-Rosales *et al.*, 2021). Moreover, NaOH has been reported as hazardous as it can be a health risk for humans and can also corrode metal and damage glass (Budimir *et al.*, 2018). KOH on the other hand, is only reported to cause skin and eye irritation and is of low cost to use (Table 1.4) (Miller *et al.*, 2017).

1.4.3 Enzymatic digestion

An enzyme (Proteinase-K) was first used to digest organic matter in a microplastic extraction experiment in 2014, and was found to be more effective than an acid (HCl) and a base (NaOH) and had no effect on the plastic (Cole *et al.*, 2014). Multiple studies have used proteinase-K since, finding high recovery rates (>97%) in fish samples (Correia and Loeschner, 2018) and in sediment and water (Karlsson *et al.*, 2017). The basic enzymatic purification protocol (BEPP) combines multiple enzymes to produce a high extraction efficiency from surface waters. However each enzyme requires an optimum pH and temperature, making it a time consuming method (Loder *et al.*, 2017). Industrial enzymes are an alternative that come pre-buffered and less expensive. Digestive enzymes such as papain, trypsin and collagenase are less expensive and can break down tissues in biological samples such as those in bivalves (Courtene-Jones *et al.*, 2017).

Table 1.4 **Advantages, disadvantages, cost per litre and hazards of commonly used digesting reagents**

Advantages, disadvantages, cost per litre (Fisher Scientific, 2022) and hazards (SDS of Fisher Scientific (2022)) of the most commonly used oxidising agents (hydrogen peroxide, Fenton's reagent), acids (nitric acid, hydrochloric acid), bases (potassium hydroxide, sodium hydroxide) and enzymes (papain, trypsin, collagenase) used to digest environmental media and extract microplastics, N/A denotes no SDS.

Reagent	Advantages	Disadvantages	Cost (£) per L per strength (%/M/g)	Hazards	References
Hydrogen peroxide (H_2O_2)	Low cost, frequently used	Degrades nylon at 50 ˚C; changes the colour of PET	£56.78 35%	Oral toxicity, skin corrosion	(Karami *et al.*, 2017a)

Fenton's Reagent	More effective than H_2O_2 alone	Optimum pH needed; reaction will increase temperature of solution	£56.78 35% (H_2O_2) + £1.30 20g (Iron)	Oral toxicity, skin corrosion	(Munno *et al.*, 2018) (Yan *et al.*, 2020)
Nitric acid (HNO_3)	Increase in temperature will increase speed of digestion drastically	Degrades nylon; melts LDPE, HDPE, and PP; reduces the weight of nylon; discolours most polymers	£34.40 65%	Skin corrosion, eye irritation/ damage	(Yan *et al.*, 2020)
Hydrochloric acid (HCl)	Very high digesting efficiency if used at high concentrations (37%)	Melts PET fragments: makes polymers stick together	£45.20 1M	Skin corrosion, serious eye damage, organ toxicity	(Cole *et al.*, 2014)
Potassium hydroxide (KOH)	Can be combined with acids and density separations; does not effective many polymers	Does not break down organic matter efficiently; can degrade polystyrene	£5.50 100g	Skin irritation, eye irritation	(Thiele *et al.*, 2019)
Sodium hydroxide (NaOH)	Can be combined with acids and density separations	Does not break down organic matter efficiently	£2.80 100g	Skin corrosion, serious eye damage	(Hurley *et al.*, 2018)
Proteinase-K	More effective than HCl and NaOH and has no effect on the plastic	Requires an optimum pH and temperature to work, therefore time consuming	£28.80 (500 µg/mL)	N/A	(Cole *et al.*, 2014)
Digestive enzymes: papain, trypsin, collagenase	Come pre-buffered, therefore less expensive and less time consuming	Digesting efficiency could vary been environments tested	Papain: £14.7 Trypsin: £52.80 (10g)	N/A	(Courtene-Jones *et al.*, 2017)

1.5 Other methods

New methods and techniques from varying fields are regularly being developed and tested for extracting microplastics from environmental matrices. Some of the equipment used in these methods are not widely available for general use. From the recycling industry, the use of electrostatic separation has been adapted to separate microplastics from the environment. Felsing *et al.* (2018) developed on the Korona-Walzen-Scheider (KWS) (corona-roller-separator) and used the separator to divide non-conductive microplastics from conductive sediments. They found recovery rates of 100% for larger microplastics (2-5 mm) and almost

100% for the size category of 630 µm-2 mm. However, a drawback to this method is that samples must be completely dry before use.

Another technique that has been used is magnetism. Grbic *et al.* (2019) magnetised plastics with hydrophobic iron (Fe) particles and then extracted the microplastics with a magnet. They found recovery rates of 93% from seawater, 84% from freshwater and 78% from sediments. Similarly, Rhein *et al.* (2019) used a magnetic seed of magnetite (equal amounts of Fe(I) and Fe(II)) to attract the microplastics to the magnet. They found recovery rates of 95% from an ultrapure water suspension. The high recovery rates found by Grbic *et al.* (2019) could be due to many of their trials resulting in >100% recovery rate because of the large and medium sized microplastics fragmenting during the method process. This type of method would be difficult to extract microplastics from environments other than water due to the substrate interference with the magnets.

Solvents are often used to extract microplastics from the environment. La Nasa *et al.* (2021) used dichloromethane (DCM) and a microwave solvent extraction to extract microplastics from sand. Recovery rates of 97% were found for PS. Fuller and Gautam (2016) used a pressurised fluid extractor and methanol, hexane and DCM to extract microplastics from municipal waste and found recovery rates of between 85-94%. However this method is destructive to the plastics, so it is recommended to sort and categorise the plastics prior to extraction if possible. Nguyen *et al.* (2019) discussed the potential of techniques from other fields such as gel electrophoresis and size exclusion chromatography; drawbacks included plastics becoming embedded in the gel.

Spectroscopic methods such as Fourier Transform Infrared (FTIR) spectroscopy and Raman spectroscopy are frequently used to identify the plastic polymers of microplastics in a sample. FTIR exposes microplastics to infrared light and the resultant radiation is then measured in transmission or reflectance modes (Käppler *et al.*, 2016). In transmission mode, light must pass through the sample before being measured, whereas in reflection mode the beam is reflected by an infrared reflective surface (Xu *et al.*, 2019). Limitations with these methods include issues with disturbance of reflected signals. Additionally ATR-FTIR (attenuated total reflectance) can be adopted. During this mode, the microplastic to be studied must cover the whole ATR crystal and then the surface of the sample is illuminated by an evanescent wave (Xu *et al.*, 2019). However, over time the ATR crystal can become scratched and damaged which may affect the results produced (Xu *et al.*, 2019). Raman spectroscopy differs to FTIR by scattering photons of a sample using a laser source, which then produces a spectrum to be analysed (Käppler *et al.*, 2016). Often microplastics are placed on a filter before being scanned with Raman. However, to scan a whole filter in this way would be very time consuming, therefore subsets of a filter are usually scanned (Xu *et al.*, 2019). This way of examining microplastics is sometimes contested due to its low representation of the microplastics on the filter (Xu *et al.*, 2019). Moreover, it is thought that substrates left on the filter could cause spectral interference, which will affect the resulting microplastic spectra (Xu *et al.*, 2019).

1.6 Thermo-analytical identification methods.

During sample preparation, when the environmental matrix is removed or digested, there is high chance of losing or degrading microplastics. More recently, researchers are exploring options to either reduce sample preparation or remove it entirely, thus decreasing the chance of losing microplastics to the method used. One of these options is the use of thermo-analytical methods. Thermo-analytical methods refer to thermal desorption (TD) and pyrolysis (Pyr). Both of these methods decompose macromolecules under an inert atmosphere, into low molecular weight chemicals (Yakovenko *et al.*, 2020). The resulting products are then separated by gas chromatography (GC) and subsequently identified by mass spectrometry (MS). Although these techniques have, to date (2022), been rarely used compared to the use of FTIR and Raman spectroscopy in the field of microplastic research, thermal desorption and pyrolysis have been used occasionally to study microplastics in environmental matrices. For example, Dümichen *et al.* (2017) used TD-GC-MS to study microplastics in riverine freshwater and biogas residue. They explained how this method was beneficial due to how quickly samples could be measured due to the low amounts of sample preparation, however the low sample amount of 0.5mg was a large drawback as it is only representative of a small section of the environment matrix studied. Dekiff *et al.* (2014) used Pyr-GC-MS to study beach sediments; however they reported that the library database to identify the plastics needs to be extended to include lesser common polymers.

Thermo-analytical methods have several advantages. Although these methods cannot measure the size of particles, they can detect microplastics down to the nm scale, which has always been a large drawback of routine spectroscopic methods. It is also possible to detect and identify multiple types of polymers in a single sample/run. Furthermore, these methods are often automated, meaning large sample numbers can be set up to run, and makes the methods highly reproducible, and more efficient in regards to time and resources (Yakovenko *et al.*, 2020). However, there are some drawbacks to using thermo-analytical methods. Often very small sample volumes can be used, with pyrolysis usually only a few milligrams can be used per sample. This issue could have further implications when examining heterogenous environmental matrices for microplastics. It has been hypothesized that the results found could be affected by the degree of weathering of the tested plastic. Weathering essentially oxidises the polymer backbone, which changes the distribution of the product produced by pyrolysis (Yakovenko *et al.*, 2020).

As with the methods described in section 1.4, method validation and quality assurance/quality control (QA/QC) measures should be ran alongside thermo-analytical methods to ensure the method is reliable and as accurate as possible. Such measure should include limits of detection and quantification, running blank samples, recovery rate studies and inter-laboratory comparisons (Schymanski *et al.*, 2021).

1.7 Methods used to extract microplastics from fishmeal

As mentioned in section 1.3, researchers are beginning to study fishmeal for microplastic contamination. However, as with much of the research in to microplastics, there is a lack of standardisation when it comes to the methods used to extract microplastics from fishmeal. For example, Hanachi *et al.* (2019) used a KOH digestion, NaI density separation and centrifugation to digest the fishmeal; Karbalaei *et al.* (2020) used the same method, minus the centrifugation; Gündoğdu *et al.* (2021) used a KOH:NaClO digestion and a NaI density separation; Thiele *et al.* (2021) used a NaCl density separation and overflow; Yao *et al.* (2021) used a NaCl density separation followed by a H_2O_2 digestion; and Wang *et al.* (2021a) used both a H_2O_2 and a KOH digestion.

It is clear that the results from these studies will be difficult to compare due to the large differences in methods used. All but one of these studies (Thiele *et al.*, 2021) validated the methods proposed. Without this validation and subsequent recovery rate, it is difficult to assess the effectiveness of each method used, thus the accuracy of the results found. Furthermore, the recovery rates/effectiveness of each of these methods, could show whether each study is over or underestimating how many microplastics are truly in fishmeal.

1.8 Key knowledge gaps

1.8.1 Validating extraction methods

Although finding the amount of microplastics in the environment is one of the most important aspects to microplastics research, the method used to extract the microplastics is the key foundation and building block to collecting accurate results that show a true representation of the amount of microplastics in the environment.

With microplastics being so ubiquitous in the environment, it brings about the issue of having to extract microplastics from new, complex environments; often consisting of complex organics and carbonates to separate. Furthermore, researchers are often having to develop new methods to combat this, as one method will not suit all environmental matrices. Due to there now being a large range of different methods used in this field, researchers are calling for there to be some degree of standardisation used, to allow for easier comparison of results between studies. For example, Lusher *et al.* (2021) explains that the lack of understanding of the risk of plastic pollution is due to the very little standardised methods in the field.

To validate a method, many researchers use a process of "spiking" an environmental matrix with known amounts of microplastics, run the method as they plan to for the main study and then retrieve and count the recovered spiked microplastics. This is called a recovery rate and is often given as a percentage. There is currently a lack of information on how frequently this form of method validation is adopted in the field of microplastic research and if there are any consequences for poorly validated methods.

1.8.2 Standardisation within method development

Standardisation is of utmost importance in this field. However, to allow for the widespread use of similar methods, there are certain criteria that should be met. One example is the use of representative microplastics when developing a method. Currently, the most common types of microplastics used are virgin microplastic standards, these are the pre-production plastic pellets used to mould plastic products, or other spherical type beads (Weis and Palmquist, 2021). The issue with using these as a baseline for methods, is that they do not represent the microplastics that are found in environmental samples. Environmental microplastics would have been exposed to some level of weathering (Alimi *et al.*, 2022), which ultimately will affect how they will behave in reagents and methodological processes. Weathering of microplastics for this reason is not commonplace in this research field, and there are limited studies that have shown the difference between using virgin and weathered microplastics in methods of extraction.

1.8.3 Risks, hazards and costs of methods

Microplastic extraction methods need to be accessible for all, meaning the costs of reagents and equipment need to be kept as low as possible whilst maintaining robust, high quality, reproducible, reliable and consistent results. This way a method can be available to use for a wide array of researchers and laboratories. Furthermore, whilst studying a pollutant in the environment, it is important to not also be using and potentially releasing other harmful reagents into the environment. Thus reagents used when extracting microplastics in the environment should not be toxic to users or the surrounding environment.

Some of the reagents used in microplastic research have been reported to be toxic to aquatic life and harmful to users. For example, zinc chloride has been shown to produce growth defects in zebrafish embryos (*Danio rerio*) (Salvaggio *et al.*, 2016), reduce hatching and increased mortality of red sea bream (*Pagrus major*) embryos (Huang *et al.*, 2010) and compromised the function of the spleen and liver of the javelin goby (*Synechogobius hasta*) (Zheng *et al.*, 2011). Li *et al.* (2019) found that as aquatic pH decreases, the toxicity of zinc to organisms increases. Bellasi *et al.* (2021) reviewed several methods papers and classified sodium iodide, potassium iodide, zinc bromide and potassium fluoride to be extremely hazardous to either or both users and aquatic life. Some of the effects to users include skin and eye damage, organ damage after prolonged exposure and can cause breathing issues (Bellasi *et al.*, 2021).

As mentioned in section 1.4, reagents commonly used in microplastic extraction methods can have high costs, making them difficult to be replicated by others. In particular, saline solutions can be costly when trying to make a solution of high density as more salt is needed than the lower-density solutions. Furthermore, in these types of experiments, high numbers of replicates are expected to produce accurate and reliable results, further increasing the costs of these experiments. An example of this cost was provided by Claessens *et al.* (2013) who explained how a kg of sodium iodide was 70 times the cost of a kg of sodium chloride. Often, these solutions are only used once before being disposed of, due to them being contaminated with environmental samples, making this process largely wasteful and costly.

Another way in which to reduce cost is to limit sample preparation as much as possible. This has the additional benefit of reducing the samples' exposure to contamination, and minimising loss of target microplastics. For example, Dimante-Deimantovica *et al.* (2022) found that with increased sample preparation, there was a decrease in recovery of microplastics. Thermo-analytical methods, as described in section 1.6, require very limited sample preparation and should be considered as an option to reduce cost and speed up sample processing time.

The concept of developing methods that are of low cost and low harm to users or the environment is not widely adopted in this field. There is a need to encourage researchers to find a balance between effective methods and creating a method that can be easily accessed by researchers worldwide.

1.9 Rationale of thesis

Due to the importance and severity of microplastic pollution in the environment, research on the topic is now exceptionally widespread. A consequence of this is that the methods used to extract microplastics from the environment vary considerably. This large variation brings issues with comparability and repeatability of these methods. This thesis investigates already published methods and how effectively they have been validated.

Methods used within the projects of this thesis are replicated and developed in ways that make them accessible for all without polluting the environment further. These directions are considered when investigating microplastics in a new, complex environmental medium. Fishmeal is an enormously important resource- supporting food security and nutrition globally- but faces potential issues with microplastic contamination. It is a complex medium and the study of microplastics within is still in its infancy, therefore it is imperative that established and new methods used on this medium are fully evaluated so that potential issues can be considered and addressed by the industry.

New thermo-analytical methods with realistic microplastic samples are considered, for simpler sample preparation and higher accuracy. Several challenges and advantages of optimising extraction methods are considered throughout this thesis to encourage others to develop methods that consider effectiveness, cost and environmental impact.

1.10 Thesis framework

This thesis is structured as a research paper thesis, with each main chapter presented as a published or publishable research paper. Due to this structure there may be slight overlap in introductory sections of each chapter.

Chapter Two: Evidence of underestimation in microplastic research: A meta-analysis of recovery rate studies.

> This chapter investigates whether the amount of microplastics in the environment are underestimated based on the methods used, taking into account the types of reagents used and the test medium. It also assesses the quality of recovery rate studies used to

validate the methods developed and provides recommendations for future recovery rate studies.

Chapter Three: Assessing the effectiveness of microplastic extraction methods on fishmeal with different properties.

This chapter begins to develop methods on a complex environmental medium (fishmeal). It investigates already established methods used on fishmeal and develops new methods that focus on keeping costs low and reducing the use of hazardous reagents. It uses different density saline solutions for density separations, potassium hydroxide for digestions and the use of a dispersant. This chapter also discusses the impact of the fishmeal properties on the ability to extract microplastics.

Chapter Four: Qualitatively identifying chemical indicator markers of virgin and weathered microplastics using solvent extraction and thermal desorption GC-MS.

This chapter develops thermo-analytical methods (solvent extractions and thermal desorption), to identify chemical markers of both virgin and weathered (laboratory weathered and naturally weathered) microplastics and explains why each marker is indicative of the subsequent polymer. It also discusses how the detection of the weathered microplastics is applicable to true environmental samples.

Chapter Five: General discussion of thesis

This chapter lays out a summary of the main findings of this thesis. It discusses the challenges faced and the limitations of the research. It concludes with further recommendations related to this work.

1.11 Aims and objectives

Overarching thesis aim:

To contribute to and improve the current understanding of method development when extracting microplastics from complex environmental media.

Aim i (Chapter 2): to determine the amount of underestimation of microplastics in environmental samples, dependent on the methods used.

- Identify recovery rate type studies undertaken in existing peer-reviewed literature.

- Via a meta-analysis, critically examine the most commonly used methods and type of environmental media tested.
- Estimate overall underestimation of microplastics in the environment.

Aim ii (Chapter 3) Develop a suitable, cost effective and environmentally friendly method to extract microplastics from a complex environmental medium (fishmeal).

- Determine the properties of different types of fishmeal.
- Investigate the effectiveness of new and existing microplastic extraction methods on the different fishmeal types, using spiking trials.

Aim iii (Chapter 4) Determine the suitability of thermo-analytical methods for identifying virgin and weathered microplastics.

- Develop a process to artificially weather microplastics.
- Using solvent extraction, determine suitable chemical markers that are indicative of the virgin and weathered microplastics.
- Using thermal desorption, determine suitable chemical markers that are indicative of the virgin and weathered microplastics.
- Compare the effectiveness of solvent extractions and direct thermal desorption.
- Investigate the suitability of using each selected chemical marker as identification for each subsequent microplastic polymer.

Chapter 2 Evidence of underestimation in microplastic research: a meta-analysis of recovery rate studies

This chapter is published as:

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Concept for this chapter was developed by Chloe Way and supervisors (co-authors), methodological refinement, research of literature, analysis and writing was undertaken by Chloe Way.

2.1 Abstract

Research on microplastics in the environment is of high interest to many scientists and industries globally. Key to the success of this research is the accuracy, efficiency, reliability, robustness and repeatability of the method(s) used to isolate the microplastics from environmental media. However, with microplastics now being found in new complex media, many multifaceted methods have been developed to research the quantities of these pollutants. To validate new methods, recovery studies can be undertaken by spiking the test medium with known quantities of plastics. The method is typically run as normal, and the recovered plastics counted to give a recovery rate. A current issue in this field is that methods are rarely or poorly validated in this way. Here, a meta-analysis is conducted on 71 recovery rate studies. Sediment was found to be the most studied medium and saline solutions were the most used reagents. Polyethylene and polystyrene were the most used spiking polymers, which is relevant to the most common polymers in the environment. Recovery rates were highest from plant material, whole organisms and excrement (>88%), and lowest from fishmeal, water and soil (58-71%). Moreover, all reagents but water were able to recover more than 80% of the spiked plastics. This is the first (to my knowledge) overarching indication for the underestimation of microplastics in the environment of approximately 14% across the studies reviewed, varying with the methods used. Furthermore, it is recommended that the quality, use and reporting of recovery rate studies should be improved to aid the standardisation and replication of microplastic research.

2.2 Introduction

Currently, global microplastic research has a high public profile, is of high importance and includes many research avenues within one field. Crucially, it is primarily focused on the amount of these pollutants in different environmental matrices. For example, microplastics have now been found in wastewater and sludge from China (Li *et al.*, 2018), Finland (Railo *et al.*, 2018) and Australia (Ziajahromi *et al.*, 2017), in soil samples from Chile (Corradini *et al.*, 2019) and Switzerland (Scheurer and Bigalke, 2018), and in aquatic sediments from Belgium (Claessens *et al.*, 2011), England (Horton *et al.*, 2017) and the Arctic (Kanhai *et al.*, 2019). Research has also focused on the sources of this pollutant. For instance, it has been estimated that a single washing machine load of clothing could release approximately 700,000 microplastic fibres into wastewater systems (Napper and Thompson, 2016), and similarly one use of a face wash could release up to 94,000 microbeads (Napper *et al.*, 2015). Some of this research has resulted in policy change, like the banning of facial cleansers containing microbeads (Guerranti *et al.*, 2019).

However, a key to successful microplastic research lies within the method used to extract these small pollutants. Researchers in this discipline face criticism for their lack of standardisation and comparative approaches (Underwood *et al.*, 2017). Methods can vary significantly; density separation methods use many different saline solutions such as sodium chloride (NaCl)(Nuelle *et al.*, 2014; Pagter *et al.*, 2018; Quinn *et al.*, 2017), zinc chloride ($ZnCl_2$) (Imhof *et al.*, 2012; Wang *et al.*, 2018), sodium iodide (NaI) (Nuelle *et al.*, 2014; Roch and Brinker, 2017) and calcium chloride ($CaCl_2$) (Crichton *et al.*, 2017; Stolte *et al.*, 2015); various acids, bases and oxidising agents have been used (Bianchi *et al.*, 2020; Schirinzi *et al.*, 2020; Yu *et al.*, 2019). Enzymes (Catarino *et al.*, 2017; Loder *et al.*, 2017), and oils (Radford *et al.*, 2021) are also being utilised, with or without the use of additional reagents such as dispersants. Many of the methods are used in combination – for example combining in sequence oxidising agents with density separation methods. Also, new equipment and devices are being developed to assist in the extraction of microplastics (Coppock *et al.*, 2017; Imhof *et al.*, 2012; Nakajima *et al.*, 2019b). However, with some methods inaccessible due to cost or limited access to equipment, this is not always achievable. For example, spectroscopic equipment such as Fourier transform infrared (FTIR) and Raman spectroscopy, used to identify polymers often come at very high cost, with some systems priced between US$200,000 - 300,000 (Primpke *et al.*, 2020). Similarly, saline solutions used in density separations can be expensive when needed in large quantities. For instance, NaI may cost US$69 for just 100 mL and $ZnCl_2$ can cost US$710 for just 30 litres (Crichton *et al.*, 2017). More complex matrices such as fishmeal (Gündoğdu *et al.*, 2021; Thiele *et al.*, 2021) and terrestrial soils (Corradini *et al.*, 2019) are being found to contain microplastics, thus many multifaceted methods are being developed and published to cater for this, or current methods are being developed further to combat current limitations.

For this suite of methods to be replicated and used by others, they should be verified and validated[2] in some way. However, method verification/validation is not as common as it may need to be in this developing field of research. For example, Underwood *et al.* (2017, p. 1337, p.1337) stated *"Methods used have not been analysed experimentally to determine the relative importance of the different thermal, physical and chemical techniques on rates of recovery and dissolution of different sizes and polymers of microplastic"*. To verify and validate new methods, so called "recovery rate" studies are sometimes undertaken alongside the main microplastic extraction. This entails 'spiking' the studied matrix with known types and configurations of spiking polymers, running a method considered for use in further study of that matrix, and then establishing the amount of spiking plastics recovered. This provides an indication of how effective the method is at extracting plastics from a specific matrix, typically as a percentage recovery rate. When implemented effectively, this could provide an insight in to how well a method could perform compared to others. Further to this, a recovery below 100% could suggest how using a certain method may underestimate the amount of microplastics in a matrix, and a recovery over 100% could show a potential for overestimation. Under and/or overestimating the number of microplastics in the environment could have further implications. For example, underestimating the number provides the chance of underplaying the severity of this pollution, whereas overestimating may allow for scaremongering of an issue which is not that severe. Both of these scenarios can have a negative impact if used in the media, particularly to those readers in the wider non-scientific population.

This meta-analysis aims to identify the recovery rates from multiple studies, and critically review how they vary when using different methods to extract microplastics from a wide range of matrices. The analysis is the first (to my knowledge) to provide an estimate of how much microplastic research may be under or over-estimating current levels of microplastics

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[2] *Method verification* is an evaluation of a methods suitability under authentic experimental conditions. *Method validation* is a process of evaluating the performance of a known method using laboratory tests, with the goal of ensuring that all performance parameters are compatible with the anticipated analytical applications. An analytical method should be scrutinised from a range of positions to demonstrate that the arising results are reliable, replicable, authoritative and can be used correctly for its intended purpose.

based on the methods utilised and the recovery rates found. Finally, recommended reporting criteria are provided for future recovery rate studies to allow for improved validation and simpler replication.

Hypothesis: Microplastic recovery rates will vary between studies using different environmental matrices and using different methods.

2.3 Method

2.3.1 Methodology for literature search – Identification

The methodological approach of this meta-analysis was carried out by following the guidance of the PRISMA 2020 flow diagram (Page *et al.*, 2021) (Figure 2.1). The PRISMA flow diagram allows for a visual depiction of the different stages of the systematic literature search; including how sources were identified, how many were found, how many were screened for suitability, how many were included in the analysis and reasons for those excluded. Sections of the PRISMA 2020 checklist (Page *et al.*, 2021) were also complied and followed, with the inclusion of the eligibility criteria, information sources, a full search strategy, study selection, the data collections process and the data extracted. The PRISMA approach has been widely applied to optimise methodologies and reporting quality in research studies (Liu *et al.*, 2019a).

During January 2021, a database search was undertaken using Web of Science, Scopus, GreenFILE and PubMed search engines. The search was conducted using the following search terms: “recovery rate” OR “recovery efficiency” AND microplastic OR plastic OR nanoplastic AND extraction OR identification OR validation.

The search was filtered further to only include peer-reviewed articles; however, no limit was put on date of publication. Following from the database searches, 855 records were found, and a reference manager (Endnote) was used to organise the articles. Duplicates were removed, leaving 791 papers to be screened for suitable titles and abstracts (Figure 2.1).

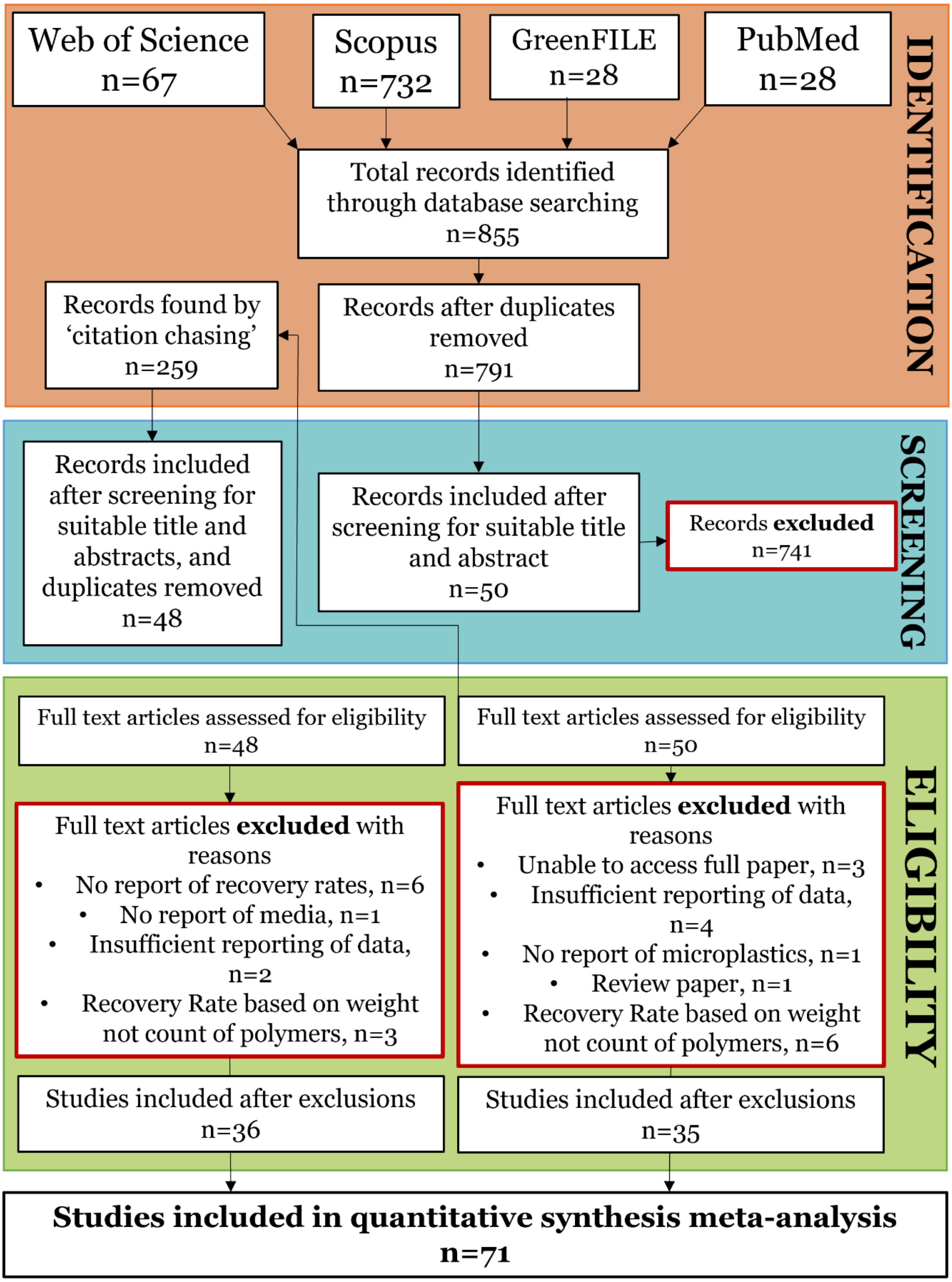

Figure 2.1 **Literature search flowchart**

Including literature identification, screening and eligibility process. Including number of articles found and/or excluded at each stage. Adapted from PRISMA flow diagram (Page *et al.*, 2021).

2.3.2 Criteria for inclusion – Screening and Eligibility

During the screening for suitable titles and abstracts, certain inclusion criteria were applied. Articles incorporated must include plastics that are either below 5 mm (large microplastics) or plastics between 1 µm-1 mm (microplastics) (International Organization for Standardization, 2020). The titles and/or abstract must also indicate that the method used was validated in some way, either by including a recovery rate or using another term such as efficiency. The media tested in the studies were not limited.

Following on from screening for suitable title and abstracts, 50 full text articles were assessed for eligibility. Articles were excluded for the following reasons: No access to the full paper and data, insufficient reporting of data (recovery rates were reported in graphs/figures, but an accurate average and/or variation could not be extracted and used), no report of using microplastics in the study or spiking trial, no report of recovery rates, any review papers and recovery rates calculated by weight difference, not count. Papers based on count largely outnumber papers published based on weight. It is also difficult to accurately convert mass related results on plastic to be comparable with those based on count. However, it is noted that this may excluded papers that have done recovery studies using smaller microplastics and thus produced recovery rates based on weight.

Due to recovery rate studies being often undertaken as a side project alongside microplastic extraction/identification studies, many recovery rate studies may have not been identified during screening titles and abstracts. Therefore, “citation chasing” (Barrett, 2005) was carried out to counterbalance this. When reading the full text articles, suitable references were identified and pooled. 259 potentially suitable articles were identified and managed within the reference manager. After duplicates were removed and abstracts and titles were screened for the same inclusions mentioned previously, 48 articles were selected to be checked for full paper eligibility.

After all articles were assessed for eligibility, including those found by citation chasing, 71 papers were included for the meta-analysis.

2.3.3 Data extracted

Data extracted from the articles included basic information such as the authors’ names, the journal name and date of publication. Other material extracted included a short detail on the method used, the test media, the types of reagent used, the spiking microplastic polymer types, the spiking microplastic shapes, the spiking microplastic sizes and the recovery rates found.

The quantitative analysis was further conducted in Microsoft Excel and RStudio (version 3.6.1). The microplastic size category was further subdivided into MP (microplastic) (any microplastics between 1 μm and 1 mm) and LMP (large microplastic) (any microplastics between 1 mm and 5 mm) (International Organization for Standardization, 2020). Similarly, the test reagents were categorised into oils, alcohols, dyes, acids, oxidising agents, bases, saline solutions, water, enzymes and solvents. The test media were categorised into plant material (vegetal plant material), air, fishmeal, biofilms, excrement, whole organisms, tissues of organisms, soil (horticultural/agricultural soil, farmland soil, compost), wastewater effluent/sludge, water and sediment (marine and freshwater sediment/beach and river sediment). All information on studies included is provided in Appendix. A.1.

Due to the lack of control samples used in recovery rate studies and lack of reported sample sizes for the recovery rate part of a study, a sample effect size was not able to be calculated. However, this limitation will be examined in the discussion.

2.3.4 Quality of selected studies

The quality of the selected studies in this analysis are assessed by ranking each study subjectively from 1 to 5 (1 being low quality, 5 being high quality). The criteria (Appendix. A.3) are adapted from Porter *et al.* (2014) and Fidai *et al.* (2020) and is based on the quality of the recovery rate method, comprising of the inclusion of the test media, the reagent used and information on the spiking plastics used. Furthermore the criteria included whether the studies have potential for replication, a sufficient number of replicates (minimum of 3 to allow for standard deviation) and the clarity and presentation of results.

2.4 Results

2.4.1 Summaries of studies included in meta-analysis

2.4.1.1 Quality of selected studies

The purpose of reviewing the quality of included studies is to highlight the areas of recovery rate studies which need improvement. The mode score for the 71 studies included in this meta-analysis is 4. With only 14 studies achieving the rank of 5, it shows there are many limitations of recovery rate studies to be discussed.

2.4.1.2 Media and reagent used

A total of 12 different types of media were studied, including fishmeal (Thiele *et al.*, 2021), plant material (Herrera *et al.*, 2018), air (Prata *et al.*, 2020a), biofilms (Peez *et al.*, 2019), excrement (Wu *et al.*, 2020; Yan *et al.*, 2020), whole organisms (Catarino *et al.*, 2017; Karlsson *et al.*, 2017; Peez *et al.*, 2019; Thiele *et al.*, 2019; Yu *et al.*, 2019), tissues of organisms (Claessens *et al.*, 2013; Dawson *et al.*, 2020; Dehaut *et al.*, 2016; Digka *et al.*, 2018; Jaafar *et al.*, 2020), soil (Büks *et al.*, 2021; Corradini *et al.*, 2019; Scopetani *et al.*, 2020), wastewater/sludge (Dyachenko *et al.*, 2017; Scopetani *et al.*, 2020; Xu *et al.*, 2020), gastrointestinal tracts (Munno *et al.*, 2018; Tsangaris *et al.*, 2020; Yu *et al.*, 2019), water (Birkenhead *et al.*, 2020; Hildebrandt *et al.*, 2019; Wiggin and Holland, 2019) and sediment (Mahon *et al.*, 2017; Mohamed Nor and Obbard, 2014; Pagter *et al.*, 2018) (for a breakdown of these media categories see section 2.3.3 Data extracted). One study did not report the medium used (N/A in Figure 2.2). The most tested medium is sediment (n=28), followed by water (n=15) and gastrointestinal tracts (n=12) (Figure 2.2).

Several different reagents were used in the studies when performing recovery rate trials. These include solvents (Peez *et al.*, 2019; Scopetani *et al.*, 2020), enzymes (Catarino *et al.*, 2017; Karlsson *et al.*, 2017), dyes (Prata *et al.*, 2020b; Vermeiren *et al.*, 2020), bases (Dawson *et al.*, 2020; Jaafar *et al.*, 2020), acids (Hernández-Arenas *et al.*, 2021; Weber *et al.*, 2021), oxidising agents (Nuelle *et al.*, 2014; Stolte *et al.*, 2015), water (Lares *et al.*, 2019; Mahon *et al.*, 2017), alcohol (Hildebrandt *et al.*, 2019; Palermo *et al.*, 2020), oil (Karlsson *et al.*, 2017; Scopetani *et al.*, 2020) and saline solutions (Büks *et al.*, 2021; Crichton *et al.*, 2017). The most frequently used reagents were saline solutions (n=39), followed by oxidising agents (n=31), oxidising agents combined with saline solutions (n=17) and bases (n=14) (Figure 2.2). The most commonly used saline solutions include sodium chloride (n=15), sodium iodide (n=10) and zinc chloride (n=10) (Appendix. A.2). Moreover, five studies did not state what reagent was used in the recovery trial (N/A in Figure 2.2).

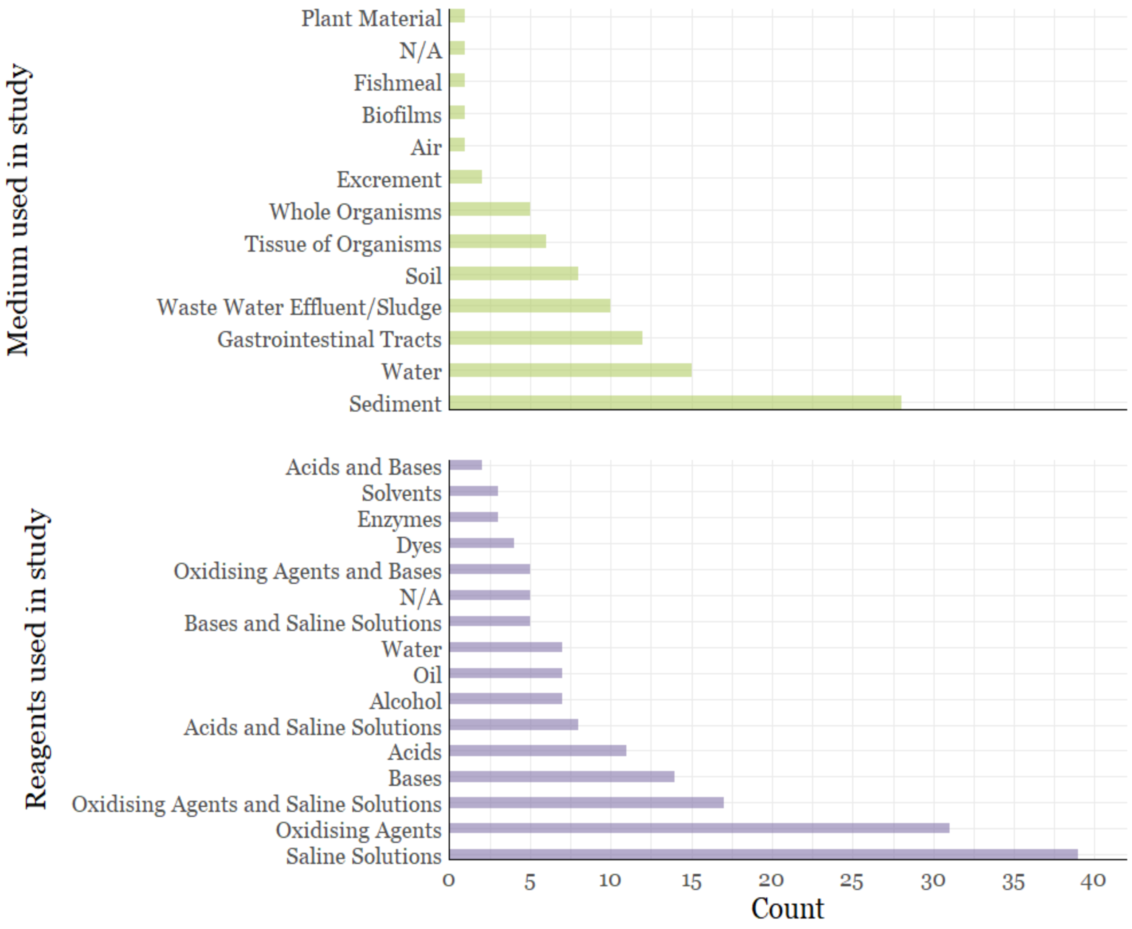

Figure 2.2 **Count of studies included in meta-analysis using different media and reagents**

The count of studies included in this meta-analysis which used each medium and each reagent during a recovery rate experiment. N/A represents number of studies which did not report the medium or reagent used.

2.4.1.3 Type of spiking polymer used

A total of 27 different spiking polymers were used in the microplastic recovery experiments reviewed. The most commonly used polymer was polyethylene (PE) (n=44), followed by polystyrene (PS) (n=36) and polyethylene terephthalate (PET) (n=35) (Figure 2.3). One study did not report the type of spiking polymer used. From here forward, the eight most used polymers (used in more than eight studies), were further analysed. These eight polymers have been further categorised into high- density (PET, PVC and PA) and low-density (PE, PS, PP, LDPE and HDPE) polymers (Figure 2.3). At least one or more of these polymers are used in 98.5% of the studies selected for this meta-analysis (70 out of 71 studies).

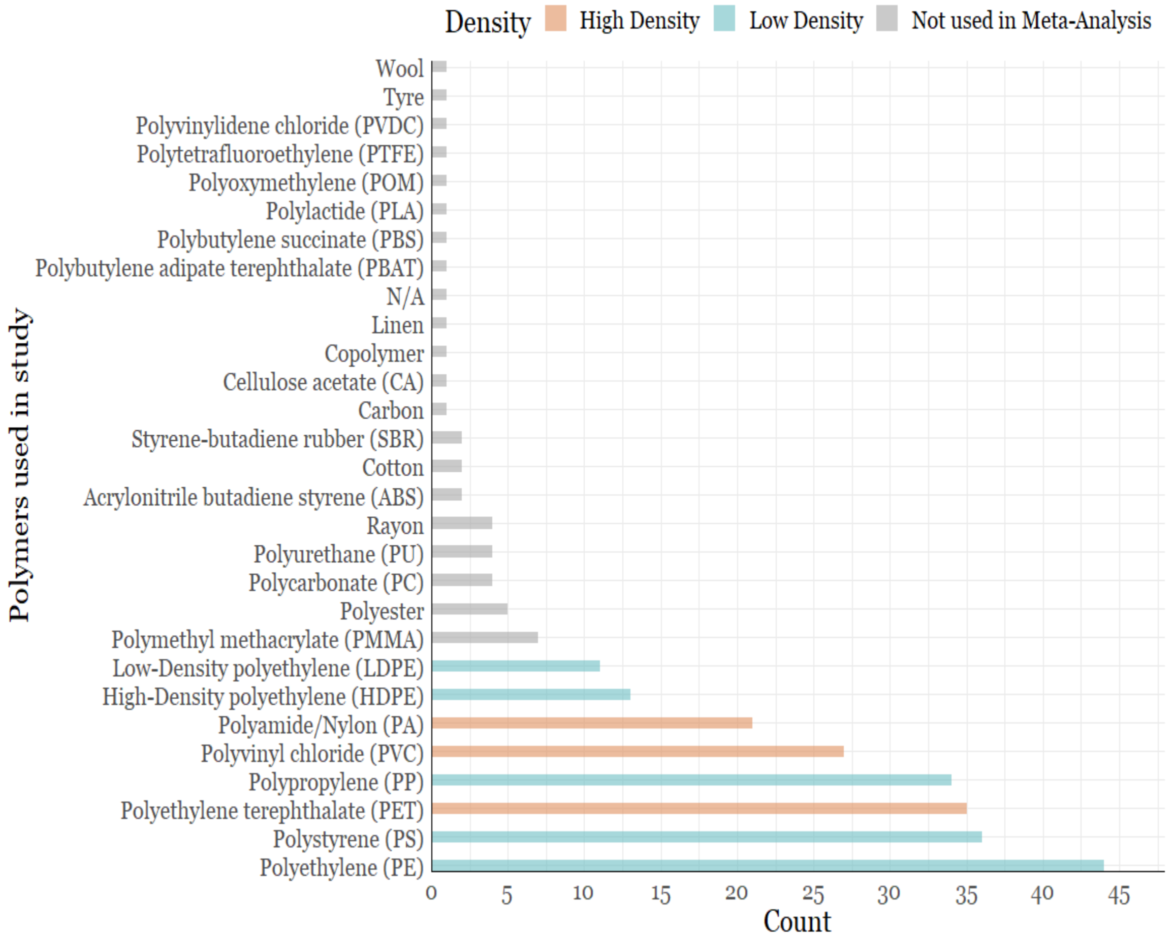

Figure 2.3 **Count of the types of spiking polymers used in the studies examined in this meta-analysis.**

Those polymers used in more than 8 studies are further split into high (red) and low-density (blue) polymers for further investigation. The polymers used in less than 8 of the studies were not used for further investigation (grey).

2.4.1.4 Shape and size of spiking polymer used

The most common shape spiking polymer used was fragments (n=27), followed by fibres (n=22) (Figure 2.4). A large number of studies did not report the shape of the spiking polymer (n=10). Furthermore, 11 studies used the word "particle" to describe the spiking polymer used. This is an ambiguous term which could be interpreted and described as many shapes, so this term was given its own category. With regard to the size of spiking polymers used, the majority of the studies (n=60) used microplastics (1 µm-1 mm) as their spiking polymers. However, four studies did not report the size of the spiking polymer used (Figure 2.4).

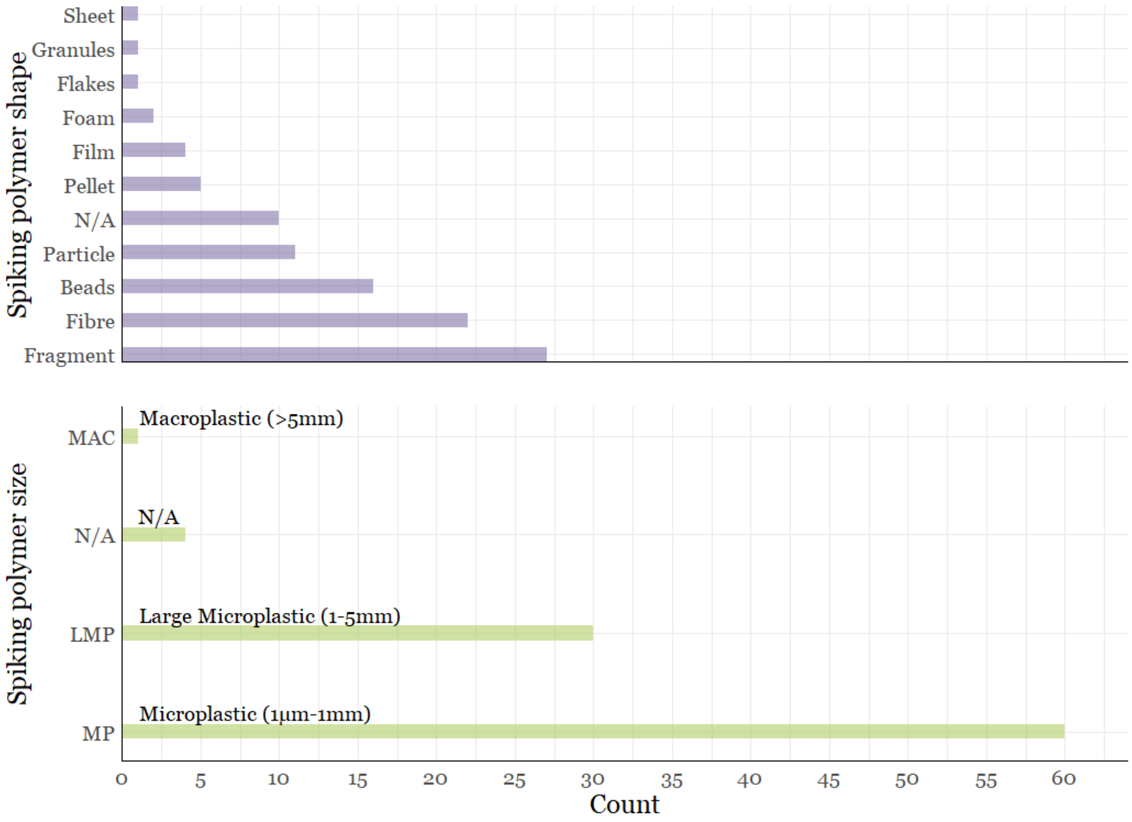

Figure 2.4 **The count of different shape and size of spiking plastics used in the studies selected for this meta-analysis.**

Large microplastics are those classed between 1 mm-5 mm, microplastics are those classed between 1 µm-1 mm (International Organization for Standardization, 2020). N/A represents the number of studies not reporting the spiking polymer shape or size. Many studies used more than one different shape and size of spiking plastics.

2.4.2 Meta-analysis of recovery rates across studies

2.4.2.1 Recovery rates of different sized spiking plastics

On average, recovery rates of spiking plastics increased with the size of the plastics (Figure 2.5). Studies using the smallest spiking plastics (microplastics (<1 mm)) recovered 84.5±22.2% on average, whereas the studies using the large microplastics (1-5 mm) and the macroplastics (>5 mm) as spiking plastics, recovered 84.8±24.5% and 100% respectively. Notably, four studies did not report the size of the spiking plastics used but achieved a recovery rate of 95.1±6.8% on average.

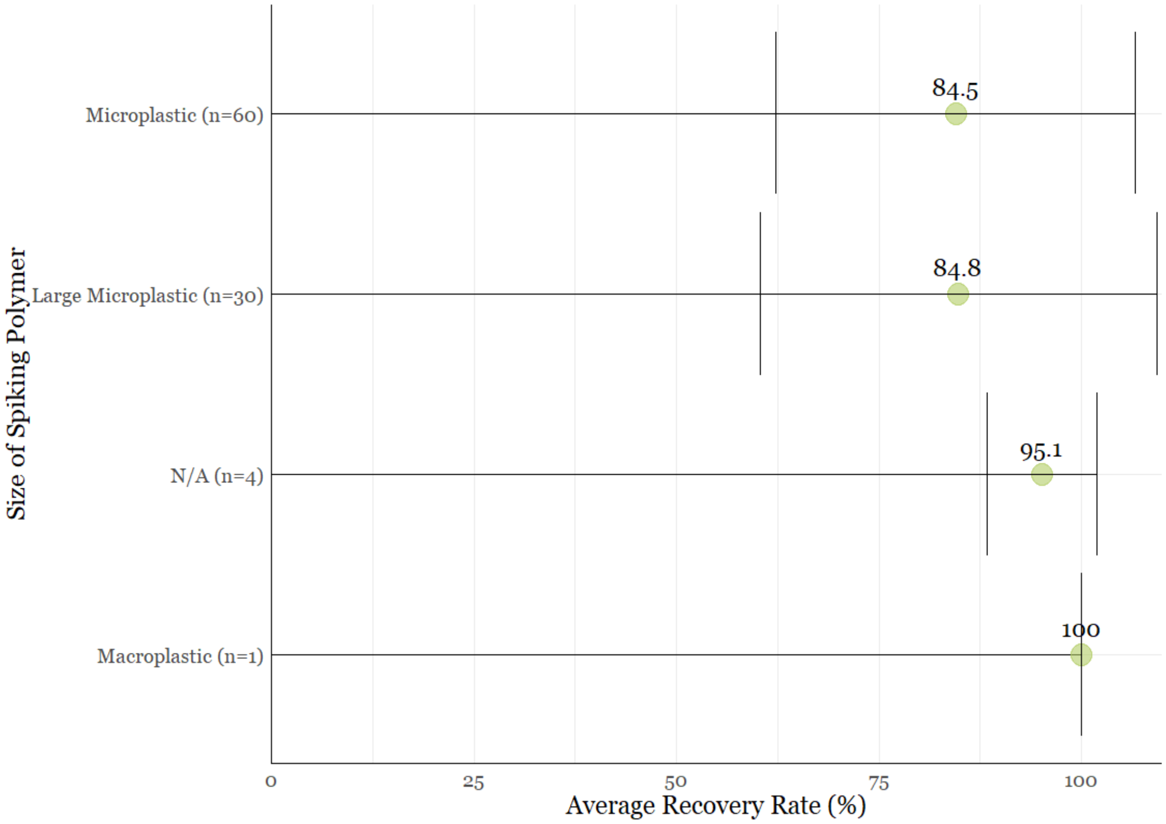

Figure 2.5 **Average recovery rates across studies of different sized spiking plastics**

Macroplastics are those plastics above 5 mm, large microplastics are those classed between 1 mm-5 mm, microplastics are those classed between 1 µm-1 mm (International Organization for Standardization, 2020). N/A represents the studies which did not classify the size of spiking plastics used. Bars around the mean represent the standard deviation of the mean.

2.4.2.2 Recovery rates of polymers from different media

The majority of the lower recovery rates in each media type came from the high-density polymers (PVC, PET and PA). This is the case for fishmeal, water, wastewater/sludge, tissues of organisms and whole organisms (Figure 2.6). However, in the studies that have used gastrointestinal tracts and excrement as the study medium, the opposite is found, with lower recovery rates of low-density polymers (PS, PP, PE, LDPE, HDPE). Overall, polymers were recovered more effectively from plant material (all 100%), biofilms (96%), whole organisms (91-95%) and excrement (88-95%); and recovered least from fishmeal (58-70%), water (67-82%) and wastewater effluent/sludge (76-89%) (Figure 2.6). The difference in recovery rates between high and low-density polymers is much larger in some media compared to others. For example, 22% more low-density polymers were recovered from soil than high-density polymers. However, from tissues of organisms only 3% more low-density polymers were recovered than high-density polymers (Figure 2.6).

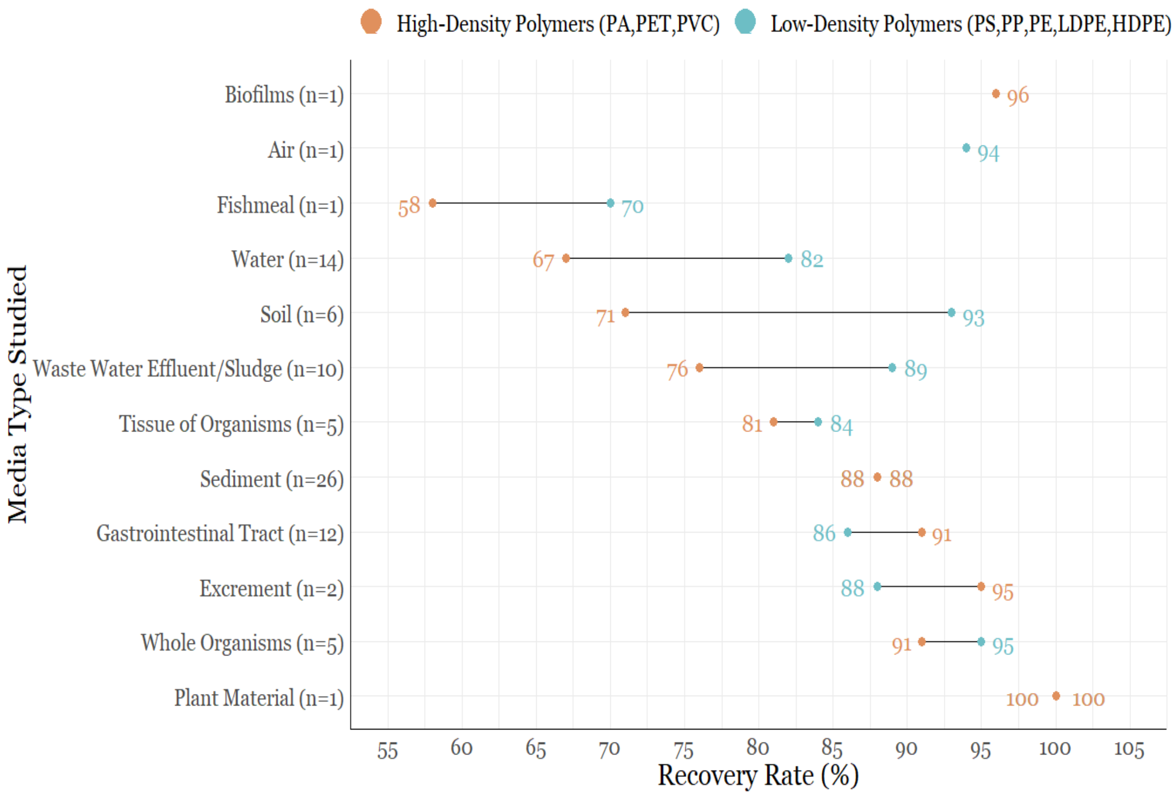

Figure 2.6 **Average recovery rates across studies of high and low-density polymers when extracted from different media.**

Numbers against the media represent number of studies in this meta-analysis using each medium.

2.4.2.3 Recovery rates of polymers using different reagents

Similarly to the trend found in the recovery of polymers in different media, most reagents recovered more low-density polymers than high density polymers, which is the case for water, saline solutions, oxidising agents, bases and dyes. However, the opposite is found when studies used solvents, alcohols, acids and oils, which removed more high-density polymers. Moreover, all but one reagent (water) recovered more than 80% of spiking polymers on average. However, the studies that used water as a reagent to recover the polymers showed the lowest recovery rates (averages 53% for high-density polymers, 65% for low-density polymers) (Figure 2.7).

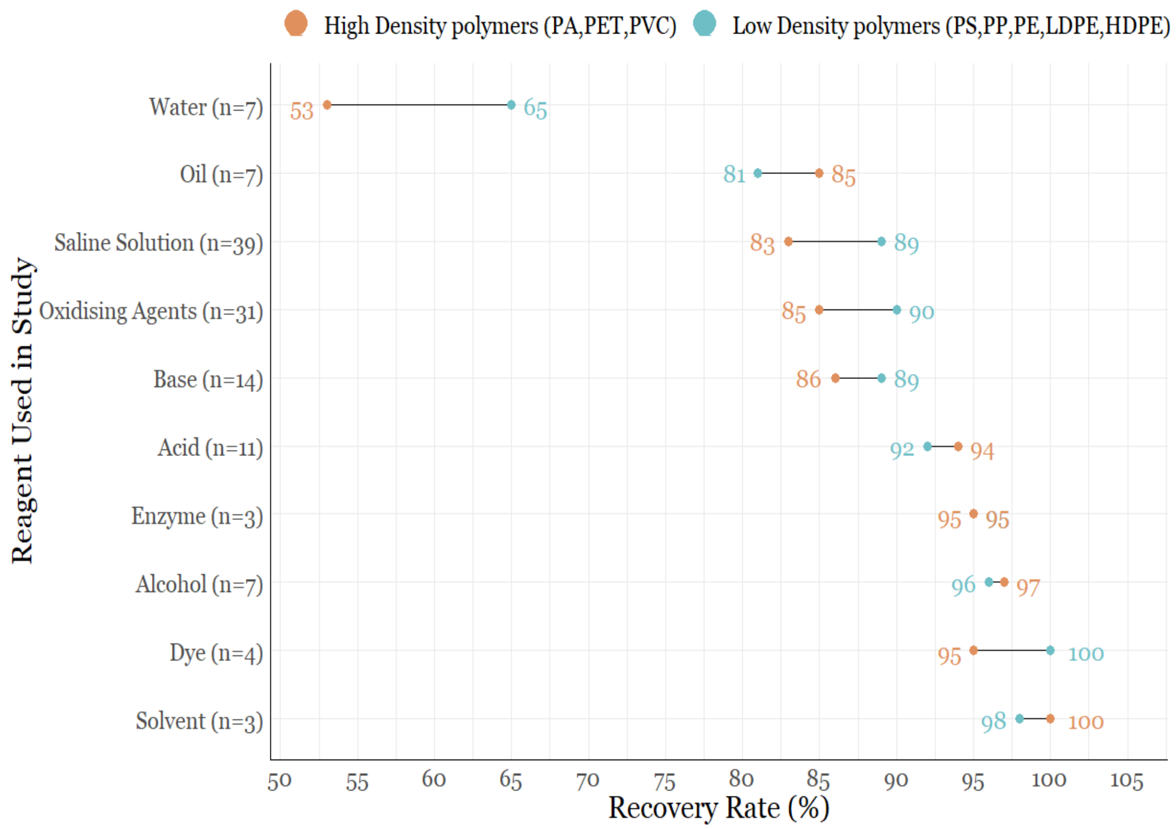

Figure 2.7 **Average recovery rates across studies of high- and low-density polymers when extracted using different reagents.**

Numbers against the reagents represent the number of studies in this meta-analysis using each reagent.

2.4.2.4 Combination of different reagents and media on the recovery rates of polymers

Individually, reagents and type of media have an effect on recovery of microplastic polymers (Figure 2.6 & Figure 2.7), however they can also have an effect on recovery when combined (Figure 2.8). For example, the use of an acid as a reagent results in higher recovery than other reagents when used in the same media. This is the case for excrement, sediment and whole organisms. However, when an acid is used to recover polymers from wastewater/sludge and water, lower recovery rates are found (Figure 2.8). The use of oxidising reagents recovered the most polymers from air, excrement, gastrointestinal tracts and plant material, however, these reagents resulted in very low recoveries of high-density polymers from soil (Figure 2.8).

Similarly, saline solutions recover high amounts of polymers from air and whole organisms, but lower amounts from media such as excrement, fishmeal, soil, tissues of organisms and wastewater/sludge (Figure 2.8).

Moreover, the use of an oil as reagent to recover plastics produced high recovery rates in soil. However, much lower recovery rates were found when using the same reagent to extract polymers from gastrointestinal tracts and tissues of organisms.

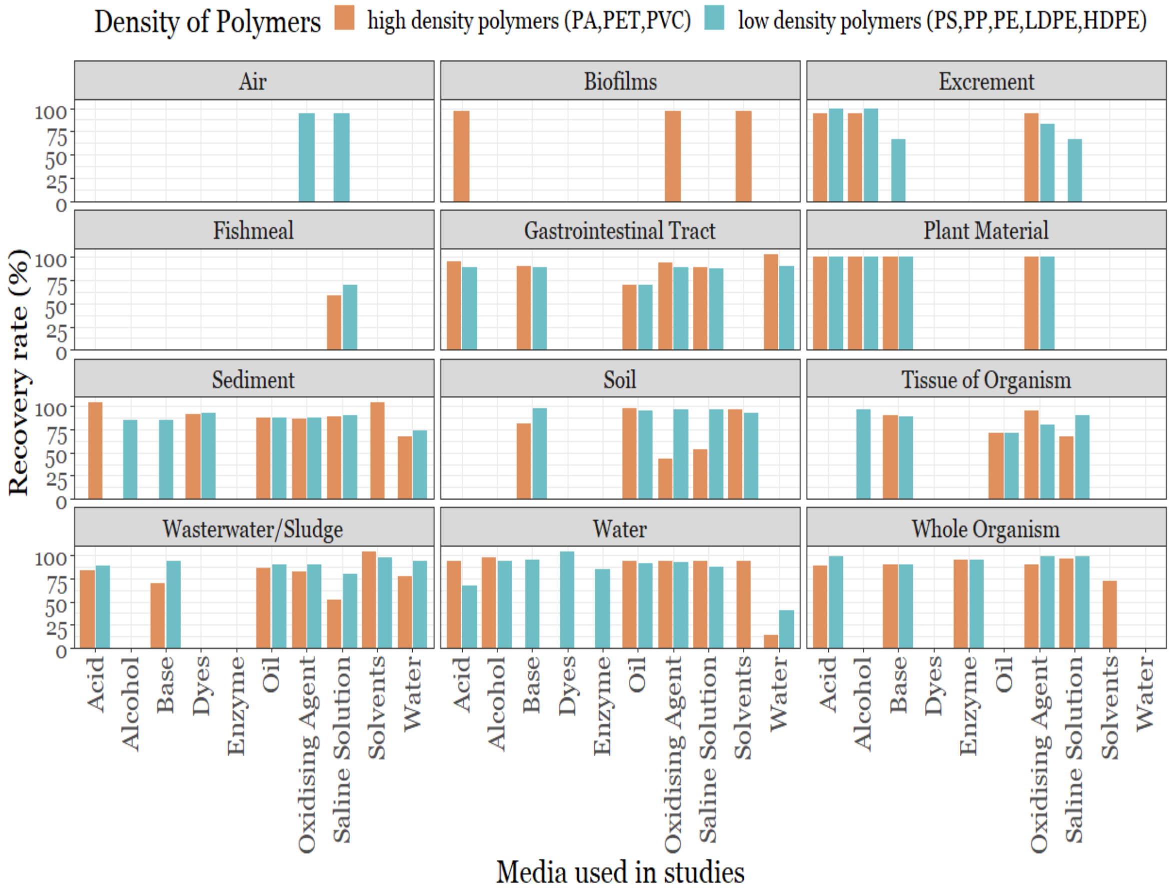

Figure 2.8 **Recovery rates of media and reagents combined**

Average recovery rates across studies of high and low-density spiking polymers when using different reagents and tested on different media.

2.4.3 Assessment of underestimation

As seen in Figures 6-8, very few combinations of reagents and media tested result in 100% recovery of spiking microplastics, meaning there is a level of underestimation when using these methods to extract polymers. Due to the lack of consistent information reported and the low importance given to recovery experiments in much microplastic research, an effect size could not be calculated for this meta-analysis. Therefore, this was counterbalanced by calculating a weighted mean based on equations provided by Gurnsey (2017). It is estimated that microplastic research could be underestimating how many microplastics are found by approximately 14% (calculation in Appendix. A.4), based on the type of reagents and medium used. It would be recommended to take any underestimations found by method validation into account when concluding how many microplastics are found in environmental samples. Underestimations may be higher or lower than 14% depending on the method used, including the medium and reagents used.

2.5 Discussion

This meta-analysis has gathered recovery rates from studies that have used a wide array of media (Figure 2.2), including plant material, fishmeal, biofilms, air, excrement, whole and tissues of organisms, soil, wastewater treatment plant products, gastrointestinal tracts, water and sediments. There are benefits to studying such different types of media as it has been increasingly evident that microplastic contamination of the environment is enormously widespread. For example, Ross *et al.* (2021) found polyester fibres in remote environments such as the Arctic. However, with regards to the method used with these new media types, problems can arise, specifically with the ability to standardise. Microplastic researchers have been calling for standardisation when it comes to methods for extraction (Skalska *et al.*, 2020). However, a "one-size-fits-all" kind of method is extremely difficult to achieve when properties of the study media vary so drastically. Lusher *et al.* (2020) explained how methods could be divided depending on their complexity and the number of steps needed.

Similarly, with new methods being developed to extract microplastics from complex media, often new reagents are used. This meta-analysis found a range of reagents including solvents, enzymes, dyes, bases, acids, oxidising agents, water, alcohols, oils and saline solutions (Figure 2.2). These were either used individually (Digka *et al.*, 2018; Thiele *et al.*, 2019) or sometimes combined (Hurley *et al.*, 2018; Yu *et al.*, 2019). With the aim of microplastic research to identify harmful microplastics in the environment to eventually find solutions for their removal, it could be argued that harmful/toxic reagents should not be used in methods. For example, zinc chloride ($ZnCl_2$) and sodium hypochlorite (NaOCl) are commonly used to extract microplastics (Collard *et al.*, 2015; Coppock *et al.*, 2017), however both of these reagents can be toxic to the environment and marine life and have multiple hazard statements in safety data sheets. For example, zinc chloride can alter bone development of zebrafish (Salvaggio *et al.*, 2016), and similarly sodium hypochlorite can cause acute toxicity on the same species (Emmanuel *et al.*, 2004). However, high recovery rates (>80%) of microplastics have been found when using less harmful alternatives such as sodium chloride

(Quinn *et al.*, 2017). Moreover, it could be the case that certain regents are more suited to extracting microplastics from certain media. For example oil works as a better reagent to recover microplastics from soil than gastrointestinal tracts and tissues of organisms (Figure 2.8). Reasons for this could be due to the majority of soils having less than 30% of organic matter, allowing oil to work well as a density separation (Radford *et al.*, 2021). Whereas oil may not work as well at separating microplastics from biological material such as gastrointestinal tracts or tissues, which often need to be digested beforehand with use of a strong oxidising agent such as hydrogen peroxide (H_2O_2) (Avio *et al.*, 2015).

As a part of a recovery rate study, spiking polymers/microplastics are used. This meta-analysis identified that a wide range of type, shape and size polymers were used (Figure 2.3 and Figure 2.4), with little explanation or justification in each of the studies. The most commonly used spiking polymers were PE, PS, PET and PP. It would be most reflective of real environmental conditions if the spiking polymers used would be the same as those commonly found in the environment. Phuong *et al.* (2016) found that most studies use more plastics in experiments than those in the environment, but the most common microplastics found in the environment are polyethylene, polypropylene and polystyrene. Therefore, the four most widely used spiking polymers in this meta-analysis are environmentally relevant if used in the correct quantity. Similarly, it is important that the shape and size of the spiking plastics is environmentally relevant. The most common shape used in the studies in this meta-analysis is fragments (Figure 2.4). A review by Phuong *et al.* (2016) confirmed that this is also the most commonly found shape in sediment and water samples, however other shapes such as fibres were also predominant depending on the type of method used. The shape of the spiking polymer is an important aspect to consider as different shape microplastics may be recovered easier than others. For instance, researchers have reported some microplastics sticking to glassware (Thiele *et al.*, 2019). Also, foam-like microplastics such as polystyrene have a low density of 0.028-0.045 g/cm^3 (British Plastics Federation, 2020) which enables it to float more readily than other denser microplastics, thus enabling easier density separation. Micro-sized plastics (1 µm- 1 mm) (International Organization for Standardization, 2020) were the most commonly sized spiking plastic identified in this meta-analysis. This is environmentally relevant. However, it is becoming apparent that smaller nano-sized (<1 µm) particles may be more abundant in the environment but have yet to be studied in depth due to technological limitations. An example of this limitation is the ability to identify and quantify such small particles. Even if nanoplastics are in high abundance, their mass could be so low that it is difficult to detect with current technology and methods, or nanoplastics may be found aggregated to other particles due to their size, making them difficult to isolate

(Jakubowicz *et al.*, 2021). This provides further evidence that smaller microplastics are more difficult to isolate, with this analysis showing that on average, macroplastics and larger microplastics were recovered at a higher rate than the smaller microplastics (Figure 2.5). However, as larger microplastics (1-5 mm) and macroplastics (>5 mm) are more easily visible to the eye, it may lead to a bias in being able to locate and identify these plastics during a recovery type study than the microplastics (1 µm- 1 mm) and nanoplastics. This could be particularly apparent during rinsing stages where the observer is visibly concentrating on clearing glassware of debris.

The environmental relevance of the types of plastics used as spiking polymers is crucial as it must represent as close to a true environmental sample as possible. Microplastics in the environment may vary in bioavailability and toxicity depending on many factors including the aforementioned type, shape and size, but also due to their colour, crystallinity and stability (Ma *et al.*, 2020). These properties will not only affect the organisms in the environment but will also affect the way in which the plastics can be extracted from the environmental medium. Furthermore, these type of spiking recovery studies typically use new or ‘virgin’ plastic to spike the sample. However, true extractions from environmental media will usually involve isolating material that has been subjected to some ageing and weathering thus will behave differently from the virgin spiking material. Routine spiking studies with weathered microplastics would be challenging to deliver but is an area that could reward some further study.

When looking at the recovery of microplastics from different media types, microplastics were recovered at higher rates from some types over others. For example, plant material, biofilms, air, whole organisms and excrement had recovery rates over 94%, whereas fishmeal, water, soil and wastewater effluent/sludge had recovery rates below 80% (Figure 2.6). This could be due to some of the properties of those media types. For example, there would be less organic material to breakdown in air than in fishmeal and soil. Radford *et al.* (2021) found organic material was one of the key factors in hindering the recovery of microplastics. Similarly, Wang *et al.* (2018) found that particle size influences the ability to extract microplastics from soil and biosolids, as some nano and micro-sized plastics take longer to float than larger sized plastics. Moreover, the range of recovery between low and high-density microplastics varies considerably between the different media types. For example, there is 22% difference between low and high-density microplastics recovered from soil (71-93%) (Figure 2.6), but only 3% different from those recovered from tissues of organisms (81-84%). This could be due to the complexity of the test media. For example, the soil used in the different studies may vary considerably in regards to particle size distribution and organic matter which depending on the quantity of each, may benefit the lower-density plastics, but hinder the high-density plastics.

Similarly, this meta-analysis has revealed that using different reagents can yield different recovery rates. The majority of the reagents (oil, saline solutions, bases, acids, oxidising agents, enzymes, alcohols, dyes and solvents) recovered more than 80% of the spiking plastics (Figure 2.7). However, in the studies which used water, recovery rates were below 65% (53-65%) (Figure 2.7). This is not surprising as the density of water is approximately 0.99 g/cm^3 (Tanaka *et al.*, 2001), which is lower than many plastics (PET: 1.37 g/cm^3, PVC: 1.38 g/cm^3 (British Plastics Federation, 2020)). In the cases when using water, saline solutions, oxidising agents, bases and dyes, the lowest recovery rates were found with the higher density plastics (Figure 2.7). In the case of saline solutions, of the 39 types used in the studies included, 16 used NaCl solution, which is a low-density solution and thus will be less likely to extract the higher density plastics. What is surprising, is that in some cases when using oils, alcohols and solvents, more high-density polymers were recovered than low density polymers (Figure 2.7). A reason for this could be due to the density of these reagents. Chloroform has a density of 1.49 g/cm^3 but is corrosive enough to attack plastics (National Center for Biotechnology Information, 2021). The high density of chloroform will allow for higher density plastics to float, however, depending on the concentration of chloroform and length of exposure, certain types and sizes of microplastics may corrode.

This meta-analysis highlights average recovery rates of microplastics from different environmental media. As seen in Figure 2.6 and Figure 2.7, it is clear that recoveries can vary between different density polymers even when using the same medium or reagent. This opens the argument of how repeatable these methods are. The variance could be simply due to the density of the polymers, but there could also be influence from human error (more experienced observers will more easily identify plastics), different laboratory equipment (glassware etc) and slight changes in the methods used. This reinforces the need for stringent QA/QC measures, particularly intra/inter laboratory method repeats when developing microplastic extraction methods.

What is overwhelmingly clear from the results of this meta-analysis is that it is rare for all spiking plastics to be recovered, thus a 100% recovery rate is seldom achieved. This meta-analysis found that on average- across all studies, microplastics could be underestimated by approximately 14% (See Appendix. A.4 for calculation). More so, studies rarely account for any underestimation brought about by the methods used. If underestimations are accounted for, the amounts of microplastics in the environment could be a lot larger than estimated to date. Although these figures show that there is an underestimation in the amount of microplastics in the environment, the true figure could be even lower if there has been contamination of plastics from the environment or methods used. This shows the potential for both under and over estimation and the need for strict QA/QC procedures such as these recovery studies and procedural blanks.

Overall, this meta-analysis has highlighted many issues within recovery rate studies and microplastic research. Firstly, recovery rate studies are rarely used to validate methods in published studies. For example, the 71 studies found and used in this analysis is a minute size compared to the large number of microplastic research papers and methods that have been published over time (Provencher *et al.*, 2020). Furthermore, those papers that are published with a recovery rate study are often poorly executed with key information missing, such as sample size and the type, shape and size of the spiking plastic used. With this missing information, it is difficult to make further inferences regarding the effect size and publication bias, also this makes it problematic for others to replicate the method used. Often recovery rate results are poorly displayed and are seen as unimportant compared to the main results of a study. A standardisation needs to be agreed on in several aspects of these studies. Firstly, it should be agreed on whether recoveries are calculated by weight difference or difference by count; and secondly, the terms used to describe the shapes of the spiking polymers, often the term ‘particle’ is used, which can be interpreted in many ways. Due to the aforementioned limitations, recommended reporting criteria were assembled specifically for recovery rate studies, with the intention of making validation of microplastic extraction methods clearer to others.

2.6 Conclusions and Recommendations

The varying range of recovery rates found in the studies included in this meta-analysis were dependent on the media types and reagents used. However, very rarely were 100% of the spiking plastics recovered, and overall an underestimation of 14% was discovered, meaning the amount of microplastics in the environment could be higher than estimated from research studies to date. From this meta-analysis it is clear that recovery studies are not utilised enough and, on the occasion, when they are, they are often poorly executed. It could be argued, that with a more holistic approach to validating methods, by studying the properties of the test medium, and clearly and concisely reporting the recoveries, it could help with the ever-growing issue of standardisation in microplastics research. This meta-analysis flagged several limitations within recovery rate studies, which the following improvements are recommended:

Report all raw or average recovery rates with variance in both tabulate AND graphical form. Include this in supplementary material if needed. Many studies either reported a single percentage in the text or displayed recovery rates in graphical form, often making it difficult to extract an exact percentage, thus making it difficult for others to accurately assess the effectiveness of the method.

Calculate the recovery rate by count of recovered plastics. Few studies calculated the recovery rate by change in weight, these studies were removed from this meta-analysis as they were not comparable to the majority which use counts. If this is adopted by all, it allows for standardisation.

Use triangulation: have multiple researchers count recovered plastics in a study. If counted by eye, counts of recovered microplastics could be different depending on the observer’s experience carrying out this task.

Report the number of samples used in the recovery rate study. Many studies did not report the sample size, making it difficult for further analysis.

Report the shape, size, type and size of spiking plastics used. The reporting style of the spiking plastics across the studies varied considerably. For example, one study did not state the type of polymer used, ten studies did not state the shape of the polymer used, eleven studies used the word ‘particle’ to describe the shape, which could be interpreted differently by others, and four studies did not report the size of the polymer used. It is recommended that reporting these properties clear enough for replication and to use environmentally relevant quantities which are reported in the literature for each test medium.

Do the recovery rate study on the same media which is to be tested for the main experiment. Methods will work differently on media with different properties, thus different recovery rates will be found.

The aim of this meta-analysis is to highlight the importance to researchers of using a recovery rate study/trial to validate their methods, with the proposal that in the future this becomes a “new normal” during method development, and the quality of these types of studies are up to a standard that can be replicated. Figure 2.8 can be used as a matrix by other researchers as a way to decide which solutions would work best to extract microplastics from the variation of environmental media included. Furthermore, if the amount of underestimation, brought about by the methods used is accounted for in each study, the amounts of microplastics reported will probably be higher but more realistic, which can offer more robust evidence for policy makers

Chapter 3 Assessing the effectiveness of microplastic extraction methods on fishmeal with different properties

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Concept for this chapter was developed by Chloe Way and supervisors (co-authors), methodological development (including laboratory work), analysis and writing was undertaken by Chloe Way.

3.1 Abstract

Microplastic presence in fishmeal is an emerging research area because of its potential to enter food chains, and the importance of fishmeal within global food security. However, fishmeal is a complex medium dependant on fish composition. This study measured properties (organics, carbonates, protein and density) of five fishmeal types (trimmings, sardine and anchovy, krill, tuna and salmon), sourced from locations worldwide (Norway, South America, Antarctica, Spain and Scotland). Microplastic recovery rates were compared for existing methodologies using sodium chloride overflows and potassium hydroxide digestions and then compared to newly developed methods. These methods included dispersants and calcium chloride density separations which were developed and designed to be environmentally conscious and affordable, which arguably should become an international standard approach for researchers. A calcium chloride overflow with dispersant and potassium hydroxide digestion provided highest recovery rates in sardine and anchovy fishmeal (66.3 %). Positive correlations with recovery rate were found with protein content, and negative correlations with organic content. Low recovery rates found here suggest microplastics in fishmeal reported in the literature are underestimated. With complex media such as fishmeal, attention must be paid to variation between types and composition when choosing methods and interpreting results.

3.2 Introduction

Plastic pollution is a concern worldwide. Tides, rivers and currents such as the North Atlantic current (Winther and Johannessen, 2006), the Norwegian Coastal current (NCC) (Winther and Johannessen, 2006), the Humboldt current (Kämpf and Chapman, 2016b), the Canary current (Kämpf and Chapman, 2016a), and the melting of sea ice around the Antarctic peninsula (Nicol *et al.*, 2012) provide pathways for plastics to enter the marine environment. It is thought than an estimated 1.15-2.41 million tonnes of plastic enter the ocean from rivers alone (Lebreton *et al.*, 2017). Once in the marine environment, plastic debris is subject to fragmentation into secondary microplastics by ultraviolet radiation, and mechanical and microbial degradation (Andrady, 2015a). Other forms of microplastics include primary microplastics that are manufactured as a small size, such as those in toiletries, cosmetics, tyre wear particles and synthetic fibres from washing clothes (Boucher and Friot, 2017). A definition of microplastics which includes their physiochemical properties was proposed by Frias and Nash (2019) : "Microplastics are any synthetic solid particle or polymeric matrix, with regular or irregular shape and with size ranging from 1 μm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water". However, others believe large microplastics are between 1-5 mm (International Organization for Standardization, 2020).

Due to the widespread nature of marine microplastics, there is a high potential for them to infiltrate the human food chain. Many studies have identified microplastics in the gastrointestinal tract (Guven *et al.*, 2017; Lusher *et al.*, 2013; Roch and Brinker, 2017) and gills (Brennecke *et al.*, 2015; Fabbri *et al.*, 2020) of marine life; however, few have studied either the whole fish or the tissue used as food for humans. Ribeiro *et al.* (2020) investigated the edible sections of commonly eaten seafood such as oysters, prawns, squid, crabs and sardines, and found sardines had the highest amount of microplastic by mass (0.3 mg g^{-1} tissue). Similarly, Karami *et al.* (2017b) found more MP in the flesh of dried fish than the organs. There are many avenues microplastics may enter this pathway. For example, in areas where microplastics concentrations are high, it is more likely that some will be ingested by organisms (non-selective feeding) (Scherer *et al.*, 2017). Moreover, some marine organisms have shown an ability to selectively ingest microplastics of certain sizes (Cole *et al.*, 2013). Many marine organisms exposed to microplastics are harvested for fishmeal production, which indicates the potential for microplastic-contaminated fishmeal to get into the human food chain.

Fishmeal is a foodstuff made of whole fish or fish trimmings that is broken down, cooked, strained and milled (Food and Agriculture Organization of the United Nations, 1986). It has a high nutritional content including proteins, omega-3 fatty acids, amino acids and vitamins, that can support the diet of many animals (IFFO The Marine Ingredients Organisation, 2020). The majority of landings in certain fisheries around the world supply primarily to the fishmeal sector. For example, 98% of landings of Peruvian anchovies are used to produce fishmeal and fish oil (Wijkstrom, 2010). Fishmeal is mainly used as feed in aquaculture, pig and poultry farming (Shepherd and Jackson, 2013). Furthermore, aquaculture provided 171 million tonnes of fish in 2016, with 88% being used as food for humans (Food and Agriculture Organization of the United Nations, 2018). The fish provided by aquaculture are a cheap source of protein and in 2018, aquaculture was the main supply of fish for 52% of the world's population (Food and Agriculture Organization of the United Nations, 2018), which showcases the importance of aquaculture with respect to global food security (Pradeepkiran, 2019). Fishmeal is of considerable economic value, with Peruvian fishmeal pellets alone selling for £1,126 per metric tonne in 2009 (World Bank Commodity Price Data, 2020). Therefore, in the light of growing public concern surrounding microplastics, it is necessary to evaluate the production of fishmeal and food as a potential exposure pathway.

Fishmeal is a considerably complex medium, which will bring about issues when creating a method to isolate the microplastics within. Previously, other media including: seawater (Cole *et al.*, 2014; Guven *et al.*, 2017; Grbic *et al.*, 2019); freshwater (Grbic *et al.*, 2019; Rodrigues *et al.*, 2018); estuaries (Anderson *et al.*, 2018; Stead *et al.*, 2020); sediments (Crichton *et al.*, 2017; Guven *et al.*, 2017; Nakajima *et al.*, 2019b); soils (David *et al.*, 2018; Hurley *et al.*, 2018; Steinmetz *et al.*, 2020); sewage/wastewater (Hurley *et al.*, 2018; Wang *et al.*, 2018); and biota (Guven *et al.*, 2017; Lusher *et al.*, 2013; Lusher *et al.*, 2017; Phillips and Bonner, 2015) have been assessed for microplastics using various different methods. Studies use density separation techniques involving saline solutions (Nuelle *et al.*, 2014; Wang *et al.*, 2018), and acidic and basic solutions to digest a media, making the polymers more easily available for extraction (Bianchi *et al.*, 2020; Schirinzi *et al.*, 2020). An aim of many of these studies is to develop and standardise methodologies within each medium. Fishmeal is yet to be studied in much depth, with few studies at present being able to isolate and identify microplastics, and few validating methods with a recovery study to show how effective they are at recovering microplastics. Underwood *et al.* (2017) also noted this issue of many studies not validating methods with a recovery experiment. Moreover, studies that have extracted microplastics from fishmeal, have used widely different methods applied to different kinds of fishmeal, which vary considerably with regard to source material and composition.

Hanachi *et al.* (2019) and Karbalaei *et al.* (2020) have reported similar methodologies (potassium hydroxide (KOH) digestion) albeit with slight differences in amounts of sample and spectroscopic method used. Also, the fishmeal used is different, with Hanachi *et al.* (2019) using fishmeal from Iran, composed of salmon, sardines and kilka caught in the Persian Gulf and Caspian Sea, whereas the study by Karbalaei *et al.* (2020) used Malaysian fishmeal containing Indian mackerel (*Rastrelliger kanagurta)* and fish waste from the China Sea. Thiele *et al.* (2021) investigated microplastics in fishmeal but used a very different method than the previous studies; concluding that a sodium chloride (NaCl) soak and density separation was the most suitable method to extract microplastics from fishmeal, as applied to whitefish fishmeal, and sardine and anchovy fishmeal. This study was the only fishmeal focused study that undertook a recovery study (producing recoveries between 49 and 71%). More recently, Gündoğdu *et al*. (2021) assessed 26 different fishmeal types including fishmeal composed of; pilchard, blue whiting, sandeel, krill, anchovy, sprat, sardines, and mixed fish. They separated the microplastics from the fishmeal using a 30% KOH:NaClO solution as a way to digest the organic material before using NaI as a density separation.

Research into microplastics is fundamentally about studying its effects in/on the environment. Therefore the study of this pollutant should not contribute harm to the environment either, including the use of chemicals. Many chemicals are known to be toxic to aquatic life, for example, zinc chloride can affect the growth of fish embryos (Salvaggio *et al.*, 2016). Similarly, the cost of studying microplastics should be kept to a minimum where possible to maximise opportunities for research and monitoring globally. Microplastic research is evolving at such as rate that standardisation should be of high importance so that studies can be comparable. However, for many researchers, this cannot be adhered to if the cost of equipment/chemicals used are high. Therefore, the aim of this study is to use equipment and chemicals that are affordable, environmentally friendly and easily accessible.

What is clear from the literature is that many methodologies are being investigated on many types of fishmeal, with no clear reason as to why certain methods are being chosen over others. Fishmeal has a range of different properties, from protein and oil content, to organic content, carbonate content and different bulk densities. Consequently, it could prove difficult to apply one universally effective method to all different types of fishmeal to extract microplastics reliably and consistently. Therefore, this study aims to: i) investigate whether different methods used to extract microplastics (density separation, chemical digestion and dispersants) are more suited to fishmeal with certain characteristics (protein content, organic content, carbonate content and bulk density) and ii) aims to consider practicality, environmental impact and cost-effectiveness when developing new methods.

Hypothesis: The properties of fishmeal and the type of method used will affect the recovery of microplastics.

3.3 Methods

Methods from previously published studies looking into microplastics into fishmeal (Gündoğdu *et al.*, 2021; Karbalaei *et al.*, 2020; Thiele *et al.*, 2021) were gathered and assessed with regard to the effectiveness of extracting microplastics from fishmeal, while remaining cost effective and using environmentally friendly reagents. High-cost methods are those which use a reagent that is over USD$100 per litre (Table 3.1). Environmentally friendly methods are those which do not have a report of aquatic toxicity on the respective safety data sheets (Table 3.1). The method by Gündoğdu *et al.* (2021) was investigated but ruled out due to the inclusion of large amounts of high-cost reagents which are not environmentally friendly. The method by Karbalaei *et al.* (2020) was tested as only a small amount of expensive reagent (NaI) is required. The method by Thiele *et al.* (2021) was tested, and due to it being the most environmentally friendly and cost-effective method, it was further developed using commonly used methods in microplastic extraction such as chemical digestion with KOH, the use of a dispersant (Sodium hexametaphosphate), and an increased density saline solution of low-cost calcium chloride (Table 3.1). These methods are detailed in Table 3.3. The effectiveness of each method on each fishmeal was assessed by determining the recovery of spiked microplastics. Polymers were not assessed for signs of degradation: KOH at a temperature of 40 °C was the only digestion solution used and has already been tested for its ability to degrade polymer fragments (Karami *et al.*, 2017a) and fibres (Treilles *et al.*, 2020; Radford *et al.*, 2021) at this temperature, with no effect found.

Table 3.1 **Different types of saline solution commonly used in the literature**

With common densities in the solution, its effect on the environment and approximate costs as a salt and in solution. Environmental friendliness based on whether an aquatic toxicity hazard is listed on the safety data sheets of Fisher Scientific (2022). N/A (Not applicable).

Separating solution	***Density of salt in solution (g/cm^3)**	**Solution density in literature (g/cm^3)[3]**	**Environmentally friendly?[2]**	**Approx. Cost[1] (USD/kg)**	**Approx. cost per litre* (USD/L)**
Seawater	1.02	N/A	Y	N/A	N/A
Sodium Chloride (NaCl)	1.19 (26 wt% @ 25°C)	1.2 [a]	Y	~$60.54	~$15.74

Separating solution	*Density of salt in solution (g/cm^3)	Solution density in literature (g/cm^3)[3]	Environmentally friendly?[2]	Approx. Cost[1] (USD/kg)	Approx. cost per litre* (USD/L)
Calcium Chloride ($CaCl_2$)	1.39 (40 wt% @ 20˚C)	1.46 [b], 1.4 [c]	Y	~$60.69	~$24.27
Sodium Bromide (NaBr)	1.41 (40 wt% @ 20˚C)	1.37 [d], 1.55 [e]	N	~$96.14	~$38.45
Zinc Chloride ($ZnCl_2$)	1.7 (60 wt% @ 20˚C)	1.5 [f]	N	~$87.31	~$52.38
Sodium Iodide (NaI)	1.8 (60 wt% @ 20˚C)	1.566 [d], 1.8 [g]	N	~$533.98	~$320
Sodium Polytungstate	3.1 (85 wt% @ 20 ˚C)	1.5 [h]	N	~$623.42	~$497.94

[1] Cost per kg listed on Fisher scientific (Fisher Scientific, 2022), in US Dollars (USD)

[2] Sodium Iodide hazards includes aquatic toxicity. Zinc Chloride hazards include chronic aquatic toxicity. Sodium Bromide should not be released into the environment. Sodium Polytungstate may cause long term adverse effects in the aquatic environment.

[3] Literature: a (Thiele *et al.* (2021)), b (Crichton *et al.* (2017)), c (Stolte *et al.* (2015)), d (Quinn *et al.* (2017)), e (Liu *et al.* (2019c)), f (Coppock *et al.* (2017)), g (Kedzierski *et al.* (2017)), h (Corcoran *et al.* (2020)).

3.3.1 Spiking microplastics

Microplastic polymer types, sizes and amounts used for spiking were based on the methods used by Radford *et al.* (2021). Materials used to create the spiking plastics were from common consumer products and consisted of the main six plastic resin codes (American Chemistry Council, 2020) (Table 3.2). Each polymer was either sorted into fibres and fragments (PET and PP) or sorted into two size categories (0.25-0.5 mm (small) and 0.5-1 mm (big)) (HDPE, PVC, LDPE and PS). Plastic fragments were sized using a household coffee grinder and sized metal sieves (1 mm, 0.5 mm, 0.25 mm), and fibres were manually cut. The spiking plastics were chosen due their specific characteristic and/or colours to aid straightforward identification when mixed with a sample and included polymers that could be broadly categorised as high (> 1 g/cm^3: PET, PVC) and low (< 1 g/cm^3: HDPE, LDPE, PP, PS) density. The spiking plastic polymer types were confirmed with high matches (>85% for all polymers) using Attenuated Total Reflectance Fourier-Transform Infrared spectroscopy (ATR FTIR) (Frontier, Perkin Elmer). Each fishmeal sample was spiked with a total of 60 microplastic particles (five of each type of spiking plastic created). The samples were spiked by manually placing the microplastics into the glass jars containing the fishmeal using microscopes and tweezers. The jars containing the fishmeal were inverted several times to ensure the microplastics were evenly distributed throughout the fishmeal prior to any reagent being added.

Table 3.2 **Spiking plastics**

Spiking plastics used in this method with corresponding resin code, shape (fibre/fragment), size, colour, origin product and density (g/cm^3).

Resin Code	Abbreviation	Shape	Size (mm)	Colour	Original Product	Density (g/cm^3)[1]
1	PET	Fragment	0.5-1	Blue	Drinks Bottle	1.37
		Fibre	1-5	Green	Craft Ribbon	
2	HDPE	Fragment	0.25 – 0.5	Pink	Cleaning Product Bottle	0.944-0.965
		Fragment	0.5-1			
3	PVC	Fragment	0.25- 0.5	Red	Tablecloth	1.38
		Fragment	0.5-1			
4	LDPE	Fragment	0.25-0.5	Purple	Carrier Bag	0.917-0.930
		Fragment	0.5-1			
5	PP	Fragment	0.5-1	White	Storage Bottle	0.905
		Fibre	1-5	Purple	Carpet	
6	PS	Fragment	0.25-0.5	White	Packaging	0.028-0.045
		Fragment	0.5-1			

[1] Densities of plastics gathered from British Plastics Federation (2020)

3.3.2 Fishmeal

Commercial fishmeal samples were bought from online UK suppliers, with focus on collecting fishmeal made from various fish caught from different locations around the world. Fishmeal collected included Norwegian LT94 fishmeal, South American sardine and anchovy fishmeal, Antarctic krill meal, Spanish tuna fishmeal and Scottish salmon fishmeal. Properties of the fishmeal are detailed in Table 3.4. Protein and oil content of fishmeal was listed on their product specification sheets. The organic matter content was calculated using loss-on-ignition (LOI) at 550 °C and carbonate content was calculated using LOI at 950 °C. Bulk density of the fishmeal was calculated by weighing 1 cm^3 of dried fishmeal.

Each fishmeal sample was weighed in triplicate according to the amount needed for each method (Table 3.3). Methods used include those from existing literature (Karbalaei *et al.*, 2020; Thiele *et al.*, 2021) and new methods based on steps commonly used for other media (density separation (NaCl) with digestion and two density separations (NaCl and $CaCl_2$) with dispersant and digestion), which use environmentally friendly chemicals and solutions, with minimal steps to avoid loss of microplastics.

3.3.3 Method by Thiele *et al.* (2021) (Method 1)

Glass jars (550 mL) were used to accurately weigh 40 g of fishmeal in triplicate. NaCl (1.2 g/cm^3) was added to the fishmeal in 550 mL jars up to approximately 1 cm (50 mL) from the top, the lid was added, and the jar was shaken for 30 seconds. Thiele *et al.* (2021) stated jars must be left to stand to settle for a minimum of 30 minutes, in the case of this study, samples were left for 24 hours. Once settled, the jar was placed in a larger beaker and lid was removed. NaCl was slowly poured into the jar to allow the supernatant to overflow into beaker. The outside of the jar and the lid was rinsed with pure water into the overflow liquid. Each piece of equipment (jar, lid and filtering apparatus) was completely rinsed three times with continuous flow of pure water. This "overflow method" was repeated three times for each sample, filtering each overflow separately. The supernatant was vacuum filtered through 20-25 µm filter paper and stored in a petri dish for analysis.

3.3.4 NaCl density separation with KOH digestion (Method 2)

This method was created with similarities to the steps used by Thiele *et al.* (2021), to maintain levels of standardisation. 40 g of fishmeal was placed in 550 mL jars in triplicate and NaCl was added up to 1 cm (50 mL) from the top, before being shaken for 30 seconds and left to settle for 24 hours. The overflow method was applied; however, supernatant was filtered on to 25 µm metal filters. The metal filter was placed in glass jars with 200 mL 10 % KOH and heated to 40 °C and agitated at 100 rpm for 1 hour. The sample was then vacuum filtered through a 25 µm filter paper and stored in a petri dish for analysis.

3.3.5 NaCl density separation with dispersant and KOH digestion (Method 3)

This method was followed the same as the density separation with KOH digestion (Method 2), with one difference. Before NaCl is added to the sample, 50 mL dispersant (5 % Sodium hexametaphosphate) was added.

3.3.6 Method by Karbalaei *et al.* (2020) (Method 4)

This method was followed as closely as possible to the method reported. Glass jars were used to accurately weigh out 20 g of each fishmeal, in triplicate. Following this, 200 mL of 10 % KOH was added to the glass jars, which were then incubated at 40 °C for 72 hours. The contents of the jar were then vacuum filtered through 149 µm metal filters. This metal filter was then placed in 10 mL of 4.4 M sodium iodide (NaI) and sonicated at 50 Hz for 5 minutes, before the filter was removed, and the sonication step was repeated once more. The mixture was centrifuged at 500 x g for two minutes before allowing the supernatant to be filtered through an 8 μm filter membrane.

3.3.7 $CaCl_2$ density separation with dispersant and KOH digestion (Method 5)

This method was followed the same as the density separation with dispersant and KOH digestion (Method 3), with one difference; the saline solution was changed to a higher density (1.4 g/cm^3) solution of calcium chloride. Note the solution was filtered through a larger pore size filter (149 μm) due to the viscosity of the calcium chloride solution.

3.3.8 Calculating spiked plastic recovery rates

Recovered microplastic particles were manually counted under a Nikon SMZ100 microscope (x40 magnification) and percentage of microplastics recovered (recovery rate) was calculated.

3.3.9 Statistics

Statistical analysis was undertaken via RStudio (1.3.1093). Distribution of data were shown using histograms and Shapiro-Wilks normality tests. Non-normal distributions were observed in all data sets. Therefore, Kruskal-Wallis tests were used for the recovery rates of microplastics using different methods, and Dunn's test (without p value adjustments) to look for pairwise comparisons between fishmeal types and to identify which groups are statistically significantly different to one another. Kruskal-Wallis tests were used to analyse recovery rates of specific polymers between methods, and to analyse the recovery rates of different size and shape microplastics between methods used, followed by post hoc analysis with Dunn's test (without p value adjustments). Correlations between recovery rate and all four fishmeal properties were estimated using Spearman's rank.

Table 3.3 **Summary of methods used**

Five methods used in this study, consisting of two from existing literature (Thiele *et al.*, 2021; Karbalaei *et al.*, 2020) and three newly developed.

Author/Method	**Method Details**

Thiele *et al.* (2021) (Method 1)	- 40 g fishmeal to 550 mL glass jar. - Add NaCl (1.2 g/cm^{-3}) (99.5%, Acros Organics) to sample up to a cm (50 mL) from top of 550 mL jar. - Add lid and agitate for 30 seconds. - Leave for a minimum of 30 minutes. Overflow method - Place jar in larger container and remove lid. - Slowly pour NaCl into jar to allow supernatant to overflow into container. - Rinse outside of jar and inside of lid with pure water into overflow liquid. - Repeat overflow three times for each sample, filtering each overflow separately. - Filter supernatant through 20-25 µm filter paper and place in petri dish
NaCl Density Separation and KOH Digestion (Method 2)	- 40 g fishmeal to 550 mL glass jar. - Add NaCl (1.2 g/cm^{-3}) to sample up to a cm (50 mL) from top of 550 mL jar. - Add lid and agitate for 30 seconds. - Leave for a minimum of 30 minutes. - Follow Overflow method. - Filter supernatant onto 25 µm metal mesh. - Place metal mesh in 200 mL 10% KOH (>85%, Fisher Scientific) and heat to 40 °C at 100 rpm for 1 hour. - Filter over 20-25 µm filter paper.
Dispersant, NaCl Density Separation and KOH Digestion (Method 3)	- 40 g fishmeal to glass 550 mL jar. - Add NaCl (1.2 g/cm^{-3}) and 50 mL dispersant (5 % Sodium hexametaphosphate) (General purpose grade, Fisher Scientific) to sample up to a cm (50 mL) from top of jar. - Add lid and agitate for 30 seconds. - Leave for a minimum of 30 minutes. - Follow Overflow method. - Filter supernatant onto 25 µm metal mesh. - Place metal mesh in 200 mL 10% KOH and heat to 40 °C at 100 rpm for 1 hour. - Filter over 20-25 µm filter paper.
Karbalaei *et al.* (2020) (Method 4)	- Place 20 g fishmeal sample into 250 mL DURAN glass bottle. - Add 200 mL KOH to each sample. - Incubate sample at 40 °C for 72 hours. - Filter sample over 149 µm filter paper. - Place 149 µm filter paper in 10-15 mL NaI (≥99.5%, Sigma-Aldrich) and sonicate for 5 mins at 50 Hz by ultrasonic bath. - Remove filter papers and repeat sonication process. - Centrifuge solution at 500 x g for 2 mins at room temperature. - Filter the supernatant though 8 µm filter paper and place in petri dish.
Dispersant, $CaCl_2$ Density Separation and KOH Digestion (Method 5)	- 40 g fishmeal to 550 mL glass jar. - Add $CaCl_2$ (1.4 g/cm^{-3}) (93%, Fisher Scientific) and 50 mL dispersant (5% Sodium hexametaphosphate) to sample up to a cm (50 mL) from top of jar. - Add lid and agitate for 30 seconds. - Leave for a minimum of 30 minutes. - Follow Overflow method. - Filter supernatant onto 149 µm metal mesh.

	- Place metal mesh in 200 mL 10% KOH and heat to 40 °C at 100 rpm for 1 hour. - Filter over 20-25 µm filter paper.

3.4 Results

3.4.1 Fishmeal properties

Fishmeal properties measured include organic content (%), carbonate content (%), bulk density (g/cm^3), protein (%) and oil (%) (Table 3.4). Antarctic krill meal had the highest organic content (87.5%), the lowest bulk density (0.47 g/cm^3) and lowest protein content (56%). The South American sardine and anchovy fishmeal had the lowest organic content (74.7%), the lowest carbonate content (3.4%) and the highest bulk density (0.83 g/cm^3).

Table 3.4 **Properties of fishmeal**

Properties of five fishmeal types (Norwegian LT94, South American sardine and anchovy, Antarctic krill, Spanish tuna and Scottish salmon), including organic content (%), bulk density (g/cm^3), protein content (%) and oil content (%). Protein and oil contents were provided by the respective product specification sheets.

Fishmeal	Type of fish used	Organic Content (%)	Carbonate Content (%)	Bulk Density (g/cm^3)	Protein (%)	Oil (%)
Norwegian LT94	Species unknown, mix of whole fish and trimmings	81.75±0.04	5.47±0.03	0.74±0.01	71	12
S American Sardine & Anchovy	Whole sardines and anchovies	74.69±0.05	3.419±0.006	0.827±0.007	68	N/A
Antarctic Krill	Antarctic Krill	87.49±0.01	3.554±0.004	0.47±0.01	56	N/A
Spanish Tuna	Whole Tuna	77.89±0.23	3.46±0.04	0.69±0.01	60	12
Scottish Salmon	Whole Salmon	76.49±3.41	5.38±0.58	0.752±0.009	66	9

3.4.2 Recovery rates of polymers in fishmeal

The five methods used to extract the spiked microplastics from each fishmeal type produced significantly different recovery rates ($p<0.05$, Kruskal Wallis). The NaCl density separation method (method 1), the density separation with KOH digestion method (method 2), the NaCl density separation with dispersant and digestion method (method 3) and the $CaCl_2$ method (method 5) all recovered significantly more spiked microplastics overall than the method outlined by Karbalaei *et al.* (2020) (method 4) ($p<0.05$, Dunn's Test) (Figure 3.1).

The NaCl Density separation (method 1) recovered significantly different amounts of microplastics from the five different fishmeal types ($p<0.05$, Kruskal Wallis). This method was more effective at recovering microplastics from the Norwegian LT94 (48.3% (11.7 IQR) RR (recovery rate)) and sardine and anchovy (33.3% (19.2 IQR) RR) than the Spanish tuna (5% (3.3 IQR) RR) ($p<0.05$, Dunn's Test), and more effective at recovering microplastics from the Scottish salmon (56.7% (1.7 IQR) RR) than the Antarctic krill (8.33% (3.3 IQR) RR) and Spanish tuna (5% (3.3 IQR) RR) fishmeal ($p<0.05$, Dunn's Test).

The method using a NaCl density separation with a KOH digestion (method 2) recovered significantly different amounts of spiked microplastics from the five fishmeal types ($p<0.05$, Kruskal Wallis). This method recovered significantly more microplastics from Norwegian LT94 and Sardine and anchovy fishmeal (46.7% (8.3 IQR) RR and 43.3% (5.8 IQR) RR respectively), than Antarctic krill meal (5% (2.5 IQR) RR) ($P<0.05$, Dunn's Test), and this method was more effective at recovering spiked microplastics from Scottish salmon fishmeal (48.3% (7.5 IQR) RR) than Antarctic krill meal and Spanish tuna meal (18.3% (5 IQR) RR) ($p<0.05$, Dunn's Test).

The addition of a dispersant (sodium hexametaphosphate) to NaCl density separation and KOH digestion (method 3) resulted in significant differences between the recovery rate of spiked microplastics extracted from the five fishmeal types ($p<0.05$ Kruskal Wallis). Using this method, significantly more spiked microplastics were recovered from the Scottish salmon fishmeal (60% (6.6 IQR) RR) and the Norwegian LT94 fishmeal (53.3% (3.3 IQR) RR) than the Antarctic krill meal (15% (5.8 IQR) RR) and the Spanish tuna fishmeal (38.3% (15.8 IQR) RR) ($p<0.05$, Dunn's Test).

The method developed by Karbalaei *et al.* (2020) (method 4) did not affect the recovery rate of spiked microplastics between the fishmeal types ($p>0.05$, Kruskal Wallis). However, the Norwegian LT94 fishmeal, the sardine and anchovy fishmeal and the Scottish salmon fishmeal had the same median recovery rate of 16.7%.

When using an increased density saline solution of calcium chloride with a dispersant and a KOH digestion (method 5) (Figure 3.1), a significant difference in the recovered microplastics was found between the five fishmeal types ($p<0.05$, Kruskal Wallis). Significantly more microplastics were extracted from the sardine and anchovy fishmeal (66.3% (11.6 IQR) RR) than the Norwegian LT94 fishmeal (13.33% (5 IQR) RR) and the Antarctic krill meal (10% (4.2 IQR) RR) ($p<0.05$, Dunn's Test). Also significantly more microplastics were recovered from the Scottish salmon fishmeal (30% (10.8 IQR) RR) than the Antarctic krill meal using this method ($p<0.05$, Dunn's Test).

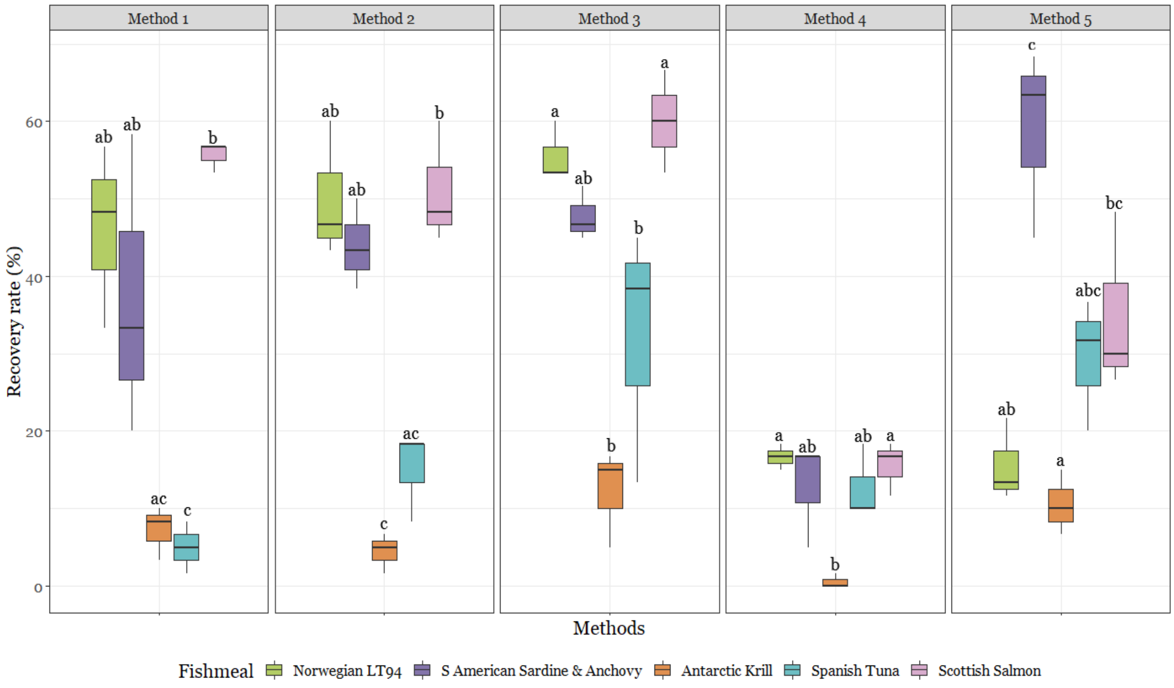


Figure 3.1 **Spiked microplastic recovery rates (%) from five fishmeal types, using four methods.**

Spiked microplastic recovery rate (%) from five fishmeal types (Norwegian LT94, South American Sardine and Anchovy, Antarctic Krill, Spanish Tuna and Scottish Salmon), using four extraction methods (NaCl density separation (Method 1), NaCl density separation followed by a KOH digestion (Method 2), NaCl density separation with Sodium hexametaphosphate dispersant followed by KOH digestion (Method 3), a previously published method by Karbalaei *et al.* (2020) (Method 4) and a Calcium Chloride density separation with Sodium hexametaphosphate dispersant followed by KOH digestion (Method 5)). Boxes represent median values with the interquartile range, whiskers represent min and max values. Boxes with different letters are significantly different (Dunn's test, $p<0.05$).

3.4.3 Effect of fishmeal properties on recovery rates

All methods but the method by Karbalaei *et al.* (2020) (method 4) produced strong significant positive correlations between spiked microplastic recovery rates and bulk density ($r_s = 0.71$ (method 1), $r_s = 0.73$ (method 2), $r_s = 0.63$ (method 3), $r_s = 0.75$ (method 5), $p<0.05$, Spearman's rank) (Figure 2). The NaCl density separation with added KOH digestion method (method 2), the density separation with dispersant and KOH digestion method (method 3) and the method by Karbalaei *et al.* (2020) (method 4) all had the strongest significant positive correlation between spiked microplastic recovery rate and protein content ($r_s = 0.76$, 0.71, 0.59 (respectively), $p<0.05$ Spearman's rank) (Figure 3.2). These three methods and the method with $CaCl_2$ used as a saline solution (method 5) shared the strongest significant negative correlation between recovery rate and organic content ($r_s = -0.52$, -0.38, -0.41, -0.89 (respectively), $p<0.05$ Spearman's rank). Moreover, there was no significant correlation between spiked microplastic recovery rate and organic content when using the NaCl density separation (Method 1) ($r_s = -0.46$, $p>0.05$, Spearman's rank) (Figure 3.2).

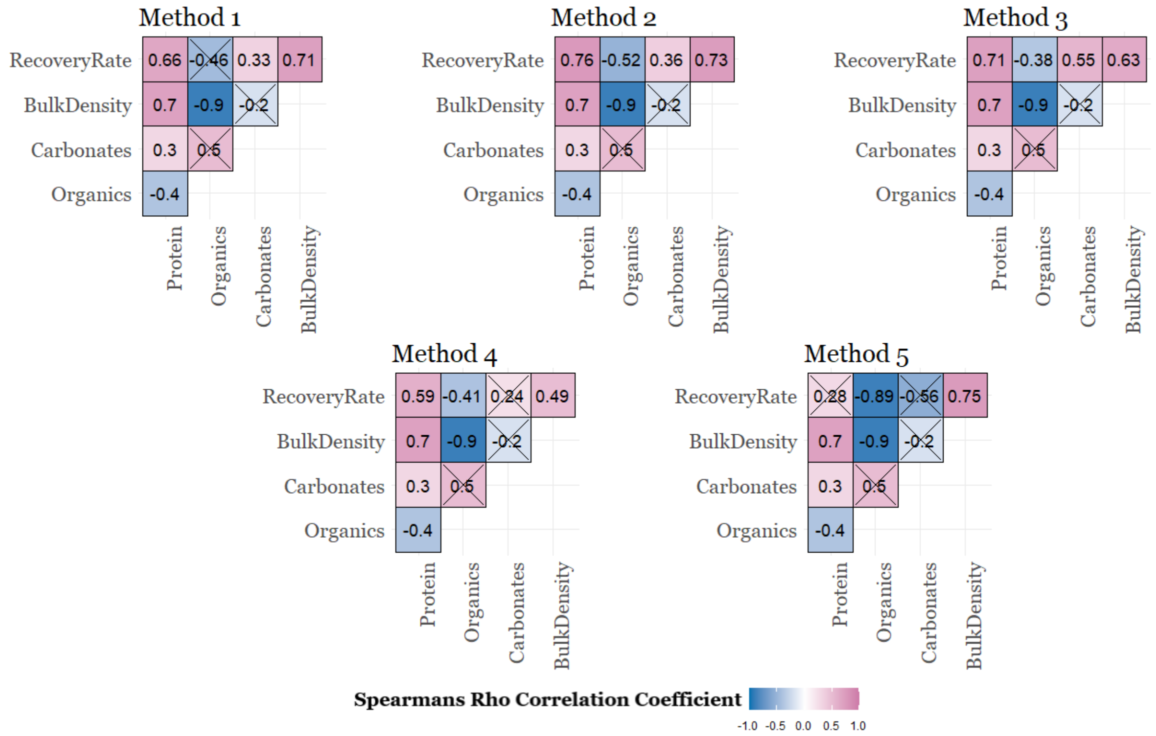


Figure 3.2 **Spearman Rho correlations between fishmeal properties and spiked microplastic recovery rate.**

Correlogram showing Spearman Rho correlation coefficients between fishmeal properties (organic content, carbonate content, protein content and bulk density) and spiked microplastic recovery rate. -1 indicates strong negative correlation, +1 indicates strong positive correlation. Squares including a black cross represent those correlations with no significance ($p > 0.05$). The five methods include: NaCl density separation (Method 1), NaCl density separation followed by a KOH digestion (Method 2), NaCl density separation with Sodium hexametaphosphate dispersant followed by KOH digestion (Method 3), a previously published method by Karbalaei *et al.* (2020) (Method 4) and a Calcium Chloride density separation with Sodium hexametaphosphate dispersant followed by KOH digestion (Method 5).

3.4.4 Recovery of individual polymers

All five methods used recovered significantly different amounts of spiked microplastic polymer types ($p < 0.05$ for all, Kruskal Wallis) (Figure 3.3). The NaCl density separation method (method 1) extracted significantly more low-density polymers such as HDPE (48% RR), LDPE (56.7% RR) and PS (42.7% RR) than high-density polymers such as PET (4.7% RR) and PVC (0.7% RR) ($p < 0.05$ for all, Dunn's test). This method also extracted significantly more LDPE than PP (28.7% RR) (< 0.05, Dunn's test).

The methods with added KOH digestion (method 2) and added dispersant (method 3) recovered significantly more low-density polymers [such as HDPE (KOH: 57.3% RR, Dispersant: 70.7% RR), LDPE (KOH: 60% RR, Dispersant: 75.3% RR), PP (KOH: 32% RR, Dispersant: 44.7% RR) and PS (KOH: 41.3%, Dispersant: 50.7% RR)] than high-density PET [(KOH: 4% RR, Dispersant: 6% RR) and PVC (KOH: 2.7% RR, Dispersant: 2% RR)] ($p < 0.05$, Dunn's test).

The method by Karbalaei *et al.* (2020) (method 4) recovered significantly more low-density polymers [such as HDPE (14.7% RR), LDPE (32.7% RR), PP (7.3% RR) and PS (10.7% RR)] than high-density PET (0.7% RR) ($p < 0.05$ Dunn's test). However, this method only found significantly more low-density HDPE and LDPE than high-density PVC (4% RR) (< 0.05, Dunn's test). This method also recovered significantly more LDPE polymers than any other polymer ($p < 0.05$, Dunn's test).

The method with an increased density saline solution of calcium chloride, a dispersant and a KOH digestion (method 5) also recovered significantly more low-density polymers of HDPE (62% RR) and LDPE (60.6% RR) than the higher density polymers of PET (11.3% RR) and PVC (20.6% RR) ($p < 0.05$, Dunn's test). However, polystyrene (15,3% RR), which has the lowest density, was recovered significantly less than the other low-density polymers of LDPE and HDPE ($p < 0.05$, Dunn's test). This method also recovered the highest amount of the high-density polymers such as PET and PVC compared to the other four methods, with recovery rates of 11.3% and 20.6% respectively (Figure 3.3).

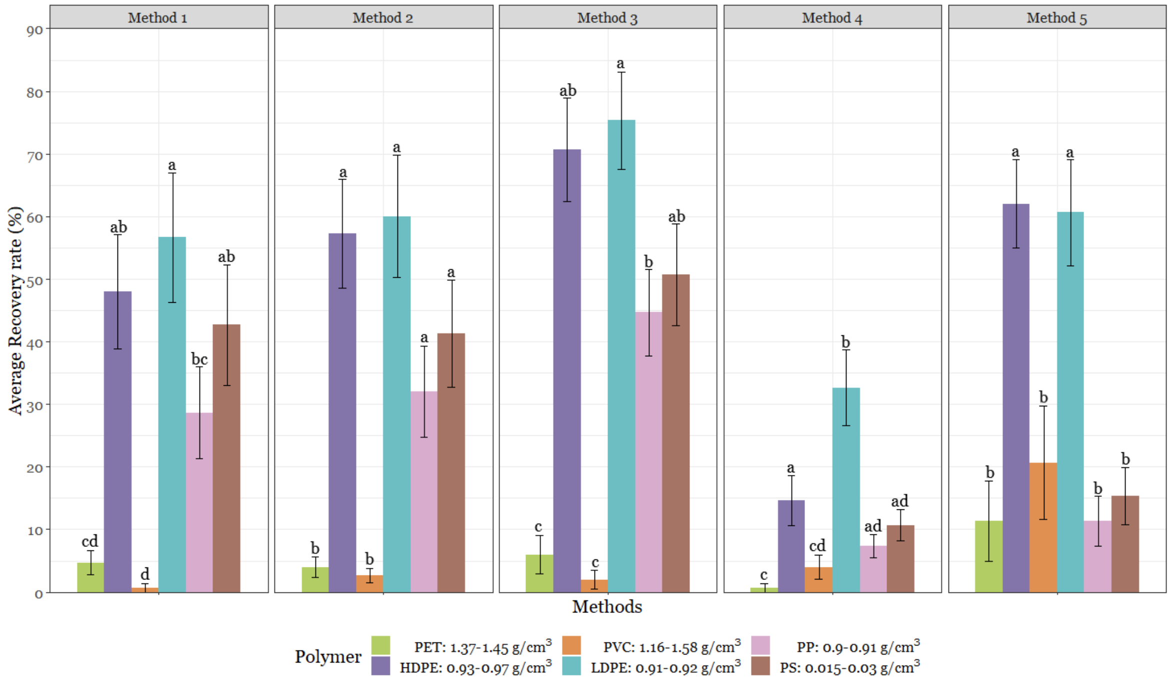


Figure 3.3 **Average recovery rates of six common microplastic polymers extracted from fishmeal using five separation/digestion methods.**

Average recovery rates (%) of 6 common microplastic polymers (first six plastic resin codes), extracted from fishmeal, using five separation/digestion methods used in existing literature (NaCl Density Separation (method 1), NaCl separation with a KOH digestion (method 2), NaCl separation with Sodium hexametaphosphate dispersant followed by KOH digestion (method 3), a previously published method by Karbalaei *et al.* (2020) (method 4) and a Calcium Chloride density separation with Sodium hexametaphosphate dispersant followed by KOH digestion (Method 5)). Error bars represent standard error of the mean. Bars with different letter notations within each method are significantly different (Dunn's test, p<0.05).

3.4.5 Individual polymer properties

All methods that include a NaCl (methods 1, 2 and 3) or a $CaCl_2$ density separation (method 5) recovered significantly more big (0.5-1 mm) microplastics (41.7%, 42%, 51.3%, 47% RR respectively) than the method by Karbalaei *et al.* (2020) (14.3% RR) (p<0.05, Dunn's test) (Figure 3.4 A). These four methods also recovered significantly more fragments (RR= method

1: 32%, method 2: 34.8%, method 3: 43%, method 5: 31.3%) than the method by Karbalaei *et al.* (2020) (method 4) (RR= 10.7%) ($p<0.05$, Dunn's test for both) (Figure 3.4 B).

However, Method 4 (Karbalaei *et al.*, 2020) recovered on average more small (0.25-0.5 mm) microplastics (16.7% RR) than big microplastics (14.3% RR) which is an opposite trends to all other methods which recovered more big microplastics than small.

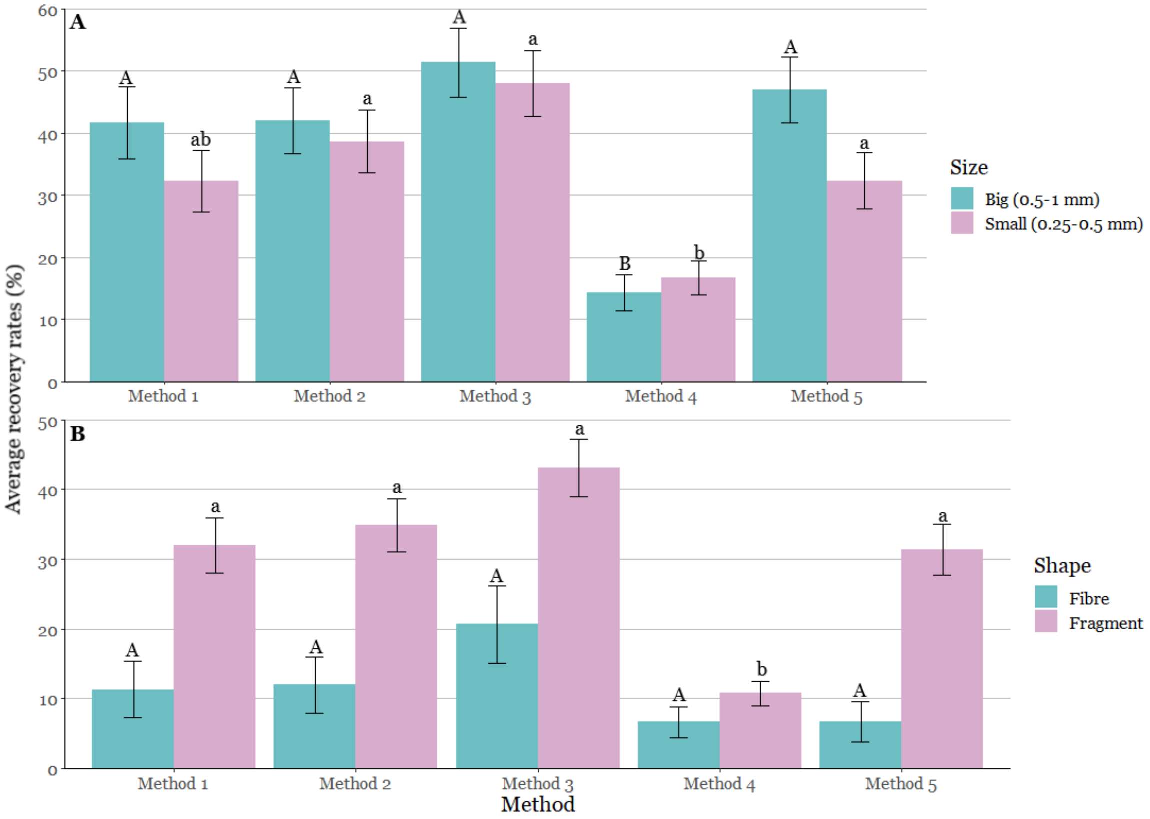


Figure 3.4 **Average recovery rate of big, small, fibres and fragments spiked microplastics from fishmeal using five methods.**

Average recovery rate (%) of big (0.5-1 mm) (A), small (0.25-0.5 mm) (A), fibres(B) and fragments(B) spiked microplastics extracted from fishmeal, using five different methods (NaCl Density Separation (method 1), NaCl separation with a KOH digestion (method 2), NaCl separation with Sodium hexametaphosphate dispersant followed by KOH digestion (method 3), a previously published method by Karbalaei *et al.* (2020) (method 4) and a Calcium Chloride density separation with Sodium hexametaphosphate dispersant followed by KOH digestion (method 5)). Bars with different letter notations are significantly different (Dunn's test, $p<0.05$), different case of letters represents different tests in each plot.

3.4.6 Recovery of polymers comparing shape, size and method used

Figure 3.5 highlights the recovery rates found when combining shape (Figure 3.4), size (Figure 3.4) and the individual polymer types (Figure 3.3) when extracting the microplastics from the fishmeal, using the five different methods. The highest recovery rate (78.7±7.4%) was found from big (0.5-1 mm) LDPE fragments using method 3. The lowest recovery rates (0%) were found from small (0.25 -0.5 mm) PVC fragments using method 1, and big PET fibres using method 4 (Figure 3.5).

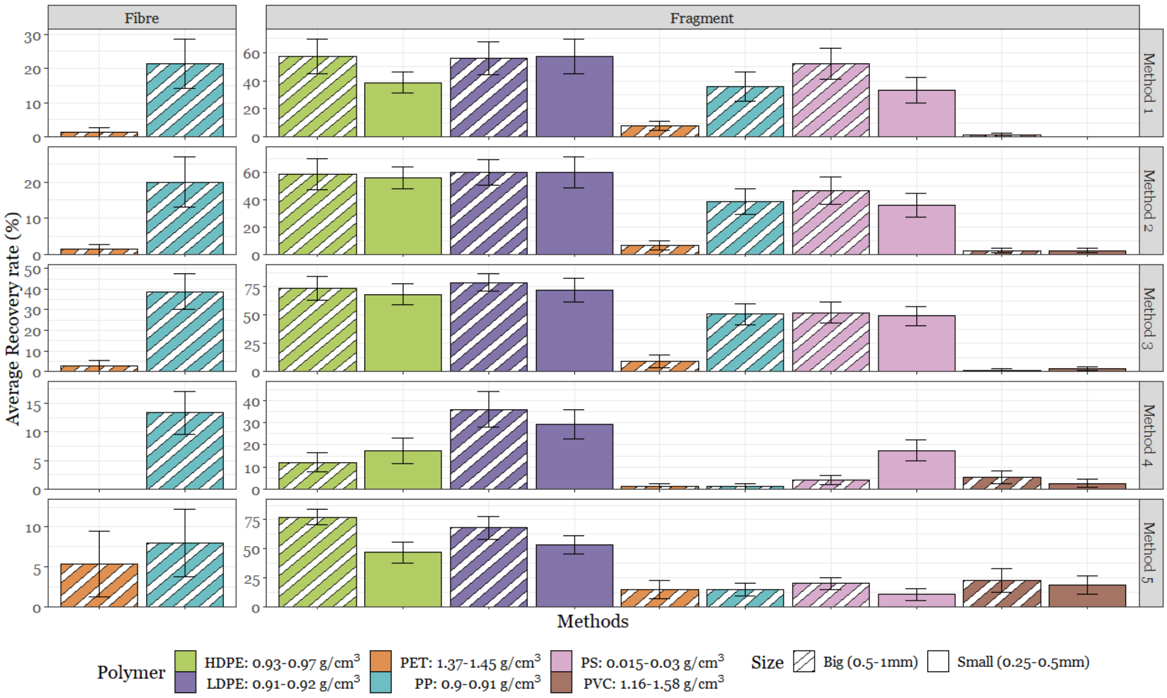


Figure 3.5 **Average recovery rates of six common microplastic polymers, split by size and shape, extracted from fishmeal using five separation/digestion methods.**

Average recovery rate (%) of big (0.5-1 mm), small (0.25-0.5 mm), fibres, fragments of 6 common microplastic polymers (first six plastic resin codes) extracted from fishmeal, using five different methods (NaCl Density Separation (method 1), NaCl separation with a KOH digestion (method 2), NaCl separation with Sodium hexametaphosphate dispersant followed by KOH digestion (method 3), a previously published method by Karbalaei *et al.* (2020) (method 4) and a Calcium Chloride density separation with Sodium hexametaphosphate dispersant followed by KOH digestion (method 5)). Error bars represent standard error of the mean.

3.5 Discussion

When investigating microplastics in a new medium, it is paramount to understand the properties of the medium and whether these will have an effect on extraction of plastic particles. Here, four properties of five commercially available types of fishmeal were measured and subjected to five different methods to establish recovery rate of spiked microplastics. The method of $CaCl_2$ density separation with dispersant and KOH digestion recovered the most microplastics in the sardine and anchovy fishmeal. However, the NaCl density separation with dispersant and a KOH digestion stage recovered the most microplastics from the four other fishmeal types. Moreover, the organic content of fishmeal was found to be negatively correlated with microplastic recovery rate. Overall, recovery rates varied across fishmeal types when using the same method (Figure 3.1), suggesting that the properties of the fishmeal could influence the amount of microplastics recovered. In addition, recovery rates were also low (0-66.3%), suggesting a potential for general underestimation of microplastics reported in fishmeal literature.

Sodium chloride density separation has been used as a method to separate microplastics from a matrix for a long time (Thompson *et al.*, 2004). More recently, it has been utilised to recover microplastics from fishmeal. Thiele *et al.* (2021) used a NaCl density separation 'Overflow' method (Table 3.3) to extract microplastics from two fishmeal types. They found a recovery rate of 49.3±1.2% in sardine and anchovy fishmeal, whereas this study found 33.3% recovery rate with the same fishmeal type (but obtained from a different source). This difference in recovery rate suggests there is a variability in the same fishmeal when manufactured in different places, or that the fish is sourced from different locations. This in turn may influence the effectiveness of the method. The study by Thiele *et al.* (2021) used different spiking polymers consisting of PS, PP, PET, PA and rayon, which have different densities than the polymers used in this study (PET, HDPE, LDPE, PVC, PS and PP), making it difficult to compare recovery rates. Even when comparing the recovery rates of the polymers used in both this study and the study by Thiele *et al.* (2021), there are differences. For example with PS, Thiele *et al.* (2021) found average recovery rates of 76.7%, whereas this study found 42.7%. With PET, Thiele *et al.* (2021) found an average recovery rate of 30%, whereas this study found 4.7%. With PP, Thiele *et al.* (2021) found an average recovery rate of 46.7%, whereas this study found 28.7%. This further highlights the potential of fishmeal variability effecting the ability to extract microplastics.

Sodium chloride is frequently used when studying microplastics. For example, Hanvey *et al.* (2017) compared studies looking into microplastics in sediments, and almost half (19/43) used NaCl as a saline solution. Similarly, the meta-analysis looking into recovery rate studies in Chapter 2, found that 16 out of the 71 studies included used NaCl, which was the most frequently used reagent in the analysis. Using NaCl as a density separation is also recommended by the Marine Strategy Framework Directive (MSFD) (Galgani *et al.*, 2011). There are several reasons as to why this method is widely used and accepted: ease of use, affordability, and its non-toxic properties (Table 3.1). Although the studies which use zinc chloride ($ZnCl_2$) (Imhof *et al.*, 2012) and NaI (Claessens *et al.*, 2013) have found high recovery rates (95.5-100% and >98% respectively), the use of the more expensive and hazardous saline solutions involve multiple steps (repeat extractions) to reduce sample mass, allowing for less of the solution to be used (Nuelle *et al.*, 2014). Moreover, many studies do not use these higher-density, expensive saline solutions at the highest density the salt can reach at 20˚C (Table 3.1), suggesting that it is much more economically viable to use the lower-density, lower expense saline solutions. For these reasons, this study used and developed methods with NaCl over other more expensive and toxic reagents such as $ZnCl_2$ and NaI, in order to encourage replication and standardisation from others.

This study combined NaCl with KOH to facilitate digestion and found recovery rates of between 5% and 48.3%, depending on the fishmeal type. Many studies have reported KOH an effective digestion reagent, which depending on the incubation temperature, it can have little effect on the polymer properties. For example, Karami *et al.* (2017a) found that using KOH at 40˚C had no effect on the microplastic fragments and was effective at digesting fish tissues. Thiele *et al.* (2021) trialled the use of KOH in recovering microplastics and found fishmeal that was digested in 10% KOH was not filterable through 25 µm filter papers. Although these studies found using KOH at 40°C did not degrade fragments, they did not test this method on fibres. However other studies (Treilles *et al.*, 2020; Radford *et al.*, 2021) have found this method does not have an effect on microplastic fibre stability. This study used KOH to digest residual fishmeal after density separation with 5% sodium hexametaphosphate as a dispersant, allowing for easier filtration. This method showed variable effectiveness in extracting the spiked microplastics with recovery rates between 15% and 60%. Other studies have used various surfactants/dispersants as an effective way of dispersing microplastics in a solution (Renzi *et al.*, 2019; Sussarellu *et al.*, 2016; Salaberria *et al.*, 2020).

When a method was trialled using a higher density salt solution ($CaCl_2$) with added dispersant and a KOH digestion (method 5), spiking plastics were recovered at a higher rate of between 10-66.3%. Similar recoveries of 69% and 55.5% have been found when using calcium chloride to recover microplastics from sediment (Stolte *et al.*, 2015; Crichton *et al.*, 2017). The calcium chloride solution has a higher density than sodium chloride, so is expected to recover plastics with a higher density. However, it was observed that using this solution often caused the lower density fishmeal to rise in the beaker, which caused issues with the overflow technique and following filtration (Figure 3.6). This could explain how the highest recovery (66.6%) was found in the sardine and anchovy fishmeal which also has the highest bulk density (0.83 g/cm^3) (Table 3.4) and thus less likely to float in the calcium chloride solution. Moreover, this method did recover more high-density polymers such as PET and PVC than other methods using NaCl. Using this method, significantly less PS was recovered than other polymers. Crichton *et al.* (2017), who also used calcium chloride as a density separation similarly found higher recovery rates of PVC (86.6%) than the category of polymers containing polystyrene (42.2%). They explained that the low recovery rates could be due to the calcium chloride settling overnight.

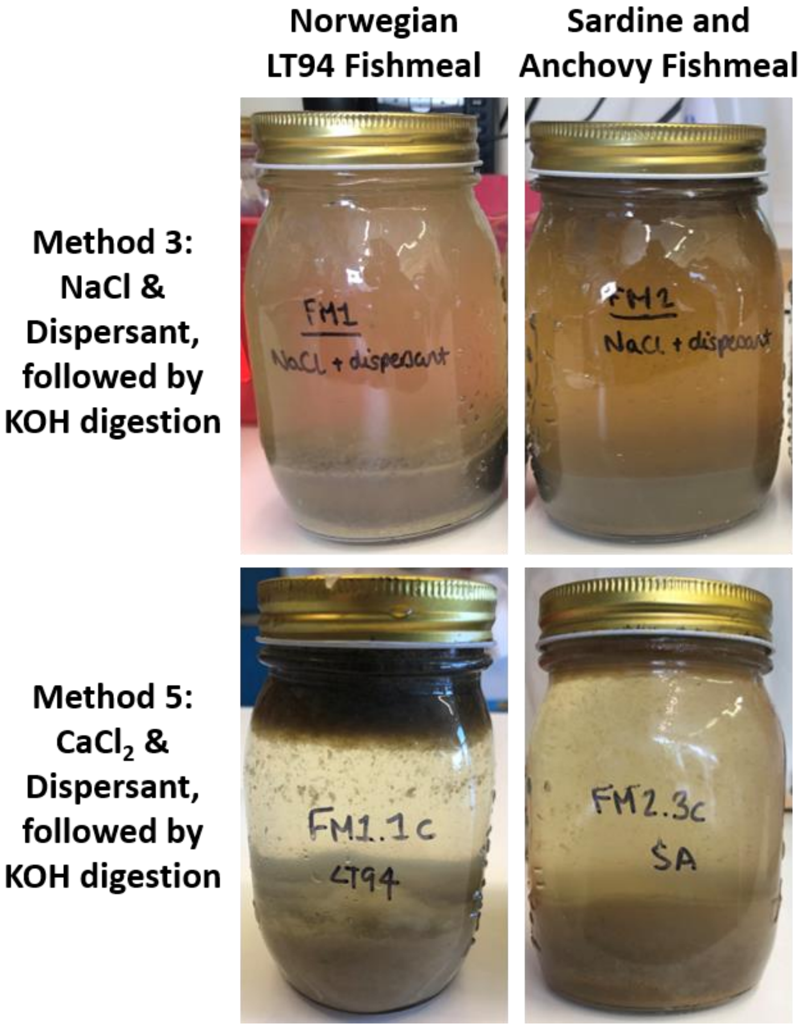


Figure 3.6 **Comparison of two similar methods using different saline solutions.**

> Comparison of two similar methods using different saline solutions (method 3: NaCl and method 5: $CaCl_2$) and the effect of these on two different fishmeal types (Norwegian LT94 and South American sardine and anchovy).

The chemistry/properties of calcium chloride may provide another explanation for the behaviour of the fishmeal in the beakers and the results found. Unlike sodium chloride, calcium chloride is hygroscopic- meaning it can absorb the moisture from air, and is deliquescent, so the salt will readily dissolve from the moisture absorbed from the air (Ropp, 2013; Peters Chemical Copmany, 2021). In solution calcium chloride may attract more water until equilibrium is reached between the ambient and solution vapor pressure. Having properties that readily absorbs water from the surroundings could provide an opportunity for water to be drawn out from the fishmeal, allowing the fishmeal to rise – thus causing the issues found with overflowing and filtering mentioned previously. Moreover, the calcium chloride solution at a density of 1.4 g/cm^3 has a viscous texture, making the solution difficult to filter. Although this method recorded the highest recovery rate, it would not be recommended, due to the issues of overflowing and filtering, making it difficult to locate the recovered spiking plastics. However, if the aim of a study is to recover high density microplastics, this method may prove useful if large pore-sized filters are used.

KOH is commonly used as a digesting agent in microplastic extraction methods. KOH digestion works by saponifying fatty tissues causing them to stop functioning, and due to its hygroscopic nature, it pulls water from cells in the surrounding medium (Drugbank Online, 2021). Many researchers have used digesting reagents (KOH) as a first step to extract microplastics from fish tissues (Foekema *et al.*, 2013; Karami *et al.*, 2017a; Thiele *et al.*, 2019). Which is the opposite to what is used in this study. However, this is not commonly used in the literature when extracting microplastics from fishmeal. Thiele *et al.* (2021) trialled using 10% KOH to digest fishmeal at 60°C for 48 hours, and found that it failed at digesting fishmeal enough to be filtered through 25μm filter paper. Therefore this order of methods was not considered for this microplastic extraction process.

Microplastics were more difficult to recover from the fishmeal with the highest organic content, shown with a significant negative correlation with the recovery rate of the spiked microplastics (r_s=-0.52, -0.38, -0.41, -0.89) (Antarctic Krill organic content = 87.5 %) using all methods. Similar trends are found with other media. For example, Radford *et al.* (2021) found lower recovery rates of microplastics from soils with higher organic matter. Hurley *et al.* (2018) mostly found higher extraction efficiencies in soils with lower organic content than in the higher organic content sludge samples. Some studies have succeeded in removing large amounts of organic matter, thus achieving high recovery rates, by using digestion steps (Vermeiren *et al.*, 2020). However, this often entails using hazardous/toxic reagents such as hydrogen peroxide or Fenton's reagent.

Bulk density (g/cm^3) often refers to the density of polymers and the saline solution. The bulk density of the fishmeal types was measured (Table 3.4). Significant correlations were found between the bulk density of fishmeal and recovery rate of spiked microplastics (r_s = 0.71, 0.73, 0.63, 0.49, 0.75). In this study, the fishmeal with the highest bulk density (sardine and anchovy: bulk density = 0.83 g/cm^3) sank in NaCl solution, making it easier for the microplastics to rise and overflow the glass jar. However, it is known that microplastics have the ability to lower the bulk density of a matrix, such as soil (de Souza Machado *et al.*, 2018). If this is the case, it may become more difficult to extract microplastics from a sample that is highly contaminated with the particles. The powder/particle size of the fishmeal could also be an impacting factor in the extraction process of microplastics. Smaller grain sized fishmeal will have smaller interstitial spaces, and a larger number of particles, thus a larger surface area to volume ratio. This would vary across different fishmeal types, therefore becoming a variable that could affect how microplastics are contained and extracted from the fishmeal. This is a variable that should be monitored in method development studies in the future.

Some studies have investigated the use of enzymes to digest material when extracting microplastics (Catarino *et al.*, 2017; Loder *et al.*, 2017; Thiele *et al.*, 2019), as they can be effective for reducing fats and proteins. Lipase enzymes are one of the main digestive enzymes found in animal organs and work by hydrolysing triacylglycerols into fatty acids (Schaechter, 2009). Considering this, it would be thought that lipases would work well to extract microplastics from highly organic fishmeal. However, studies that have used lipase enzymes have found they did not digest the organic samples further than the other enzymes (protease) used (López-Rosales *et al.*, 2021). Moreover, this study found a significant positive correlation between fishmeal with a high protein content (Norwegian LT94 fishmeal) and the recovery rate of spiked microplastics (r_s = 0.66, 0.76, 0.71, 0.59), showing that a reduction in protein content may not benefit the extraction of microplastics from fishmeal. Furthermore, the use of some enzymes, such as Proteinase-K can be expensive due to the high purification (Loder *et al.*, 2017).

Here, more low-density polymers (HDPE, LDPE, PS and PP) were extracted than the high-density polymers (PET and PVC). Similar findings have been found by Thiele *et al.* (2021), who extracted more spiked PS fragments than PET and rayon from sardine and anchovy fishmeal. This finding is comparable across other media. For example, Radford *et al.* (2021)

found PET had the lowest recovery rates in soil, whereas LDPE had the highest recovery rates. In some cases, the high-density polymers can be recovered with the higher-density solutions, such as zinc bromide ($ZnBr_2$) (Quinn *et al.*, 2017). However, this study did not utilise these solutions due to their hazardous nature and expense, but a slightly higher density, non-toxic reagent of $CaCl_2$ was tested and found high recovery rates of PET and PVC than the methods using NaCl. Attention must be noted when comparing recovery rates of polymers between studies as polymer densities and thus their floatability can be affected by the addition of plasticisers and additives (Wang *et al.*, 2014). If the aim of a study is to target high density polymers, for example in bottom feeder fish/invertebrates, then using high density saline solutions may be beneficial. To avoid the high cost of these saline solutions, some researchers have begun looking into recycling saline solutions (Kedzierski *et al.*, 2017). However, recycling the solutions by evaporation could be energy-intensive and very time-consuming, depending on the number of samples and amount of solution used.

This study showed that when using a NaCl or $CaCl_2$ density separation method, more ‘big’ (0.5-1 mm) microplastics were recovered than the ‘small’ (0.25-0.5 mm) microplastics, and more fragments than fibres. The opposite trend was found when utilising the method by Karbalaei *et al.* (2020). With few recovery studies published using fishmeal as a medium, it is difficult to compare trends. Other studies have shown that smaller microplastics are easier to find than large when using NaCl and water (Quinn *et al.*, 2017), whereas large microplastics are easier to recover when using higher density solutions such as $ZnCl_2$ (Imhof *et al.*, 2012). It should be noted here that the recovery of different sized microplastics could be influenced by human error, as larger microplastics are easier to see visually. This could be mitigated by introducing triangulation and having multiple researchers count the recovered microplastics, as suggested in the recommendations of Chapter 2 (Section 2.6).

The shape and size of microplastics recovered could depend on the number of steps used during the methodology. The method by Karbalaei *et al.* (2020) had several steps, with different equipment, ultimately giving higher chance of losing microplastics between stages. This could be a reason for finding less of the larger spiking plastics, which may have been lost through the multiple stages of the method. Alternative methods that minimise stages of preparation include the use of pyrolysis-GC-MS. Pyrolysis-GC-MS involves heating (pyrolysis) a small sample which produces pyrolysates which move into a gas chromatography (GC) column, are separated and then detected by a mass spectrometer (MS) (Pipkin *et al.*, 2021). Pyrolysis-GC-MS has the benefits of being able to detect the presence of additives and phthalates of microplastics, is less restricted by the size of the microplastic to be identified, has lower chance of contamination and is more reproducible given access to equipment

(Pipkin *et al.*, 2021). This technique is emerging as an option for identifying microplastics in environmental samples. For example, Ribeiro *et al.* (2020) used a KOH digestion followed by accelerated solvent extraction and then pyrolysis to identify microplastics in common seafood. If this technique could be adopted to identify microplastics in fishmeal, large numbers of samples could be processed, with higher accuracy and with less chance of contamination.

For future applications of these methods it would be worth evaluating the reproducibility between different operators and different laboratory settings to see whether similar results could be reproduced. When developing a method to extract microplastics from an environmental medium, there must be a fine balance between performance (recovery rate), cost and environmental impact. Although calcium chloride and sodium chloride are usually reported as having lower performance than other high density saline solutions, the significantly lower cost and environmental impact make them a preferred solution to use in most investigations of fishmeal samples. Seeing as microplastics are a pollutant themselves, this balance is something all microplastic researchers should consider when developing a method they hope to be universally accepted.

3.5.1 Future applications

The research from this study has scope to be used in other future applications. However, it is firstly imperative to improve the recovery of microplastics from fishmeal. No methods tested in this study reached 100% recovery, which means that all spiked microplastics were not recovered. Having a 100% recovery rate could be considered a 'perfect' method as all microplastics in the environmental medium tested were effectively extracted. However, as evidenced in Chapter 2, this is rarely achieved in the literature and often leads to over and/or underestimations. There is also the potential for procedural contamination and researcher bias (Section 2.6 in Chapter 2), making the goal of a 'perfect' method extremely difficult to achieve. There are small adjustments that could be made that could help when improving methods in the future. For example, only 3 replicates were used in this study. Although this is the minimum needed for sufficient statistical analysis to be undertaken, more replicates would allow for results of higher accuracy. However, if the goal is to find as many microplastics as realistically possible in the environment, then using a method which has been verified as being 100% effective is the best way to achieve this.

To make the results of this study more applicable to animal and human health, further study would need to test methods using smaller sized microplastics, such as those below 10 µm.

Many studies have now shown the presences of microplastics in humans (Schwabl *et al.*, 2019; Ragusa *et al.*, 2021; Leslie *et al.*, 2022; Huang *et al.*, 2022; Amato-Lourenço *et al.*, 2021; Jenner *et al.*, 2022; Ibrahim *et al.*, 2021), however these plastics range in sizes from 1.6 µm - 1.6 mm (Jung *et al.*, 2022). Although the health effects of microplastics are uncertain due to paucity of studies in this area, some information on *in vitro* studies is partially applicable and shows the potential dangers of microplastics. However, even within these studies some researchers have found that it is not the size of the microplastics that cause issues, but the concentration of microplastics in an area (Tan *et al.*, 2020). This is a key area of research that needs to be of high focus in the future.

Furthermore, future application of this research could investigate the comparison of multiple fishmeal variables and their effect on each other and the recovery of microplastics, using multivariate statistics. This would build on the results found comparing each fishmeal variable to recovery of microplastics used in this study. This would decrease the potential of creating incidental correlations.

3.6 Conclusions

Fishmeal is a globally important feed in aquaculture and agriculture. Consequently, microplastic presence in fishmeal is concerning and analytical methodologies are emerging. This study highlights the variability of fishmeal media, the complexity this brings when attempting to extract microplastics, and the importance of using environmentally conscious and affordable methods.

It is recommended to use methods with a dispersant, NaCl density separation and a KOH digestion; and to analyse the fishmeal properties: lower recoveries may be anticipated from fishmeal types with higher organic and lower protein content. This method is of low cost and is environmentally friendly, which is a balance that should become an international standard approach for researchers to allow for a method that is widely accepted (philosophically and scientifically) and easy to replicate.

As discussed in Chapter 2, methods with low recovery rates used in real application to the environment, present the risk of underestimating how many microplastics are truly in that medium. For example, if the method recommended above is used, based on the highest recovery rates found (60%), the amount of microplastic in fishmeal could be underestimated by 40%. If this figure is not accounted for in studies reporting microplastic contamination in fishmeal, it could pose significant risk to those animals that consume fishmeal. This is an issue that applies to other complex media (as seen in Figure 2.6 of Evidence of underestimation in microplastic research: a meta-analysis of recovery rate studies (Chapter 2), albeit at varying levels of underestimation, and must also be accounted for if the method is used for microplastic extraction in the future.

Chapter 4 Qualitatively identifying chemical indicator markers of virgin and weathered microplastics using solvent extraction and thermal desorption-GC-MS.

Concept for this chapter was developed by Chloe Way and supervisors (co-authors), methodological development (including laboratory work) was undertaken by Chloe Way with assistance from Professor John Langley (supervisor), analysis and writing was undertaken by Chloe Way.

4.1 Abstract

Microplastics are now found in many complex environmental matrices worldwide. Researchers are striving to develop the most effective methods to accurately extract and identify these small particles. Consequently, there are many different, often multi-faceted methods published in the literature relating to quantification of microplastics. Thermal desorption GC methods hold the potential to be a type of method that could allow for little to no sample preparation, thus low contamination, and a straightforward automated process. This study investigated the effectiveness of solvent extraction and thermal desorption in conjunction with GC-MS to identify chemical markers of ten types of microplastics. Virgin microplastic standards were initially used to seek out potential chemical markers. These standards were also artificially weathered, using a novel and low-cost process to allow for ease of replication. Identified markers in both treatments were compared with those frequently found in the literature using similar methods, and the suitability of the markers at identifying the plastic polymer were discussed. In some samples, the suitable chemical markers were found across all weathered and virgin microplastics. Whereas in other samples, the suitable chemical markers were more commonly found from weathered microplastics than from virgin microplastics. Chemical markers were more commonly found in the samples with no pe-preparation (direct thermal desorption). If future methods could accurately identify microplastics without solvent extractions, methods would become more simplified and would reduce user and environmental exposure to harmful reagents.

4.2 Introduction

The need for plastic products and the processes for producing these materials have been around for over 100 years. In the 1840s, “plastic” was produced partially from natural products, examples of the natural substances used include gutta-percha (liquid from Malaysian gutta trees), lac (produced by insects in India and Burma) and cemented asbestos mixed with adhesives. In 1869, the first commercially used "plastic" was produced; this material was celluloid and was made by combining camphor with cellulose nitrate. Although celluloid was durable, it was difficult to mould into shapes and was flammable. Therefore, scientists continued to develop more suitable materials. In 1909, the first fully synthetic plastic resin, made from phenol formaldehyde, was developed (Anderson, 1989). This plastic was Bakelite, which was far superior to the "plastics" produced by natural substances and is similar to the plastic in use today. The thermoplastics used presently (polyolefins, polystyrene, polyvinyl chloride *etc.*) are produced from products of fossil fuels and are considerably cheaper to produce, more durable and considerably more versatile than plastics produced previously (Crespy *et al.*, 2008). Consequently, the production of plastics has enormously expanded, with an average growth of 8.5% each year since the 1950s (Chalmin, 2019).

To date, approximately 8,300 million tonnes of virgin plastic have been produced globally (Geyer *et al.*, 2017). In 2020, 55 million tonnes of plastic was produced in Europe alone (Plastics Europe, 2020). Of this 55 million tonnes, only 10 million tonnes were recycled, 6.2 million tonnes were sent to landfill and 1.6 million tonnes were sent to other countries outside the EU (Plastics Europe, 2021). This shows there is a large proportion of plastic waste that is mismanaged. Geyer *et al.* (2017) estimated that by 2050 approximately 12,000 million tonnes of plastic could end up in the environment, which could explain how the environment is the endpoint for mismanaged plastic waste. Plastics are prone to mechanical degradation during use (*e.g.* tyre wear, physical weathering, clothes washing), and prone to degradation due to environmental forces such as ultraviolet radiation (sunlight) and biological/microbial activity (*e.g.* organism ingestion) (Horton and Dixon, 2018), which ultimately breaks down the plastics into microplastics (1 μm-5 mm) and possibly further into nanoplastics (1 nm-1 μm). Not only can microplastics cause harm when ingested, *e.g.*, in some studies microplastics in high concentrations have caused blockages and subsequent starvation (Alimba and Faggio, 2019). Also, the smaller the microplastic particle becomes, the larger its surface area to volume ratio. These factors mean that such a particle is more likely to absorb harmful persistent organic pollutants (POPs) to its surfaces from the surrounding environment (Guzzetti *et al.*, 2018), and potentially transport these to organisms. For these reasons, it is paramount that the amount of microplastics in the environment is consistently and accurately monitored.

Microplastics have been found in marine systems, freshwater systems, terrestrial systems, the atmosphere and in food and drinks (Hu *et al.*, 2022). However, even within each of these environments, the amounts of microplastics found by different researchers vary substantially. This variance could be the result of low standardisation in the methods used by each of these researchers to extract the microplastics from the environmental matrices. For example, Way *et al.* (2022) (Chapter 2) reviewed several studies looking at recovery of microplastics. They found that of 6 studies investigating soil, recoveries of microplastics varied from 71-93%, and of 14 studies investigating aquatic environments, recoveries varied from 67-82%. This lack of methodological standardisation is one of the largest concerns for researchers in this field of research, particularly when it comes to comparing results. The methods by which microplastics are identified often vary considerably (Zarfl, 2019). In earlier publications, microplastics were typically counted visually under stereomicroscopes, however identifying smaller microplastics (*e.g.* <100 µm) in this way had proven difficult (Luo *et al.*, 2022). As time progressed, the most commonly used way of identifying microplastics is with vibrational spectroscopy such as Fourier Transform Infrared (FTIR) and Raman laser. When using FTIR, a sample is exposed to infrared light and the given radiation is measured in either transmission or reflection mode, providing a spectral fingerprint to identify the polymer of the plastic (Käppler *et al.*, 2016). Raman spectroscopy differs by using a laser source to scatter photons of a sample which results in a spectrum similar to that provided by FTIR (Käppler *et al.*, 2016). Both analytical methods have several limitations. FTIR can only measure particles down to 10-20 µm and resultant spectra can be altered if there is water in the sample (Xu *et al.*, 2019). Unwanted fluorescence from the environmental matrix may also interfere with identification of microplastics when using Raman spectroscopy. The time needed to process samples and identify plastics using either of these methods vary. For example, Käppler *et al.* (2016) found in took 20 minutes to identify 32 plastics with FTIR, and 38 hours to identify 49 plastics with Raman. Although, automation processes are being developed which hope to shorten the time of identification by Raman (Leung *et al.*, 2021).

Researchers are now seeking methods that can efficiently and accurately process large numbers of samples and replicates, to ultimately determine the most realistic numbers of microplastics in the environment. Similarly, whilst studying an environmental pollutant, researchers want to undertake as little sample preparation as possible, to avoid sample loss, reduce contamination risk and to limit the use of harmful reagents. For example, Picó and Barceló (2021) explained the use of green analytical chemistry, which involves limiting the use of toxic reagents and reducing waste. This ultimately creates microplastic extraction methods that are safe for the user and the environment.

Spectrometry, in particular gas chromatography-mass spectrometry (GC-MS) is growing in popularity in the field of microplastic research as it tackles some of the issues faced when using spectroscopy methods. For example it reduces sample preparation which reduces contact points for interference, easier to identify smaller sized plastic particles and is highly reproducible (Pipkin *et al.*, 2021). It can also identify the polymer and any associated additives at the same time (Shim *et al.*, 2017). Essentially, chromatography is the separation of components by distribution between a stationary phase and a mobile phase (Poole and Poole, 2012). In gas chromatography (GC) a sample is vaporised in the inlet and flushed on to the column (Kitson *et al.*, 1996). A gas is used as the mobile phase to move the components through the column that contains the stationary phase. The components are separated based on their solubility in the stationary phase, their vapour pressure and their volatility (CHROMacademy, 2022). Through an electron ionisation process, these separated components are converted to gas phase ions, which are then further separated and elute from the column. The resultant mass spectrum is then recorded. (Kitson *et al.*, 1996). Samples can be introduced to the GC-MS system in several ways, one way that was explored was thermal desorption (TD). During TD samples are heated and any resulting volatile components are transferred directly to the GC column (Marsili, 1996). Microplastic samples can be introduced in this way. Different plastic polymers are manufactured with different materials and compounds (additives, plasticisers, fillers, dyes) and these will produce different volatiles and degradation products that will be indicative of the polymer itself or from unwanted contaminants introduced during manufacture (Dümichen *et al.*, 2015). From here on, these products will be referred to as chemical indicator markers (CIMs). Selection of CIMs need to be carefully considered as some plastics may leach compounds or share chemical indicator markers with those of natural material found in the environment (Primpke *et al.*, 2020).

Methods used in the field of microplastic research vary largely, including different types of sampling, extraction and identification methods (Hidalgo-Ruz *et al.*, 2012). When a method is validated by researchers, often the plastics used are virgin microplastics. These plastics may not be representative of those that are found in the environment because the latter have been exposed to some level of weathering. Xu *et al.* (2019) found that the chemical structure of some plastics could be changed by forms of weathering, by undergoing oxidation reactions.

This study investigates the suitability of thermo-analytical methods, such as thermal desorption-GC-MS, for identifying chemical indicator markers for virgin microplastics, as well as critically assessing this approach for artificially and naturally weathered microplastics. The application of these methods to environmental samples is then discussed.

Hypothesis: The effect of weathering will impact the ability of thermo-analytical methods to detect suitable CIMs of microplastics.

4.3 Methods

The microplastics included in these experiments were exposed to three types of sample preparation: solvent extraction, solvent extraction with sonication and no sample preparation. All samples were then analysed using thermal desorption GC-MS. These methods were tested for their ability to detect the chemical indicator markers of virgin microplastic standards, artificially weathered microplastics and a microplastic nurdle collected from the intertidal environment.

4.3.1 Virgin microplastic standards

Microplastic standards (polyvinylidene chloride (PVDC), high-density polyethylene (HDPE), polycarbonate (PC), polyamide (PA6), polyethylene terephthalate (PET), polystyrene (PS), low-density polyethylene (LDPE), polymethylmethacrylate (PMMA), polypropylene (PP) and polyurethane (PU)) were purchased online from Goodfellow (Table 4.1). These different polymers were selected as they are representative of some of the most common plastics to be used in the industry (de Haan *et al.*, 2019). They contain the first six plastic resins of the resin identification code that was created to indicate the recyclability of each type of plastic (American Standards for Testing Materials, 2020; So *et al.*, 2016). The sizes of the standards ranged from <180 µm to 5 mm.

Table 4.1 **Microplastic standards**

Microplastic standards used in the experimental trials: PVDC, HDPE, PC, PA6, PET, PS, LDPE, PMMA, PP and PU, including the mean particle size and form/shape according to the supplier (Goodfellow).

Polymer	Mean particle size	Form
PVDC	<180 µm	Powder
HDPE	3 mm	Granule
PC	3 mm	Granule
PA6	3 mm	Granule
PET	3-5 mm	Granule
PS	3.5 mm	Granule
LDPE	5 mm	Granule
PMMA	600 µm	Powder

PP	3 mm	Granule
PU	3-5 mm	Granule

4.3.2 Artificially weathered microplastics

One set of the microplastic standards (Table 4.1) was individually weighed and approximately 0.3 g of each placed in individual glass vials with 5 mL of water collected from Southampton docks on the River Itchen (14.6 psu, 7.6 pH). The seawater was not filtered or sterilised, adding a microbial aspect to weathering the microplastics. The glass vials were placed on a bottle roller (Stuart roller mixer SRT9, roller speed: 33 rpm) and rolled continuously for 1 month under a UV light bulb (Osram ultra vitalux UVA/UVB bulb), which emitted for 12 hours on, 12 hours off for the duration of the weathering experiment, and was screened from other light sources. See Figure 4.1 for set up of the artificial weathering experiment. After weathering, the plastics were examined with a JEOL JCM-6000 SEM, and pictures were taken to record signs of weathering (Supplementary Material Appendix. C.1). Clear visual signs of weathering were observed with the virgin microplastics after the one-month period. The SEM images in Appendix. C.1 show signs of cracks, bumps, small fractures and the light microscopy image of the PVDC shows signs of yellowing. All of these features are the signs of weathering that are discussed in the literature (Dong *et al.*, 2020; Alimi *et al.*, 2022; Turner and Holmes, 2011; Brandon *et al.*, 2016; Ogata *et al.*, 2009).

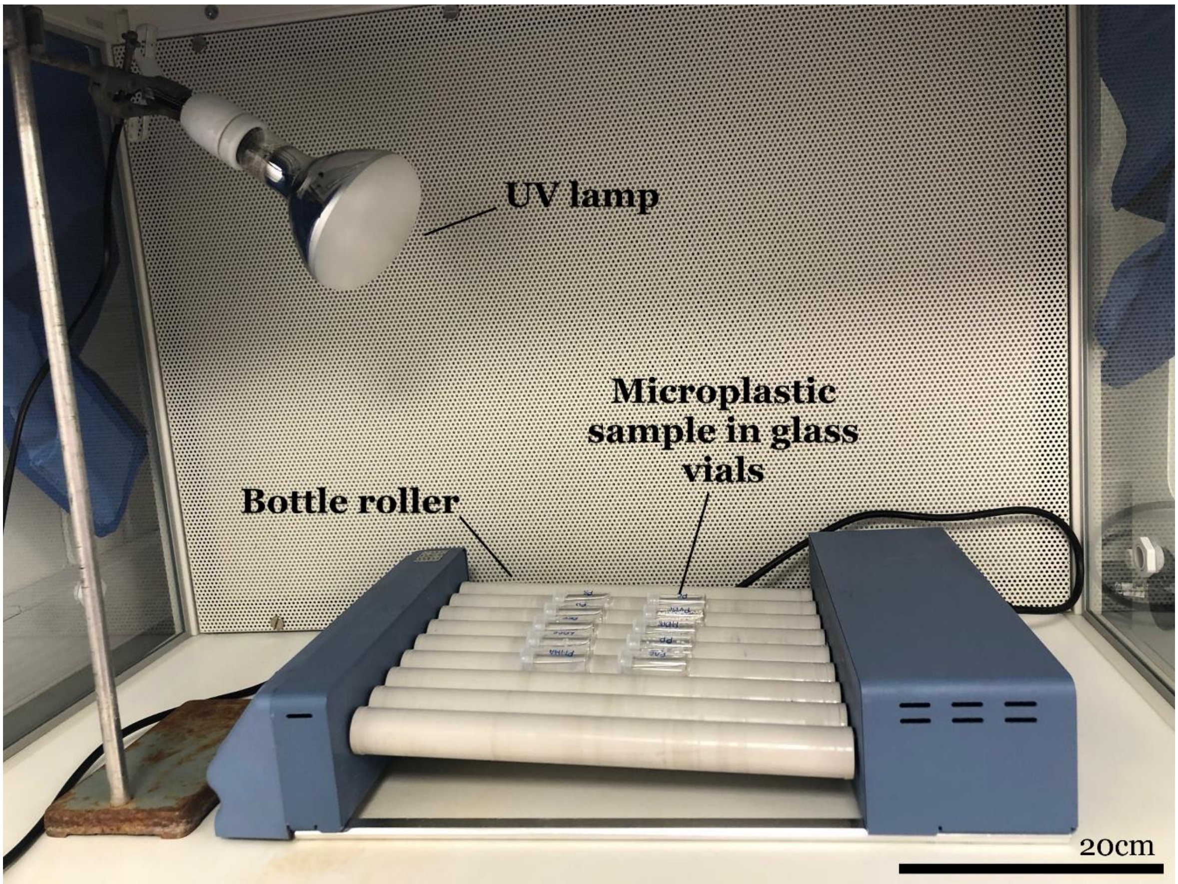


Figure 4.1 **Artificial weathering experiment set up**

Artificial weathering experiment set up with microplastics in glass vials on the bottle roller, below the UV lamp. Experiment was undertaken in a covered laminar flow cabinet.

4.3.3 Naturally weathered microplastics

Naturally weathered microplastic nurdles were collected in March 2022 from the upper intertidal zone of Chessel Bay Nature Reserve, Southampton, in the Itchen estuary (Red pinpoint in Figure 4.2). A specific green coloured nurdle was selected to be tested due to its high abundance in this environment and known approximate weathering period. To our knowledge this type of nurdle was first observed at Chessel Bay in June 2021, meaning this microplastic had been weathering in the environment for at least nine months before collection. These nurdles were confirmed as HDPE with high matching (>85%), using Attenuated Total Reflectance Fourier-Transform spectroscopy (ATR-FTIR) (Perkin Elmer Frontier at 600-4000cm^{-1} wavenumber with 10 accumulations).

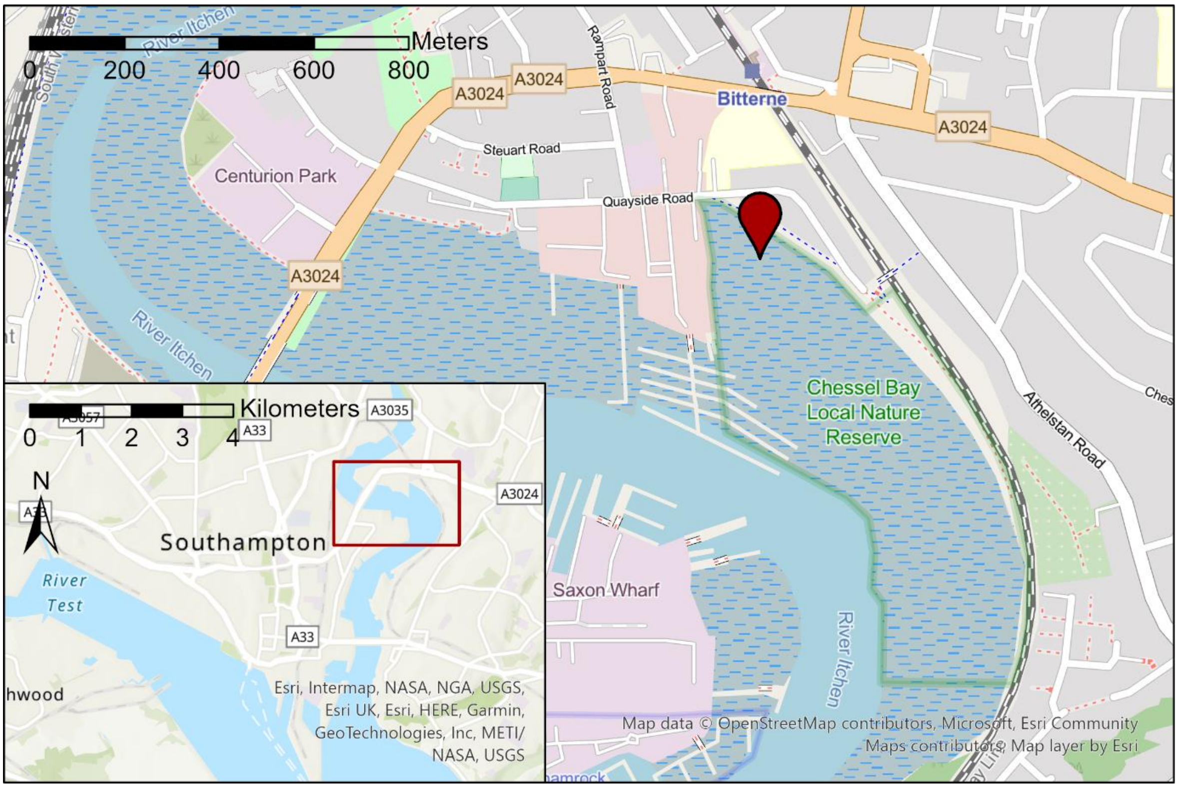


Figure 4.2 **Location of naturally weathered microplastic used in experiment**

Location of Chessel Bay, Southampton. Location of the collected naturally weathered nurdle.

4.3.4 Method justification

Dichloromethane was used as the solvent for liquid extraction as it has been used for similar experiments in the past (Hermabessiere and Rochman, 2021; La Nasa *et al.*, 2021; Okoffo *et al.*, 2020) with high recoveries (83-97%). It also poses less harm to the environment and users than other chlorinated solvents such as chlorobenzene or chloroform. For example Tobiszewski and Namieśnik (2015) gave DCM a total hazard score of 39.3, chloroform a score of 70.7 and chlorobenzene a score of 58. Although Tobiszewski and Namieśnik (2015) suggested the use of alcohols as a “greener” alternative solvent, alcohols such as methanol often do not work well at extracting microplastic fractions and are only commonly used to remove organic components of a sample (Fuller and Gautam, 2016). DCM is also considered a low cost solvent (Lynch *et al.*, 2023). An aim (Aim 3) (Section 1.11) of this thesis is to create methods that were of low cost and of little harm to the environment, using DCM in small quantities over other solvents achieves this.

The liquid extraction method was chosen over other methods due to its low cost and quick extraction time. Other methods such as the Soxhlet extraction can use large volumes of solvents and could take up to 17 hours to work (Santana-Viera *et al.*, 2021). Thermal desorption - GC-MS was the analytical method of choice as it was hoped to achieve the results using the lowest temperature possible while still achieving results that would allow for the identification of markers for the plastic polymers. Furthermore, other studies have used similar methods in the past (Dümichen *et al.*, 2015; Dümichen *et al.*, 2017; Braun *et al.*, 2020). These method choices further supports the aims of this thesis to remain low cost and allowing for easy replication. The parameters outlined in Table 4.2 were chosen based on similar experiments used in the literature (Dümichen *et al.*, 2015; Dümichen *et al.*, 2017; Jones *et al.*, 2021). With this being a preliminary study, parameters could be tweaked in the future to allow for better optimisation of results. Blank samples were run prior to the plastic samples to ensure the instrument was calibrated and working correctly.

4.3.5 First and second method of sample preparation: Solvent extraction and solvent extraction with sonication

In preparation, 10-30 mg of each type of plastic (standard and weathered) were placed in separate glass vials and 10 mL of dichloromethane (DCM) was added. Solvent solutions from a full set of the plastics were sampled immediately, and another full set were sonicated in an ultrasonic bath at 50 Hz for 30 minutes. When sampled, 1 mL of solution was transferred to the glass GC-MS vial in preparation for liquid extraction. When processed, 1 μL of the solution is transferred from the GC-MS vial into the injector.

4.3.6 Third method: Samples without preparation (Direct thermal desorption)

Glass sample tubes, GC liners and glass wool were cleaned prior to experiments by heating them at 310 °C for 4 mins. Once cleaned, single pieces of each microplastics (both virgin and weathered) were placed inside individual glass sample tubes. To avoid contamination, where possible all equipment used was glass or metal. Direct touching was avoided by always wearing nitrile gloves or using metal tweezers to transport microplastics. Sample tubes were sealed immediately after use.

4.3.7 TD GC-MS parameters

The samples were analysed using a LECO Pegasus BT 4D GCxGC-TOFMS coupled to an Agilent Technologies 7890B GC and a GL Sciences multimode GC inlet. Parameters were the same for the solvent extraction samples and different for the direct thermal desorption samples (Table 4.2). The GC column used (RTX-5MS) was 30 m length x 0.25 mm i.d. x 0.25 μm film thickness.

Helium was used as a carrier gas at a flow rate of 1 mL/min, with a split ratio of 2:1. The mass spectrometer was used in scan mode from 35-520 *m/z* (ion source temperature 250 °C). Solvent delay is shown in Table 4.2.

Table 4.2 **TD GC-MS parameters**

Parameters used for the TD GC-MS method.

Parameters	**Solvent Extractions**	**Direct Thermal Desorption**
Inlet Parameters		
Temperature	200 °C for entire run	50 °C for entire run
Inlet flow	1 mL/min	1 mL/min
GC Oven Parameters		
Start temperature	40 °C for 2 mins	40 °C for 50 s
Ramp Rate	20 °C/min	20 °C/min
Hold Temp/Time	N/A	250 °C for 2 mins
Ramp Rate	N/A	60 °C/min
Final Temp	310 °C	310 °C
Mass Spectrometer Parameters		
Solvent Delay	180 s	200 s
Mass Range	35-520 *m/z*	35-520 *m/z*
Ion Source Temperature	250 °C	250 °C
Ionisation	70 eV electron ionisation	70 V electron ionisation

4.3.8 Selecting a suitable chemical indicator marker (CIM)

Currently, there are no published standardised sets of chemical indicator markers to identify plastics, particularly when using thermal desorption-GC-MS. Therefore those that are published are somewhat subjective. The mass spectral library used to identify the chemical indicators markers was a NIST/EPA/NIH EI and NIST tandem mass spectral library (version 2.3, built May 4th 2017). The suggested potentially suitable CIM indicators provided here are suggested based on the presence found in the virgin microplastics, artificially weathered plastics and the naturally weathered plastics. A chemical marker was selected dependent on how well it matched with compounds found in the mass spectral library (similarity index (SI)). A similarity index above 90% shows greater confidence that the mass spectrum produced by these experiments matched the compound in question. The CIMs are then compared to those commonly used in the literature to see if there are similarities or differences. Where possible a potential CIM will only be suggested if the compound cannot be found naturally occurring in the environment. However, in cases where this is not possible, the best alternative that is used by others in the literature is offered.

4.4 Results and Discussion

4.4.1 Chemical indicator markers

Initially virgin microplastics were analysed using liquid extraction, liquid extraction with sonication and then analysed using thermal desorption. The TICC (Total ion current chromatogram) produced by each microplastics sample was investigated and the mass spectrum of each peak was examined, the resultant compound (CIM) was identified and listed in a table (Appendix. C.2). The same plastics were then artificially weathered and subjected to same methods (liquid extraction and thermal desorption). The compounds were identified and added to the table in Appendix. C.2. The same procedure was undertaken for the naturally weathered microplastic nurdles. Below, potentially suitable chemical markers for each plastic are discussed and any differences in CIMs dependant on degree of weathering and type of sample preparation undertaken is noted. Suitability of the CIMs is discussed with reference to their presence found in these experiments, their frequent use in the literature and their abundance in the environment, thus leading to a subjective potential CIM being suggested for each type of plastic. In some cases no suitable CIMs were found in these experiments and therefore the suggested potential CIM is based on those frequently found and used in the literature. The presence of the suggested chemical markers found in these experiments for each microplastic is shown in Figure 4.13 and Table 4.3.

4.4.1.1 Polystyrene

In these experiments, the most frequently observed chemical marker for polystyrene is styrene. This marker was consistently found in both virgin and weathered microplastic samples (Table 4.3) and with all the different types of sample preparation carried out. When polystyrene is broken down by heat, it undergoes chain end scission which ultimately produces styrene monomers (Dümichen *et al.*, 2017). Styrene has been used as a chemical marker by many researchers when identifying polystyrene using thermal desorption or pyrolysis (Fabbri *et al.*, 1998; Fabbri *et al.*, 2000; Dümichen *et al.*, 2017). However, it has been reported that styrene can act as a chemical marker for compounds found in the natural environment, such a chitin (Ivleva, 2021). Styrene has also been observed as a product of interference from material used during sample preparation, such as filters and their packaging (La Nasa *et al.*, 2020). Furthermore, styrene is also present in in acrylonitrile-butadiene-styrene (ABS) and styrene butadiene rubbers (SBS) (La Nasa *et al.*, 2020). Therefore, although styrene is the chemical marker in highest abundance, researchers have suggested using styrene dimers (2,4-diphenyl-1-butene) and trimers (2,4,6-triphenyl-1-

hexene) as the chemical markers as they are more specific in targeting polystyrene for identification (Dümichen *et al.*, 2017). Moreover, styrene dimers and trimers are not naturally found in the environment, so there would be high confidence that the presence of these CIMs in a sample would be due to the presence of polystyrene.

In these experiments, another chemical indicator was found that could also support in the identification of polystyrene. Ethylbenzene was found to be present in the liquid extraction and sonication of virgin polystyrene and in the direct thermal desorption of the weathered polystyrene. Ethylbenzene is manufactured from approximately 50% of all benzene produced and is used in the production of polystyrene (The Essential Chemical Industry, 2017). Moreover, methods can be employed to degrade polystyrene into ethylbenzene (Aljabri *et al.*, 2020). In the case of these experiments, styrene would be a reliable chemical marker to identify polystyrene than ethylbenzene as it is the monomer for polystyrene, is used widely in the literature and it was only found in the polystyrene microplastic samples, showing that it originates from this plastic and not from any interference. However, to be more confident in the identification, it could be recommended to use the presence of styrene dimers and trimers as markers in addition to the styrene monomer.

Potential chemical markers: styrene, styrene dimer, styrene trimer

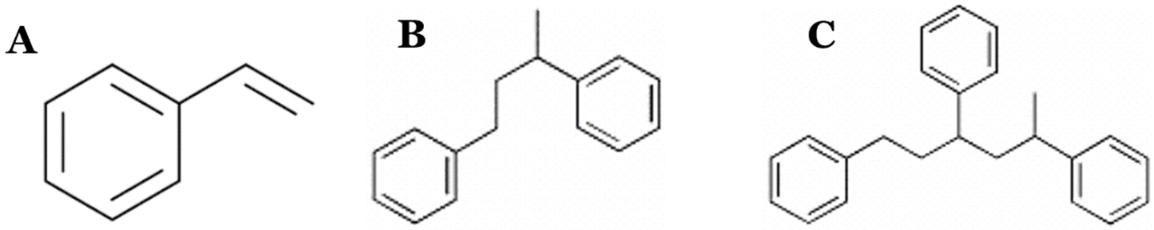


Figure 4.3 **Chemical structure of styrene (A), styrene dimer (B) and styrene trimer (C)**

4.4.1.2 Polypropylene

In these experiments there was no clear trend in the chemical markers observed for polypropylene. The thermal degradation of polypropylene is more complex than other plastic polymers (Dümichen *et al.*, 2017), due to its branched structure of unsaturated hydrocarbons (Soják *et al.*, 2007). Other researchers have claimed that the most difficult issue in finding appropriate identification markers lies with the stereoisomerism of multiple alkenes, alkanes and alkadienes found when this polymer decomposes (Soják *et al.*, 2007).

In published literature, the most commonly used chemical markers for polypropylene include series of alkenes such as 2,4-dimethylhept-1-ene, 2,4,6-trimethylnon-1-ene and 2,4,6,8-tetramethyldec-1-ene (Dümichen et al., 2019; Soják et al., 2007; Dümichen et al., 2017) (Table 4.3). However, others have reported using alkanes as indicators of polypropylene. Such as 4,6-dimethylnonane (Bockhorn *et al.*, 1999), 2,2,4,6,6-pentamethyl-heptane, 2,6-dimethyl undecane and 3,5-dimethyl octane (Mitchell *et al.*, 2014). An alkane which has been observed as a potential marker for polypropylene in the literature is 2,4,6-trimethyl decane (Mitchell *et al.*, 2014). This chemical marker was identified in our experiments during the direct thermal desorption of the virgin polypropylene microplastic. However, this is the only chemical marker found in these experiments that has also been confirmed in the literature, so would have to be used cautiously as a way of identifying polypropylene. Many types of alkenes and alkanes can be found occurring naturally in the environment, particularly originating from plants and algae (Rhew *et al.*, 2017; Zakaria *et al.*, 2018), so caution must be taken when deciding if an alkene or alkane CIM is due to the presence of a plastic or an environmental influence.

Bockhorn *et al.* (1999) found that the thermal decomposition products of polypropylene consist of approximately 84.8% alkenes, 7.6% dienes and 7.6% alkanes. Therefore, although 2,4,6-trimethyl decane could be used tentatively to identify polypropylene, using an alkene would be considerably more reliable due to the high percentage of these found when polypropylene decomposes. Bockhorn *et al.* (1999) found more alkenes at higher retention times, whereas the experiments in this study only ran for approx. 15 mins. This shows a longer run time could be more appropriate to find more suitable chemical markers for polypropylene and that methods may need to be developed separately for individual plastic polymers.

No alkenes were present in these experiments for PP, however with the widespread use of alkenes as CIM in the literature, they could be cautiously used as a CIM for PP. Confidence in the application of these CIMs would increase by limiting environmental contamination, thus proving the presence of alkenes is purely due to PP plastics.

Potential chemical markers:

2,4-dimethylhept-1-ene, 2,4,6-trimethylnon-1-ene and 2,4,6,8-tetramethyldec-1-ene

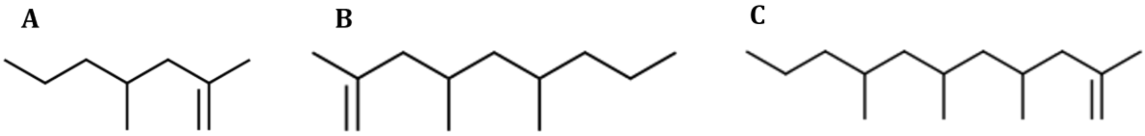


Figure 4.4 **Chemical structure of 2,4-dimethylhept-1-ene (A), 2,4,6-trimethylnon-1-ene (B) and 2,4,6,8-tetramethyldec-1-ene (C).**

4.4.1.3 Low-Density and High-Density Polyethylene

In these experiments, there were large differences in the chemical markers observed between LDPE and HDPE, between the different types of sample preparation, and between the virgin and weathered samples. Polyethylene is known to produce multiple different degradation products including alkanes, aldehydes, carboxylic acids, alkenes, alcohols, ketones and esters (Hakkarainen and Albertsson, 2004). In these experiments, more alkanes, aldehydes and carboxylic acids were found as markers for virgin LDPE microplastics and more alkenes were found as markers for the virgin HDPE microplastics. Similar studies in the literature have found similar observations. For example, Andersson and Wesslén (2003) showed how aldehydes would be appropriate chemical markers for LDPE. In these experiments, aldehydes such as nonanal and decanal were observed, which could be used as chemical markers for LDPE. However decanal can naturally occur in the environment in citrus fruit oils and plants (González-Mas et al., 2019; Zakaria et al., 2018) (Table 4.3). In the weathered LDPE samples, there were considerably more carboxylic acids, such as hexanoic acid, heptanoic acid and nonanoic acid. Carboxylic acids have been identified from thermally aged LDPE previously (Hakkarainen and Albertsson, 2004) and so could also be considered a suitable chemical markers, although with some caution, as nonanoic acid can also be found naturally in some fruit and vegetables (Pagliaro, 2019).

It the artificially weathered microplastic samples, it could be possible to have environmental interference of oil or other hydrocarbons that were present in the collected water samples. These hydrocarbons could thermally degrade into markers that may interfere with the identification of LDPE and HDPE. Zámostný *et al.* (2010) used micro-pyrolysis on many types of hydrocarbon and found hydrogen, methane, ethane and many other alkanes and alkene as the resulting products.

In the experiments with virgin and artificially weathered HDPE microplastics, alkenes were the most frequent chemical markers observed, with tetradecene present from both treatments. In the true weathered HDPE experiments, more alkanes were present. Using alkanes and alkenes as chemical indicators of polyethylene has been recognised by researchers (Primpke *et al.*, 2020). Although some alkenes and alkanes occur naturally in the environment in some waxes and fats (Ivleva, 2021), it is recognised that using alkanes and alkenes with higher carbon numbers would be preferential and more indicative of polyethylene and less likely from interfering compound from the environment (Ivleva, 2021). Therefore, the alkane chemical marker eicosane (C_{20}) which was found in the naturally weathered HDPE sample, and the alkene chemical marker eicos-1-ene (C_{20}) found in the virgin HDPE microplastic sample. Even though these compounds would be seen as the most potentially suitable chemical markers for HDPE, they are also present in the environment as a nanoparticle from diesel exhaust emissions (Kanno *et al.*, 2008), so conclusions should be met with caution.

Potential chemical markers:

LDPE: decanal or nonanoic acid

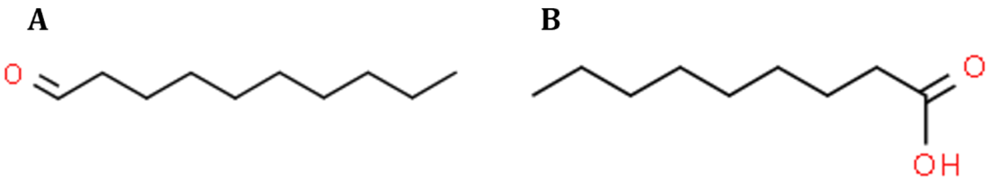


Figure 4.5 **Chemical structure of decanal (A) and nonanoic acid (B)**

HDPE: eicosane or eicos-1-ene

Figure 4.6 **Chemical structure of eicosane (A) and eicos-1-ene (B)**

4.4.1.4 Polyethylene terephthalate

The experiments with the PET microplastics produced chemical markers mainly consisting of aldehydes and carboxylic acids. The aldehydes were present in both the virgin and weathered samples, and the carboxylic acids were mainly present in the experiments using direct thermal desorption. The most common aldehyde chemical markers observed were nonanal and decanal. These compounds have been found to be the most abundant aldehyde and found in high amounts (1.25 µ/L and 5.07 µ/L respectively) in 6 month old water from PET bottles (Cincotta *et al.*, 2018). Researchers have found other aldehydes as products of thermally degraded PET, such as acetaldehyde, formaldehyde and benzaldehyde (Dzięcioł and

Trzeszczyński, 2000). Although nonanal and decanal are observed as indicative chemical markers for PET in these experiments and in the literature, they have also been observed in the experiments of other plastic polymers such as HDPE and PP (Appendix. C.2), so these chemical markers are not specific enough to reliably identify PET.

Another chemical marker that was observed throughout these experiments in both direct thermal desorption experiments of the virgin and weathered PET samples is propenoic acid. Propenoic acid is a carboxylic acid and is sometimes also referred to as acrylic acid (Banerjee and Bhattacharya, 2022). This compound can be found naturally occurring in the environment, and is known to rapidly biodegrade (Parod, 2014). However, biodegradation will be highly dependent on the amount of the compound in the environment and levels of microorganisms present. If there is still presence of this highly biodegradable compound found in a sample, it is likely due to the presence of non-biodegradable materials such as plastic. This marker was not found in the other plastic experiments and has been seen in the pyrolysis products of PET in the literature (Biale *et al.*, 2021), therefore would prove to be a potentially reliable indicator marker for PET.

Potential chemical marker: propenoic acid

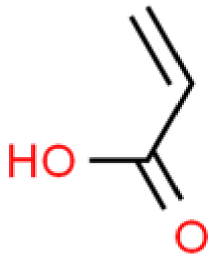


Figure 4.7 **Chemical structure of propenoic acid**

4.4.1.5 Polyurethane

The most frequently observed chemical marker for polyurethane in these experiments is butylated hydroxytoluene (BHT), which was present in both the virgin and weathered microplastic samples. BHT is not a product of the plastic itself but is actually an additive added to the plastic during it manufacture. However, it has a low molecular weight and is of low solubility, so is not often used with many of the common plastic polymers (Coleman, 2017). BHT is sometimes also used in the plastic industry as an inhibitor to prevent further polymerisation (Chu, 2006). Moreover, BHT is commonly found in the environment as it is frequently used as an antioxidant in food and cosmetics (Wang *et al.*, 2022). To our knowledge, there is no evidence in the literature of others using this compound as a chemical marker for the identification of polyurethane, so will therefore not be considered further.

Another compound that was identified in the experiments using polyurethane, specifically in the weathered microplastic samples, is butane-1,4-diol. This compound is used as a chain extending agent in the production of polyurethanes (Nakajima-Kambe et al., 1999), and can increase/strengthen some properties of polyurethane, such as the hardness of the plastic (Datta et al., 2017). Butane-1,4-diol is described in the literature for use when manufacturing polyurethane (Lee et al., 2001; Kim et al., 2003) and is only evident in the polyurethane samples of these experiments. Furthermore, butane-1,4-diol is not naturally produced (Philp and Atlas, 2017) (Table 4.3), so presence of this compound in the environment should only be indicative of pollutants such as plastic, therefore making it a potentially viable chemical identification marker for polyurethane.

Potential chemical marker: butane-1,4-diol

Figure 4.8 **Chemical structure of butane-1,4-diol**

4.4.1.6 Polyamide

In the experiments with polyamide microplastics, there were no consistent compounds that were observed through each experiment. However, in the experiments with the virgin PA microplastics, there was one observed compound of interest, this being caprolactam. When PA is thermally degraded, two processes occur. Firstly an intramolecular back biting process and then scission between the C-N bond and the amide group, due to hydrogen transfer. The major product produced by these processes is caprolactam (Ray and Cooney, 2018).

Caprolactam is the monomer of PA and provides the plastic with its properties of high stability, strength and durability. Approximately 50-70% of the produced caprolactam is used to produce nylon (PA) fibres and approximately 30% is used for nylon resins (Dahlhoff *et al.*, 2007). This shows how this compound is used primarily for the specific production of polyamides.

Many previous studies have highlighted caprolactam as the main degradation product of PA 6 (Herrera *et al.*, 2001; Lehrle *et al.*, 2000). In some cases the only peak of the resultant chromatogram from thermally degraded polyamide was caprolactam (Lehrle *et al.*, 2000). This shows the suitability of using caprolactam as a chemical identification marker for polyamide.

There was no caprolactam present in the weathered PA experiments. PA is a plastic that is extremely resistant to weathering, especially compared to other polymers such as polyester (Marjanovic, 2021). Sørensen *et al.* (2021) found that after 14 days of weathering PA with UV light in seawater, the levels of caprolactam in the water did not increase, meaning the release of caprolactam in the water was not due to degradation, but just from residues of the plastic from production. This could explain how the caprolactam from the weathered plastics in this experiment were removed with the residues in the weathering process, and further caprolactam would only be present when the plastic is fully degraded, possibly at higher temperatures than those used in these experiments.

With caprolactam being the monomer of polyamide, the main degradation product of polyamide and the fact that this compound does not occur naturally in the environment (Fortmann and Rosenberg, 1984), it would make a potentially suitable CIM for this plastic.

Potential chemical marker: caprolactam (azepan-2-one)

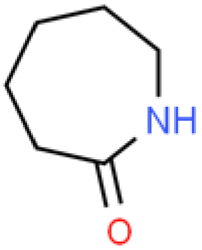


Figure 4.9 **Chemical structure of caprolactam**

4.4.1.7 Polycarbonate

Two compounds of interest were found in the experiments on polycarbonate (PC) microplastics, chlorobenzene and diphenyl carbonate. Although chlorobenzene was found in many of the samples, it would not be suitable as a chemical identification marker for PC. Chlorobenzene is mainly used as a solvent, and in the production of many different products such as rubber, adhesives, waxes and paints (Pravasi, 2014). Therefore, it would not be specific enough to identify PC. Diphenyl carbonate was found in the direct thermal desorption experiments of both the virgin and weathered PC microplastic samples.

Polycarbonate degradation involves oxidative hydrogen cleavage, scission of C-C bonds and hydrolysis of carbonate (Jang and Wilkie, 2004). One of the main compounds produced after degradation is diphenyl carbonate (Jang and Wilkie, 2004). Diphenyl carbonate is a reagent used when manufacturing PC plastics (Eckert, 2005). This plastic is produced by transesterification of bisphenol A with diphenyl carbonate (Sanders *et al.*, 2013). The use of this reagent has now been widely adopted in PC manufacturing as a replacement for the use of toxic phosgene (Fukuoka *et al.*, 2003). Many other researchers have observed the presence of diphenyl carbonate when polycarbonate thermally degrades (Davis and Golden, 1968; Chiu *et al.*, 2006; Huang *et al.*, 2018). Therefore this could be confidently adopted as a potential chemical marker to identify polycarbonate.

Potential chemical marker: diphenyl carbonate

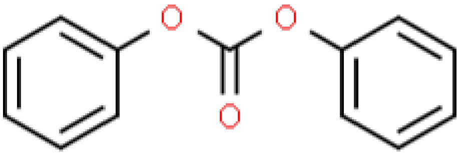


Figure 4.10 **Chemical structure of diphenyl carbonate**

4.4.1.8 Polyvinylidene chloride

Polyvinylidene chloride consists of repeating units of C-Cl bonds. Thermal degradation of this plastic involves two steps. In the first step, the C-Cl bonds break resulting in the release of hydrogen chloride (HCl). And in the second step the C-C chain is thermally broken (Nisar *et al.*, 2018). Many researchers have reported HCl as a product of thermally degraded PVDC (Fabbri *et al.*, 2000; Howell, 1987; Howell, 2015; Pasek *et al.*, 1996), showing how this could be a chemical marker for this plastic. However, HCl was only observed once in the experiments with the weathered PVDC microplastic experiments and was not present in the virgin PVDC microplastic experiments. Furthermore, HCl is not only a by-product from degraded plastic, but it can also be produced during the manufacture of many other products. For example, Hisham *et al.* (2014) explained how HCl can be produced by approximately 40 different processes, meaning this compound has widespread use. However, Hisham *et al.* (2014) also explain that 67% of the total production of HCl is used primarily for the production of vinyl chloride monomers. Therefore, although HCl is a potential chemical marker to identify PVDC, it may not be reliable, and other markers should be considered.

Similarly, another compound of interest found in these experiments with PVDC microplastics, is dichlorobenzene. This compound was also found in just one experiment of the weathered microplastic samples. Although this compound has been found as a thermal decomposition product of PVDC (Chen *et al.*, 2001), it is also not specific to PVDC and is used in many industries when producing pesticides, dyes and solvents (Centers for disease control and prevention, 2017), therefore would also not work as a reliable way to identify PVDC microplastics.

PVDC is not as commonly studied as polyvinyl chloride (PVC) in the field of microplastic research. Therefore there is a paucity of information on chemical markers used to identify this plastic polymer. Until further work is developed and published to confidently provide information on PVDC, the incidence of both hydrogen chloride and dichlorobenzene as chemical markers in a single experiment could provide some evidence for the presence of PVDC in a sample. However, this must be interpreted cautiously, and thorough sample preparation must be considered to ensure chemical markers are not from interferences.

Potential chemical markers: joint presence of hydrogen chloride and 1,4-dichlorobenzene

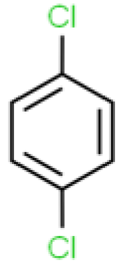


Figure 4.11 **Chemical structure of dichlorobenzene**

4.4.1.9 Polymethylmethacrylate

One of the most frequently observed chemical markers in these experiments with both virgin and weathered PMMA is methyl methacrylate. When PMMA is thermally degraded, it produces large amounts of the monomer methyl methacrylate (Popescu *et al.*, 2009; Ali *et al.*, 2015) by scission of the C-C bonds (Ali *et al.*, 2015). This compound has also been recognised as an appropriate chemical marker to identify PMMA by others in the literature (Primpke *et al.*, 2020; Fischer and Scholz-Böttcher, 2017). Methyl methacrylate is not found as a marker for any natural environmental matrices such as chitin, cellulose, wood, cotton or wool (Fischer and Scholz-Böttcher, 2017), so therefore this compound can be used confidently as a reliable chemical marker for PMMA plastic.

Potential chemical marker: methyl methacrylate (methyl 2-methylprop-2-enoate)

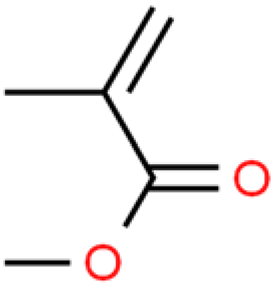
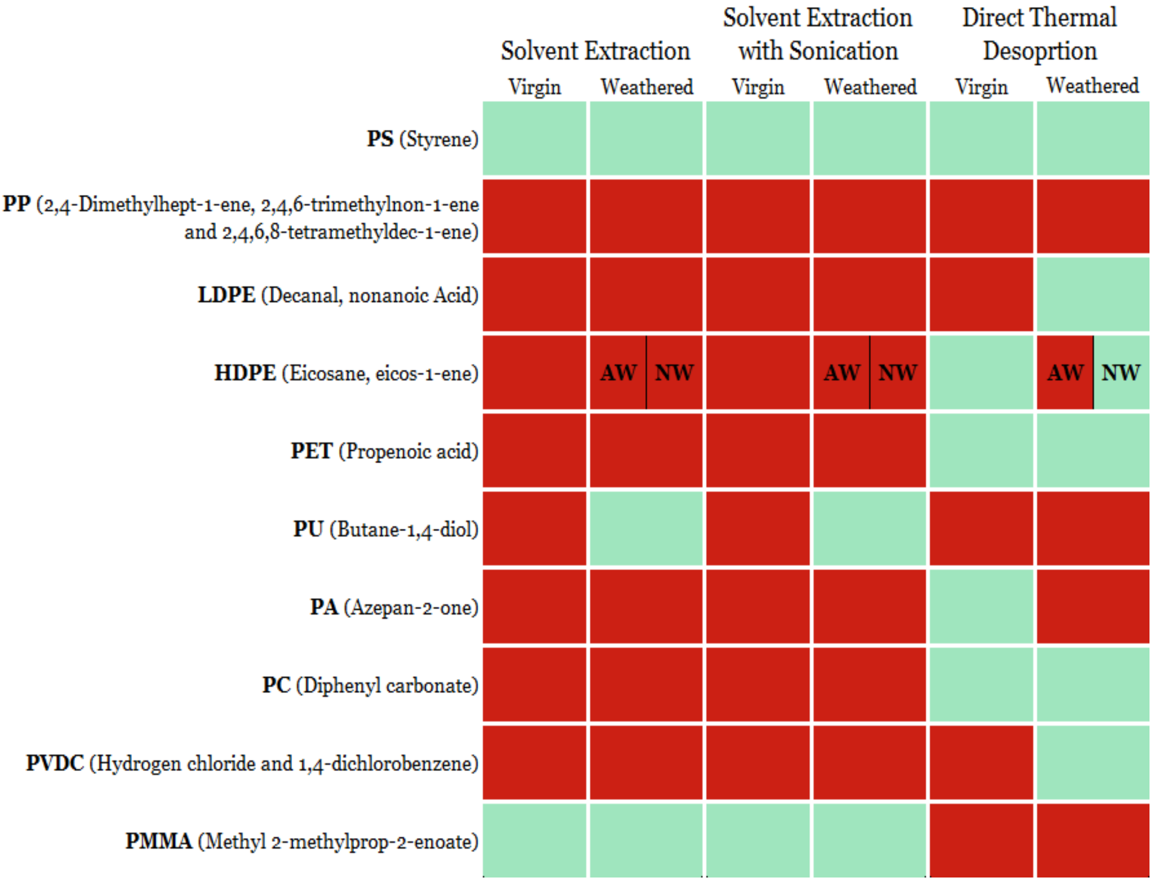


Figure 4.12 **Chemical structure of methyl methacrylate**



	Solvent Extraction		Solvent Extraction with Sonication		Direct Thermal Desoprtion	
	Virgin	Weathered	Virgin	Weathered	Virgin	Weathered
PS (Styrene)	🟩	🟩	🟩	🟩	🟩	🟩
PP (2,4-Dimethylhept-1-ene, 2,4,6-trimethylnon-1-ene and 2,4,6,8-tetramethyldec-1-ene)	🟥	🟥	🟥	🟥	🟥	🟥
LDPE (Decanal, nonanoic Acid)	🟥	🟥	🟥	🟥	🟥	🟩
HDPE (Eicosane, eicos-1-ene)	🟥	AW 🟥 NW 🟥	🟥	AW 🟥 NW 🟥	🟩	AW 🟥 NW 🟩
PET (Propenoic acid)	🟥	🟥	🟥	🟥	🟩	🟩
PU (Butane-1,4-diol)	🟥	🟩	🟥	🟩	🟥	🟥
PA (Azepan-2-one)	🟥	🟥	🟥	🟥	🟩	🟥
PC (Diphenyl carbonate)	🟥	🟥	🟥	🟥	🟩	🟩
PVDC (Hydrogen chloride and 1,4-dichlorobenzene)	🟥	🟥	🟥	🟥	🟥	🟩
PMMA (Methyl 2-methylprop-2-enoate)	🟩	🟩	🟩	🟩	🟥	🟥

Figure 4.13 **Presence/absence matrix of microplastic chemical indicator markers**

Presence of chemical markers in the corresponding experiments highlighted with a green box, absence of the chemical marker highlighted with a red box. AW denotes artificially weathered HDPE microplastic, NW denotes naturally weathered HDPE microplastic.

Table 4.3 **Summary matrix of CIMs**

Summary matrix showing potentially suitable chemical indicator markers (CIMs) for plastics discussed in these experiments; evidence of them being used as CIMs in the literature to identify plastics, or as a product of thermally degraded plastic; evidence of the CIM being used in the production of plastic; and whether the CIM occurs naturally in the environment. Green shading indicates presence of CIM in these experiments, red shading indicates absence in these experiments. V=Virgin microplastics, AW=artificially weathered microplastics, NW=naturally weathered microplastics, Not reported = No evidence reported in the literature for that column.

Plastic	Potential CIM found in these experiments			CIM used in literature to ID plastic or as a product of thermally degraded plastic?		Evidence of CIM in manufacture/production of plastic?		CIMs naturally occurring in the environment?	
PS	Styrene	🟩 V	🟩 AW	YES	Dümichen *et al.* (2017) Fabbri *et al.* (2000) Fabbri *et al.* (1998)	YES	Dümichen *et al.* (2017) Fabbri *et al.* (1998)	YES	Can be detected from chitin Ivleva (2021)
	Styrene dimers and trimers	🟥 V	🟥 AW	YES	Dümichen *et al.* (2017) Fabbri *et al.* (2000) Fabbri *et al.* (1998)	Not reported		NO	Not reported
PP	Alkenes	🟥 V	🟥 AW	YES	Dümichen *et al.* (2019) Dümichen *et al.* (2017) Soják *et al.* (2007)	Not reported		YES	Ripens crops and type of plant hormone Rhew *et al.* (2017)
	Alkanes	🟩 V	🟥 AW	YES	Bockhorn *et al.* (1999) Mitchell *et al.* (2014)	Not reported		YES	Can occur naturally in the environment (Plants, algae, cyanobacteria) Zakaria *et al.* (2018)
LDPE	Decanal	🟥 V	🟩 AW	YES	Andersson and Wesslén (2003)	Not reported		YES	Present in citrus oils and plants González-Mas *et al.* (2019) López *et al.* (1999)
	Nonanoic Acid	🟥 V	🟩 AW	YES	Hakkarainen and Albertsson (2004)	Not reported		YES	Occurs naturally in fruit and vegetables Pagliaro (2019)
HDPE	Alkenes and alkanes			YES	(Primpke *et al.*, 2020)	Not reported		YES	Occurs in waxes and fats

	🟩 V 🟥 AW 🟩 NW		Ivleva (2021)				Ivleva (2021)
PET	Nonanal and decanal 🟩 V 🟩 AW	YES	Dzięcioł and Trzeszczyński (2000)	Not reported		YES	Present in citrus oils and plants González-Mas *et al.* (2019) López *et al.* (1999)
	Propenoic acid 🟩 V 🟩 AW	YES	Biale *et al.* (2021)	YES	Parod (2014)	YES	Naturally occurring propenoic acid biodegrades rapidly Parod (2014)
PU	Butane-1,4-diol 🟥 V 🟩 AW	Not reported		YES	Nakajima-Kambe *et al.* (1999) Kim *et al.* (2003) Lee *et al.* (2001)	NO	Not naturally produced Philp and Atlas (2017)
PA	Caprolactam 🟩 V 🟥 AW	YES	Ray and Cooney (2018) Lehrle *et al.* (2000)	YES	Dahlhoff *et al.* (2007)	NO	Does not occur naturally Fortmann and Rosenberg (1984)
PC	Diphenyl carbonate 🟩 V 🟩 AW	YES	Jang and Wilkie (2004) Davis and Golden (1968) Huang *et al.* (2018)	YES	Eckert (2005) Sanders *et al.* (2013)	NO	Not reported
PVDC	Hydrogen chloride 🟥 V 🟩 AW	YES	Fabbri *et al.* (2000) Howell (1987)	Not reported		NO	But used for ~40 different processes Hisham *et al.* (2014)
	Dichlorobenzene 🟥 V 🟩 AW	YES	Chen *et al.* (2001)	Not reported		NO	But used in pesticides, dyes and solvents - Centers for disease control and prevention (2017)
PMMA	Methyl methacrylate 🟩 V 🟩 AW	YES	Ali *et al.* (2015) Popescu *et al.* (2009) Fischer and Scholz-Böttcher (2017)	Not reported		NO	But is used in many industries (industrial and medical) Casa-Resino *et al.* (2014)

4.4.2 Importance of sample preparation

In these experiments, more of the suitable chemical indicator markers were found in the direct thermal desorption samples than the samples using solvent extraction sample preparation (Figure 4.13). Using any form of sample preparation introduces room for errors, loss of information or introduction of contaminants, and particularly with solvent extractions there is the potential to analyse an un-representative portion of the microplastic sample (Akoueson *et al.*, 2021). How much of the microplastic material that is extracted by solvents is dependent on factors such as the type of solvent used, the type of plastic material and the degree of weathering (La Nasa *et al.*, 2020). Furthermore, many plastics have low solubility in solvents (Dümichen *et al.*, 2019). For example, Tsampanakis and Orbaek White (2021) found that DCM (which was the solvent used in these experiments) had a high affinity for solubility with PS and ABS, but a low affinity with PP and HDPE. In the direct thermal desorption samples, only the part of the plastic that is desorbed (volatile fraction) is delivered to the column (Randle *et al.*, 2013). This process is automated and there is very little sample preparation compared the preparation and manual handling involved in the sample preparation of solvent extraction. Therefore, this could explain how fewer chemical markers were observed in the experiments where solvent extractions were used, compared to the direct thermal desorption where the only sample preparation involved weighing the plastics into the sample tubes.

Additionally, if the step of sample preparation using solvents is not required, it could reduce exposure of these harmful reagents to users and the environment, reduce waste and lower monetary costs involved with the methods used. Picó and Barceló (2021) found that using organic solvents is one of the least "green" microplastic methods, and that DCM is toxic to the environment and difficult to dispose of (Dichloromethane Safety Data Sheet, 2022). Solvents have many toxic effects on organisms, including reduced growth and increased mortality in fish (Dave *et al.*, 1979). More specifically, DCM is potentially carcinogenic to animals and humans (Shestakova and Sillanpää, 2013). For a while, researchers have been investigating ways to avoid these toxic substances. Anastas and Warner (1998) created a set of 12 principles for green chemistry, two of which include the use of safer solvents and prevention of waste. Similarly researchers are investigating the suitability of "green" solvents as alternatives to the harmful organic solvents currently in use (Pacheco-Fernández and Pino, 2019; de la Guardia and Armenta, 2010). In the field of microplastic research, extraction methods are beginning to be tailored to less harmful reagents such as oils and KOH (Radford *et al.*, 2021; Thiele *et al.*, 2019). Not only will having less steps to sample preparation help users and the environment, but it will also reduce the complexity of the method, making it more accessible to others. This approach also fits within the United Nations draft of the 2022 plastic treaty which lists the following objective *"To promote research and development of sustainable, affordable, innovative and cost-efficient approaches"* (UNEP, 2022). With microplastic pollution a worldwide problem, it is important to ensure as many people as possible can follow the same method to ensure true representation of the microplastic pollution is achieved.

Although the use of thermal desorption on its own with no sample preparation would be ideal, and in some cases has been adopted (Jones *et al.*, 2021), this would not be as easily achieved in some scenarios. For example, environmental matrices with high organic content will need to be digested to allow the microplastics and subsequent chemical markers to be extracted and easily identifiable by the following analytical method. An example of such environmental matrix is the fishmeal described in Chapter 3. Matrix removal will first have to be performed, like the density separation and digestion (as described in section 3.3.5), before collecting the microplastics on a filter paper. However, even with this sample purification, there may still be impacts on how samples are loaded into the instrument and the background contamination received. One way researchers use thermal desorption/pyrolysis instruments with microplastic samples is by cutting small sections of quarts filters containing the microplastics and placing them into inlet liners prior to analysis (Jones *et al.*, 2021). However, even with high sample purification, the high sensitivity of these instruments means it is highly likely that compounds from the organic matrix will be detected and could overlap the CIMs of the microplastics.

4.4.3 Impact of weathering microplastics

There is a paucity of work carried out in the field of microplastic research that has focused on artificially weathering microplastic standards. In some cases solar/irradiation chambers are used to weather plastics (Simon *et al.*, 2021; Song *et al.*, 2022). These are expensive pieces of laboratory equipment which are more commonly used to test the degradability of a product, thus are not easily accessible for others to use. The artificial weathering set up developed for these set of experiments and displayed in Figure 4.1 offers a simple alternative, with equipment that is of low cost and easily accessible to others, which is an aspect of microplastic research which should be adhered to. Signs of weathering were observed in the SEM images (Appendix. C.1) of some plastics where the potentially suitable CIM were also present. For example, slight peeling was observed with the PC microplastic, cracks were observed on the PU microplastic, and yellowing was observed with the PVDC plastic (Appendix. C.1).

More suitable chemical indicator markers were found in the weathered microplastic samples rather than the virgin microplastic samples. For example with PU, butane-1,4-diol was present in weathered samples more than virgin samples, with PVDC, hydrogen chloride and 1,4-Dichlorobenzene was found more commonly in weathered microplastic samples, and with LDPE, decanal and nonanoic acid were present in the weathered samples and not in the virgin samples (Figure 4.13). A reason for this may be due to the effects of weathering reducing the temperature at which the plastic degrades, allowing the identification markers to be identified more readily, and at lower temperatures during thermal desorption. For example, it has been found that when PP is weathered and thus oxidised, the thermal stability of the plastic moved to a lower decomposition of between 100-300 °C (Czégény *et al.*, 2000; Toapanta *et al.*, 2021). Similarly, Luo *et al.* (2020) explained how the glass transition temperature of weathered LDPE microplastics reduced from 209 °C to 95 °C. Another reason as to why more CIMs were found in the weathered samples is shown by Fauser *et al.* (2022), who explained how unreacted monomers are often detected after plastic manufacture as the polymerisation reactions are rarely 100% complete. These unreacted monomers are more likely to leach from weathered plastics than virgin plastic (Fauser *et al.*, 2022).

However others have found that weathering can have the opposite effect and make it more difficult to identify the microplastic. Toapanta *et al.* (2021) looked at the impact of weathering on the ability to identify PP by pyrolysis GC-MS and found that weathering, high carbonyl index and the shape of the microplastic impacted the identification ability. They found that fragments and plastics with a carbonyl index of 18 could be underestimated by 42% and beads or microplastics with a carbonyl index of 30 could be underestimated by 49%.

High numbers of siloxanes were found in the majority of the weathered microplastic experiments (Appendix. C.2), but were not found in many of the virgin microplastic experiments. Examples include hexamethyl cyclotrisiloxane, octamethyl cyclotetrasiloxane and decamethyl cyclopentasiloxane. Siloxanes are not found naturally in the environment but have widespread industrial and consumer uses (Kaj *et al.*, 2005). The seawater used in the artificial weathering experiments was collected from the docks at the National Oceanography Centre in Southampton (Section 4.3.2). Across the river from these docks is a WWTP located on the river in Woolston. It has been found that WWTPs and its effluents are a source of siloxanes to the aquatic environment. For example a study in China found that 21% of the siloxanes found entering the WWTP processes were discharged into the surrounding river (Li *et al.*, 2016). This value may increase or decrease depending on rainfall and temperature. Therefore with Woolston WWTP in such close proximity to the water collected and used for these weathering experiments, it could explain the large numbers of siloxanes found in these experiments. An issue with this finding is that the presence of the siloxanes could be masking the presence of potentially suitable CIMs for microplastics.

4.4.4 Limitation of methodologies

One limitation of the methods used in these experiments is the possibility of missing a potential chemical marker due to overlapping peaks in the produced chromatograms. Many other researchers have found using thermo-analytical methods complicated when identifying microplastics. This is mainly due to the peaks of the plastic markers overlapping with the peaks associated to plastic additives, resulting in an overloaded and complicated chromatogram (Akoueson *et al.*, 2021; Bart, 2001). Qin *et al.* (2018) explained how when compounds are identified from thermally degraded carbon-based materials, such as plastics, many low molecular weight compounds are produced, which in turn produces numerous overlapping peaks. Additionally, if different types of plastic are to be identified together in a single sample, there is potential for the peaks of each of the plastics to overlap one another. For example, Majewsky *et al.* (2016) found that the melting temperatures for PVC, polyester, PET and PU were all between 250 and 291 °C. Therefore, the corresponding peaks for identification markers would overlap, making identification of the individual plastics challenging. Majewsky *et al.* (2016) also explained how to improve the clarity of the peaks in a chromatogram by slowing the heating rate of GC temperature programme. More recently, scientists are investigating the benefits of using two-dimensional GC-MS (GCxGC-MS) to gain clear distinction between overlapping compounds. This involves the use of two columns with different stationary phases, allowing for better peaks and a lower detection limit for compounds of a smaller size (Winnike *et al.*, 2015). Another way to improve the shapes of the peaks in a chromatogram is by cryotrapping the volatiles using liquid nitrogen prior to them being introduced to GC-MS. This will produce narrower chromatographic bands and improve the peaks (Picó and Barceló, 2020). This cryotrapping process will also ensure the system is cleaner and more stable for a longer period of time (Dümichen *et al.*, 2019).

The temperature regime used in these methods could be too low for the thermal decomposition for all of the polymers tested. For example Madorsky and Straus (1959) found that for PMMA to be 100% volatized, temperatures must reach 340°C. Therefore, higher temperatures, such as those used in pyrolysis may achieve more accurate results.

Many of the CIMs found to be ideal for identifying polymers in this study are the monomers of the selected polymer. However, in some cases, residual monomers of polymers may be released to the environment during manufacture, as seen in the case of caprolactam when producing PA (Section 4.4.1.6). These monomers would not be present again unless the polymer was fully thermally degraded at high temperatures, or may be found in the environment and not indicative of the polymer itself. This highlights a significant limitation for considering monomers as suitable CIMs and should be investigated with further study.

The methods used in these experiments provide no way to confirm confidence of the results found, other than using evidence from the published literature. One way to improve confidence would be to compare with commonly used spectroscopic methods such as FTIR and/or Raman. Researchers have used spectroscopy and spectrometry to complement each other in the past, with FTIR providing particle number information and thermo-analytical methods such as pyrolysis to provide mass related information (Käppler *et al.*, 2018). However, some researchers have explained that the type of analytical method used should be based on the research question in mind. For example, if the question asks for quantification of the microplastics in a sample, TD could be used. Whereas if a brief overview of the number of particles in a sample is required, then visual microscopy or spectroscopy could be used (Elert *et al.*, 2017). Additionally confidence and accuracy of the results would improve with inter-laboratory confirmation of the results found, similarly to the recommendation suggested in section 2.6.

In these experiments, the artificially weathered microplastics were only weathered for 1 month, and the plastics were only exposed to UV forces, mechanical rolling and submerged in brackish water (offering potential microbial growth), which could be considered simplistic compared to the weathering forces in the natural environment. Weathering in the natural environment may include other aspects of weathering such as chemical oxidation and changes in temperature (Liu *et al.*, 2021). Furthermore, by prolonging the weathering experiment, longer than one month, the results found may be amplified and more realistic. Some researchers have suggested conducting microplastic weathering experiments for longer than 3 years, and to study the changes in the bonds of the plastics (hydroxyl, carbonyl and carbon to oxygen) as a proxy for the degree of weathering achieved (Brandon *et al.*, 2016). Furthermore, there was no attempt to remove any biofilm growth on the surface of the weathered microplastics prior to examination with the SEM. This could have resulted in surface changes that could have been mistaken for weathering. Similarly, there is a possibility that microbial growth from the seawater could have impacted the intensity of the UV weathering in the artificial weathering experiment. Furthermore, depending on the size of the microplastic, those with larger surface area may be prone to higher degrees of weathering. For instance, Wu *et al.* (2022) found smaller microplastics (75 µm) were more easily colonised by biofilms and carried more biofilm on their surface than larger microplastics (4 mm). Although the process of artificially weathering microplastics is novel, the reagents used to digest environmental media (Section 1.4.2), such as KOH (Thiele *et al.*, 2019) or NaOH (Pfeiffer and Fischer, 2020) could be used to digest surface biofilm. However, this digestion must not damage the plastic as this would interfere with the effects of the weathering.

4.4.5 Future recommendations

During these experiments, the chemical identification markers found were not quantified. In the future, it would be recommended to quantify these markers, to provide an indication of the amount of these markers present and also if the weathering process impacted the amount that would be identifiable. Additionally, the MS libraries for thermo-analytical methods such as TD are still under development, and are not quite as extensive as the libraries used to identify FTIR spectra (Käppler *et al.*, 2018). Therefore, researchers spend much time searching online literature for confirmation that a compound is related to a specific plastic that is to be identified, which is what has been undertaken in this chapter. It is recommended that the information gathered in this chapter, and similar published online is to be complied and utilised to created updated MS libraries that could show matches of compounds and the related plastics, with a confidence level of the match. Furthermore, it would allow for there to be less subjectiveness between researchers as to what compound qualifies as a “suitable” CIM. Combined, this would create a much faster and streamlined analysis, ultimately allowing for more samples to be processed.

If the next steps of both quantification and detailed MS libraries are employed, these methods could be applied to identifying naturally weathered microplastics in environmental samples such as fishmeal. However a furthering step of assessing the effectiveness of the methods used would be recommended, which could be done by undertaking a recovery rate study to produce a percentage of effectiveness (similar to those discussed in Chapter 2). The recovery study can be carried out on weathered microplastics, rather than virgin microplastics, which would be more representative of the type of microplastics found in the environment. However, with a true environmental sample, there may in some cases need to be some level of sample preparation/separation (such as those tested in Chapter 3) prior to the analytical methods used in this study, to be clear of any debris which may interfere with the identification of suitable CIMs.

In future experiments of a similar nature, it would be crucial to include a plastic-free seawater control. With this control it would eliminate uncertainty of whether CIMs are present due to plastic or whether they are present in the background seawater sample. Furthermore, solvent blanks would allow for confirmation of interferences from the instrument. For example, siloxanes were present in many samples. Running blanks would rule out the solvent and/or the instrument as a source of contamination.

4.5 Conclusions

Microplastic extraction and identification methods are often criticised for not using realistic types of microplastics when in development. This study investigated the ability of thermo-analytical methods at identifying virgin and weathered microplastics and found that chemical indicator markers were more commonly present in the weathered microplastics than the virgin microplastics. Moreover potential chemical indicator markers were present in higher numbers with direct thermal desorption than with solvent extractions. Minimising a method to have as little sample preparation as possible by not using solvent extractions, will reduce the use of harmful reagents and limit waste, which are areas in which researchers are trying to achieve to produce “green” analytical methods. This work can be used as a building block to develop methods further and to ultimately quantify the chemical indicator markers for weathered microplastic in environmental samples, helping to provide accurate accounts for the number of microplastics in complex environments worldwide.

Chapter 5 General discussion

The issue of microplastic pollution is widespread and is showing no sign of decreasing. For example, microplastics are being found in new places on a regular basis, such as being found in glaciers of the Tibetan Plateau in 2021 (Zhang *et al.*, 2021) and discovered in Antarctic snow for the first time in 2022 (Aves *et al.*, 2022). Therefore, as increasing amounts of new environmental media are being found to contain these plastics, the number of new methods of microplastic extraction are likely to increase in parallel. Expanding our knowledge on the methods already published and understanding how effective they are (Chapter 2), is imperative as we seek to understand whether the amount of microplastics in the environment is accurately represented. Newly developed and replicated methods on complex media do not always produce high recovery rates (Chapter 3), showing that there is no "one-size-fits-all" method for all types of environmental media and that the properties of the media could largely influence how effective a method works at extracting microplastics.

With microplastic pollution being so widespread, it may now be affecting humans. There is evidence of microplastics in the food and drinks we consume, presence of microplastics in human lung tissue, blood and breast milk (Wright and Kelly, 2017; Leslie *et al.*, 2022; Ragusa *et al.*, 2022). Microplastic extraction methods need to be of low harm to users and the environment, be easily accessible and affordable, to not add to the threats on human health that microplastics may already be posing. Thermo-analytical methods such as thermal desorption work with little to no sample preparation, and with use of small amounts of reagents (Chapter 4) and could offer a "greener" alternative to toxic saline solutions.

This thesis highlights the variety and effectiveness of microplastic extraction methods used currently in this field of research and how challenging these are to be applied to a variety of complex environmental media. The key findings and limitations of this thesis are summarised and discussed below, and further work based on these findings are recommended.

5.1 Summary of thesis findings

The purpose of this thesis is to investigate the challenges involved with the methods used to extract and identify microplastics from complex environmental matrices. One of the largest challenges faced by researchers in the field of microplastic research is achieving standardisation within the methods used. The goal of standardisation is for there to be no variation in the methods used by all researchers, meaning the results produced will be easily comparable, and as accurate as possible. In the literature many papers call for the urgent need of standardisation (Stock *et al.*, 2019; Panti *et al.*, 2019; Mári *et al.*, 2021), but very few papers offer solutions for these urgent calls. The online repository for best ocean practices (Ocean Best Practices Repository, 2022) have gathered some publications from JPI Oceans that offer standardised protocols for extracting microplastics from sediments (Frias *et al.*, 2018), biota (Bessa *et al.*, 2019) and seawater (Gago *et al.*, 2019). Creations of similar repositories for other environmental matrices would be hugely beneficial for the goal of achieving standardisation. Standardisation is arguably one of the hardest aspects to achieve in this field due to so many varieties of methods already used and published. Chapter 2 shows this variety, with published methods already using saline solutions (10 different types), oxidising agents (2 different types), acids (3 different types), bases (2 different types), oils (3 different types), solvents (4 different types), enzymes (5 different types) and alcohols (2 different types) (Appendix. A.2). Often these different reagents are used in combination with one another (Schirinzi *et al.*, 2020; Roch and Brinker, 2017; So *et al.*, 2018; Davis and Murphy, 2015; Zobkov *et al.*, 2019) to improve efficiency of a method for a particular medium. With the pace of microplastic research progress, there is no doubt that new methods with different reagents have already been published since the completion and publication of Chapter 2. For example, Malafaia *et al.* (2022) used acetonitrile to digest filters to make the microplastics more available, but this was only carried out on PE microplastics and verified with only one species of fish. This highlights the fast-paced nature of microplastic method development, and the difficulties inherent in both capturing the 'state of play' and achieving standardisation.

Other researchers have argued that standardisation is unachievable as there will be no "one-size-fits-all" method, due to the complexity of the different environments. For example, Provencher *et al.* (2020) have explained that although there are benefits to standardisation, the sheer abundance of published methods in microplastic research, means the process of standardisation could actually hinder progression of microplastic research and is not required. However, although the overall goal of standardisation may be difficult to reach, some aspects of microplastic research would benefit from becoming standardised. An example of such is the standardisation of the size classes of plastics. Table 1.1 shows the variety of sizes in which microplastics are classed as in the literature, which ranges from 100 nm to 5000 μm. Another methodological aspect which would benefit from standardisation is the units of measurements used when reporting plastics. Even within the same environmental medium, microplastics have been reported in a variety of ways (items/m^3, n/100m^3, items/500g-dw, items/kg-dw, items/kg, particles/m^3) (Biltcliff-Ward *et al.*, 2022). One way to improve standardisation in the field of microplastic research is to use certified reference materials (CRMs). CRMs are defined by International Organization for

Standardization (2023) as "*material characterised by a metrologically valid procedure for one or more specific properties*". In recovery studies many different spiking microplastics are used, as shown in Chapter 2, and often personally made by the researchers, as shown in Chapter 3. With spiking material being different shapes, sizes and properties between studies, it makes it difficult for comparison. If all laboratories used the same CRM for their microplastic recovery trials, results would be easily comparable. This is common practice in other fields such as metals (Sahuquillo *et al.*, 1999), and is beginning to develop in the micro/nano plastic field (Seghers *et al.*, 2022), showing the possibility of largely improving reliability of results going forward.

How effective a method is at extracting microplastics will vary across environmental matrices. For example, the recovery rates in Chapter 2 were highest in plant material (100%), biofilms (96%), whole organisms (91-95%) and excrement (88-95%), and lowest in fishmeal (58-70%), water (67-82%) and wastewater effluent/sludge (76-89%) (Figure 2.6). These further highlight how one method would not work effectively for all types of environments. Additionally, there is variation found with intra-environment matrices. For example, the fishmeal used for the study in Chapter 3 had differing properties. For example, the sardine and anchovy fishmeal has the lowest organic content (74.7%) but the highest bulk density (0.83 g/cm^3), whereas the Antarctic Krill meal has the highest organic content (87.5%), but the lowest bulk density (0.47 g/cm^3) (Table 3.4). These properties had an impact on the recovery of the plastics. It was found that in four out of the five methods tested, there was a strong significant positive correlation between recovery rates of microplastics and the bulk density of the fishmeal, and a strong negative correlation between recovery rate and organic content (Figure 3.2). This shows that if a published method is to be replicated with hope of gaining similar high recovery rates, the properties of the environmental medium in question should be investigated beforehand. This will allow for comparison between the properties to see if the method will work as effectively.

It is not only the variety within and between environments that could affect how well a method works but also the type of microplastics and their properties. In Chapter 2, it was found that recoveries of microplastics increased as the plastics increased in size (Figure 3.4). Similar results were found by Avio *et al.* (2015), with higher yields of the larger size classes of microplastics than the smaller size classes extracted from gastrointestinal tracts. In Chapter 3, the polymers used are of different densities, for example PVC has a higher density (1.38 g/cm^3) than PS (0.028-0.045 g/cm^3) (Table 1.3), which in most cases meant PVC was more difficult to extract from the fishmeal (Figure 3.3). Lower recovery of higher density plastics is commonly found in the literature (Quinn *et al.*, 2017; Grbic *et al.*, 2019), and is one of the main reasons behind the use of higher density saline solutions such as $ZnCl_2$ or NaI (Rodrigues *et al.*, 2020). Furthermore, in Chapter 4, plastic indicator markers for some polymers were found to be present after a solvent extraction, whereas for other polymers no indicator markers were present (Figure 4.13). This could be due to some plastics having a higher affinity to solvents than others (Tsampanakis and Orbaek White, 2021). These results show that plastic properties such as size, density and solvent solubility could impact how well a method works, and how challenging it would be to find a single method to work effectively with such an array of differing properties. Filella (2015) explained how size, shape and density are the main drivers behind the fate of microplastics, and that these properties work alongside surface characteristics to govern how a particle interacts (coagulation and sedimentation) with the surrounding environment.

Another challenge researchers in this field face is understanding how effective a method is. Validating microplastic extraction methods using "recovery rate" studies is of extreme importance to provide others with clarity of how well a method works. This type of study is not as commonly used in this field as it may need to be. For example, the meta-analysis in Chapter 2 gathered data from 71 recovery rate studies. This is a notably low number of studies compared to the number of microplastic studies published on a yearly basis. For example, a search on Google Scholar with the term "microplastics" yields 11,000 results for 2022 (January-July). As this type of method validation essentially provides an indication of effectiveness (as a percentage), it allows for observation of either over- or underestimation of microplastics in the studied matrix, dependent on the method used. An aim of this thesis (Aim 1) was to determine the amount of underestimation of microplastics in environmental sample. Chapter 2 looked at the average recovery rates for all 71 included studies and found that on average microplastics could be underestimated in the environment by approximately 14%. In Chapter 3, the highest recovery rate of the microplastics from the fishmeal was 66% (Figure 3.1), showing that if these methods were used on fishmeal there could be an underestimation of 34%. Chapter 4 showed the presence of chemical indicator markers for microplastics. If these indicator markers were then quantified, a recovery rate could be produced. Validating a method is important for wanting to achieve standardisation or replication of a method. However, it is also important to ensure any underestimation revealed by a recovery rate must be taken into account if the method is used to assess microplastics in an environmental sample. This ensures accurate and realistic values are reported, and the proper action can be taken to develop solutions. Underestimation of the amount of microplastics in the environment may have further implications. For example, toxicological studies will not be using realistic doses in effect studies, so realistic results will not be given to policy makers for creating solutions to this environmental problem.

To improve underestimation, several aspects of methods could be improved. For instance, Pérez-Guevara *et al.* (2022) suggested that the reasons for low counts of microplastics in studies fall into six categories: Economic constraints, method of extraction, instrument availability, method of identification, method of characterisation and lack of standardised approach, and that the underestimation can occur at any or multiple stages of the method. Another aspect to improve recovery rates is to ensure the spiking polymers used in the recovery rate experiments are as realistic as those found in the environment. There are many examples of studies not using realistic types of microplastics. For instance, many studies use virgin microplastic pre-production pellets as spiking plastics (Peters *et al.*, 2022; Monteiro *et al.*, 2022), or use considerably more spiking plastics than what would be realistically found in the study environment, or report the microplastic concentrations with different units (mg/L, mg/m^2, particles/L, particles/m^2 etc) (Rozman and Kalčíková, 2022) (See page 114). Using these examples as spiking plastics would change the effectiveness of the method to how it would work on microplastics in environmental samples. In Chapter 2, the most frequently used spiking polymers used in the literature were PE, PS, PET, PP, PVC, PA, HDPE and LDPE (Figure 2.3). The spiking plastics used in Chapter 3 were made from consumer plastics such as drinking bottles, tablecloths, carrier bags, carpets and packaging (Table 3.2). Although this is an improvement from virgin microplastics, it is still not truly representative of those found in the environment. Environmental plastics, and particularly microplastics, have usually been exposed to some level of weathering, such as showing signs of yellowing, corrosion of the surface layer, surface cracks, fractures, pits, bumps and fragmentation (Jiang *et al.*, 2021; Rajakumar *et al.*, 2009; Dong *et al.*, 2020).

An aim of this thesis (Aim 3) was to determine the suitability of thermo-analytical methods for identifying artificially weathered microplastics, including developing a process to weather the plastics. In Chapter 4, a set of microplastics were weathered for one month (See Appendix. C.1), and showed signs of weathering such as cracks, yellowing and small fragments. It was found that in some cases the chemical indicator markers were observed more with these plastics than the non-weathered samples (Figure 4.13). However, this type of weathering is not common in microplastic research, so there is a paucity of information on how long plastics should be weathered for, or on the physical and chemical changes that can result. Moreover, it is difficult to accurately assess how long microplastics found in the environment have been exposed to weathering (Brandon *et al.*, 2016). Gewert *et al.* (2018) only weathered samples for 5 days, but explained how this equates to 510 days of sunlight exposure in Europe. However, Brandon *et al.* (2016) weathered samples for 3 years. This highlights the variability in the methods and techniques used to artificially weather microplastics. It would be recommended that weathered microplastics, both artificially weathered and naturally weathered, should be incorporated in spiking plastics used to validate an extraction method. Artificial weathering experiments are usually set up in a way to accelerate weathering, often with elevated temperatures (Brandon *et al.*, 2016). Therefore it would be beneficial to have the comparison of both weathering conditions (artificial and natural). However, by incorporating these types of weathered plastics, it will increase practical constraints of the methods such as time needed and costs of equipment.

It would be ideal for a spiking plastic protocol to be created for this research field to follow, including realistic amounts, types, shapes and sizes of microplastics, and a mixture of virgin, weathered and consumer plastics. Cui *et al.* (2022) recommended that spiking plastics need to consist of at least three different densities and three different shapes. To reinforce the idea of using a mixture of microplastics, Rochman *et al.* (2019) explained how microplastics are often seen as a singular inert materials, but in fact they are actually a complex mixture of a variety of chemicals consisting of different monomers and additives, and should therefore be treated as a mixture. Regardless of disagreements in methods used, and how we define microplastics, we are fairly certain that the amounts of microplastics in the environment are underestimated and it is a bigger problem than originally thought. Scientists are working on being more transparent and creating better, harmonious methods, so the results can not only be valued by others in the field, but also by the public and policy makers. Thus leading to the development of meaningful solutions.

Another large challenge microplastic researchers face when optimising methods of extraction is the issue of contamination. Contamination needs to be minimised to ensure the microplastics identified are from the environmental matrix studied and not from the methodological procedures or sample preparation. Ways to reduce contamination include clean rooms, field blanks, negative controls and positive controls (Ziajahromi and Leusch, 2022). It is possible that methods which include high levels or multiple steps of sample preparation could increase chances of contamination or loss of target microplastics. For example, in the case of Chapter 3, the method tested by Karbalaei *et al.* (2020) involved multiple steps (KOH digestion, heating, filtering, NaI separation, sonication x2, centrifugation and filtering once more) (Table 3.3) and produced the lowest recovery rate (median recovery of 16.7%) (Figure 3.1), showing how many of the spiked microplastics could have been lost during the sample preparation. Similarly, in Chapter 4, the experiments using solvent extractions had lower presence of the chemical indicator markers for the plastics than the thermal desorption, which has less sample preparation. Dimante-Deimantovica *et al.* (2022) tested this theory and did find that with an increase in processing steps of a method, there was a decrease in recovery of plastics.

An aim of this thesis (Aim 2) was to develop an environmentally friendly, user safe and affordable method for the extraction of microplastics from the environment. Microplastics are a pollutant themselves, so it would not be appropriate to add more pollutants to the environment during a sampling or analytical method or treatment/processing step. This also relates to some of the clauses set out in the United Nations plastic treaty, such as clause 3(o) which states "*To promote research into and development of sustainable, affordable, innovative and cost-efficient approaches*" (UNEP, 2022). In Chapter 2, it was found that in published studies, the reagents used most frequently were NaCl, NaI, $ZnCl_2$, H_2O_2 and KOH (Appendix. A.2). Although these reagents may be effective at extracting microplastics, some are toxic to both the environment, and users. $ZnCl_2$ is a toxic reagent with long lasting effects for aquatic animals, can cause skin irritation and is corrosive (Bellasi *et al.*, 2021). NaI is even more harmful to aquatic life than $ZnCl_2$ and can cause damage to human organs under prolonged exposure (Bellasi *et al.*, 2021). The aim of developing a more suitable method meeting these aims was achieved in Chapter 3. The method using NaCl, dispersant and KOH poses less harm to the user or the environment. This was developed further to use a higher density saline solution of $CaCl_2$ to attempt to extraction more microplastics. Both of these methods did not reach high recovery rates ($NaCl_2$: 41.6%, $CaCl_2$: 29.9%), but were substantially less damaging to the environment and the user than higher density saline solutions such as $ZnCl_2$ or NaI. In the experiments of Chapter 4 small amounts of DCM was used for the solvent extractions. DCM is a toxic solvent, which can have harmful effects on humans and on the environment. For humans, it is carcinogenic, can increase the chance of heart and lung disease, increase the chance of spontaneous abortions and fatalities have been associated with exposure (IARC, 1999). Within the environment, dissolved DCM is known to reduce growth and reproducibility of organisms (Shestakova and Sillanpää, 2013). However, in Chapter 4, it was found that more chemical indicators markers were actually found in the experiments with no sample preparation. Therefore, this indicates that the solvent extraction step using the toxic solvents is not needed.

Finding a fine balance between how effective a method is, and the environmental impact of the components of a method, is difficult, but it is a balance that needs to be considered each time a method is developed, used or replicated. A method may be effective (~100% recovery rate) but harmful to humans and the environment or be less effective and not damaging to humans or the environment. The latter could be considered as an appropriate method, as long as the lower recovery rate is taken into account when totalling the amount of microplastics found in an environmental sample.

To summarise, the findings of this thesis emphasise the challenges faced by microplastic researchers when optimising extraction methods. It highlights how the standardisation of methods is extremely difficult given the variety of environments which contain microplastics, and the sheer variety of microplastics that will need extracting. It highlights the delicate balance in public communication of the weaknesses of microplastic methods and the potential underestimations already reported. It stresses the need for methods to be affordable and of no harm to users and the environment, but to find a balance of achieving this whilst upkeeping the effectiveness. With this in mind, methods would have the potential to be accessible to as many people that need to adopt them, which is a prospect that is needed when faced with a pollution problem worldwide.

5.2 Challenges and limitations

In Chapter 2, only four search engines (Web of Science, Scopus, GreenFILE and PubMed) (Figure 2.1) were used to collect literature to use in the meta-analysis. By using more search engines, more literature may have been found. However this would have increased the time needed for the screening and eligibility processes, which was unachievable in the time frame of the project. To counteract this challenge, 'citation chasing' was performed which resulted in an additional 259 records collected. Moreover, there are many published meta-analyses which have used less than four search engines, meaning although this is a relatively low amount, it is substantial enough to gain a representative coverage of the literature (Bramer *et al.*, 2017). The search terms used to find the literature only included the terms 'recovery rate' and 'recovery efficiency' to describe the recovery type methods analysed. If a study used a different term, it would not have been included in the meta-analysis. This ties in with the ever-growing issue in this field of researchers using different definitions and terminology, creating difficulties for comparison.

Some of the records found from the literature search for the meta-analysis were inaccessible, including full papers and the associated raw data. This led to many papers having to be excluded from the analysis. The raw data was specifically needed to find accurate averages of the recovery rates found in each study. Making papers and the supplementary information/data open access should be standard good practice in the field of microplastic research to help with the comparison of results. Another reason to why some records were excluded, were due to recovery rates being reported by weight and not by count. Although, this approach may be more applicable to smaller microplastics, that are not easy to manually handle, it meant several studies were not included in the analysis. This could mean that the results of the meta-analysis may have been biased towards larger sized microplastics. However, it also shows the need for a general consensus to be made on how scientists should carry out and report the effectiveness of their methods.

In Chapter 3, there were some limitations with the methods used that came to light with progression of further research projects. For example, there were no inter- or intra-laboratory comparison of results. Having more than one person to test the method and compare results would have increased the accuracy and reliability of the results found. This is also one of the recommendations suggested in Section 2.6. Although consumer plastics were used as spiking microplastics for the experiments, and did cover a range of polymers and densities, these were not particularly realistic to plastics currently found in the environment. After completing experiments in Chapter 4, the benefits of adding weathered microplastics to a recovery experiment was observed and would be recommended in further studies. In these experiments, recovered microplastics were manually counted, which could have led to error. This is particularly the case with samples where the fishmeal was not fully digested, leaving detritus on the filter paper, thus potentially hindering the identification of microplastics. Only five types of fishmeal were used in these experiments (Table 3.4), with relatively little variance of the properties between the different types. For example, the organic content of the fishmeal only varied by a maximum of 13%. Having more types of fishmeal with a wider array of properties would have made the correlations between the fishmeal properties and the recovery rates slightly stronger. However, this improvement would increase costs and time needed for the experiments.

The project length of Chapter 4 would only allow for a weathering period of one month. A longer weathering period would have allowed for more prominent weathering effects on the plastics, which would potentially allow for clearer decisions on which CIM would be more suitable. Although the weathering set up was novel and affordable, it only accounted for UV weathering (UV light bulb), mechanical weathering (bottle rolling) and biological weathering (placed in seawater). Adding other weathering forces such as increased temperatures, microbes or periods of drying would make the artificial weathering set up more realistic. Only one type of naturally weathered microplastic was analysed (HDPE nurdle). If time and resources would have allowed, having more types of naturally weathered microplastics, would allow for comparisons of the associated CIMs with the artificially weathered microplastics of the same polymer.

In Chapter 4, some of the CIMs found in the experiments could also be found naturally occurring in the environment. This issue decreases the confidence that these CIMs are present due to the microplastics and not due to environmental interference. Mass spectrometry libraries, like the one used in these experiments, are not commonly used to identify microplastics, and are therefore not as extensive for this field as FTIR and Raman libraries. With time and more research, the identification of more suitable CIM for plastics may become a more streamlined process.

5.3 Recommendations for future work

There are several aspects of the research in this thesis that can be used as building blocks to complete further work. The average recovery rates found in Chapter 2 could be investigated further to look at their trends overs time, to see whether increasing recovery rates could mean methods are improving or achieving harmonisation.

More fishmeal types could be investigated to add to those studied in Chapter 3, covering fishmeal made from more diverse types of fish, that are sourced from a wide array of locations. The non-harmful, affordable and most effective method can be used to find actual amounts of microplastic contamination in the fishmeal collected. The amounts of microplastic in the fishmeal dependent on source location could be investigated to see if this correlates with some of the most polluted areas of the world. Future work should focus on the source of plastic contamination to fishmeal, such as looking into fishing net shedding, the production process, air contamination and packaging. This could also be tied to how this will impact the fishmeal industry and thus food security globally.

To further the work carried out in Chapter 4, the CIMs found could be quantified. This would allow for a similar recovery rate to those found in 0 to be produced, thus showing the effectiveness of the different sample preparations (solvent extraction/TD) on the different types of weathered/virgin microplastics. These methods could then be verified to detect microplastics in true environmental samples. The weathered microplastics could also be used to test Pyrolysis-GC-MS at detecting suitable CIMs. Pyrolysis would run at a higher temperature than the experiments in Chapter 4, so may result in the higher presence of monomers of the plastic polymers.

Chapter 6 Key conclusions and significant contribution

The main goal of research into microplastic extraction methods is to effectively obtain microplastics from the environment, no matter the complexity. However, the research in this thesis has found that microplastics in the environment could actually be underestimated by approximately 14% due to the methods used. Methods used and the process of method development need to be improved to accurately portray how effective a method is. As well as being effective, a method needs to be affordable, and of low harm to the user and the environment, to allow for ease of replication. An affordable method using non-harmful reagents (KOH, NaCl and dispersant) was developed and successfully extracted microplastics from a complex environment. Furthermore, environmentally realistic types of microplastics should be used during method development. Thermo-analytical methods were found to be suitable at detecting potential CIMs of virgin and weathered microplastics.

A balance must be found between achieving a highly effective method, affordability and limited used of harmful reagents. Moreover, microplastics need not to be considered a single contaminant but more as a suite of complex particles and chemicals that all behave and interact differently. This way, an ideal method can be proposed that can be adopted and replicated by others. Only with high replication can standardisation be achieved. However, if standardisation is setting out for complete deletion of variation between methods, it is perhaps harmonisation that needs to be the focus in this field, specifically within each environmental medium studied. Having a method that is completely accessible to all, is an important prospect particularly when researching an environmental pollutant that is so widespread.

Microplastic pollution in general is assessed by a UN sustainable development goal (Goal 14 – life below the water), showing how this problem has influence and impact globally. In addition, the concepts introduced by this thesis of affordable, safe, effective and realistic methods, are at the forefront of global initiatives, and contribute to some of the clauses laid out in the UN's recent (2022) plastic treaty. If these approaches are adopted and the underestimations brought about by methods are accounted for, more realistic estimates of microplastic pollution can be delivered, whilst protecting the environment and allowing for the same method to be used worldwide. We can then begin to get a clear picture of the true fate of microplastics and their effects on the world and its inhabitants, and work towards practical solutions to resolve this global pollution problem.

Appendix A Chapter 2 Supplementary material

Appendix. A.1 Compilation of recovery rate studies used in the meta-analysis

Appendix. A.1. Studies divided by those found by database searching and by 'citation chasing'. Including first authors, year of publication, journal, title of study, a brief description of method used to extract the plastics, the media studied, the solution used if applicable, the type of spiking polymer used, the shape of the spiking polymers, the size of the spiking polymers and the range of the recovery rate found (multiple individual recovery rates not included in table for ease of reporting but were included in analysis).

Authors and Year	**Journal**	**Title**	**Brief method details**	**Media studied**	**Solutions used**	**Polymer types**[3]	**Polymer shapes**	**Polymer sizes**[4]	**Range of Recovery Rates**
Studies found by database searching									
Bannick *et al.* (2019)	Water Research	Development and testing of a fractionated filtration for sampling of microplastics in water	Cascade filtering system	Water	Water	PE, PS	N/A	MP	81-110%
Birkenhead *et al.* (2020)	Scientific Reports	Validation of a method to quantify microfibres present in aquatic surface microlayers	Glass plate dipping method	Water	Water	PMMA, PET, Cotton, PP, Wool, Rayon	Fibres	LMP	17.33-37%

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[3] Polystyrene (PS), Polyethylene (PE), Polymethyl methacrylate (PMMA), Polyethylene terephthalate (PET), Polypropylene (PP), Polyamide/Nylon (PA), Polyvinyl Chloride (PVC), Low-Density Polyethylene (LDPE), Polybutylene adipate terephthalate (PBAT), Acrylonitrile butadiene styrene (ABS), High-Density Polyethylene (HDPE), Polycarbonate (PC), Polyurethane (PU), Styrene-butadiene rubber (SBR), Polybutylene succinate (PBS), Polylactide (PLA), Cellulose Acetate (CA), Polyvinylidene Chloride (PVDC), Polytetrafluoroethylene (PTFE), Polyoxymethylene (POM).

[4] Plastics between 1µm - 1mm (MP), Plastics between 1-5mm (LMP), Plastics above 5mm (MAC).

Bordós *et al.* (2021)	Water Research	Validation of pressurized fractionated filtration microplastic sampling in controlled test environment	Cascade filtering system	Water	Water	PE, PA, PET, PVC, PP	Beads, Fragments, Fibres	MP	1.8-29.7%
Büks *et al.* (2021)	Biogeosciences Discussions	Particles under stress: Ultrasonication causes size and recovery artifacts with soil derived POM, but not with microplastics	Applying ultrasonication to soil	Soil	Saline Solution	LDPE, PET, PBAT	Films	MP	96.46-97.62%
Crichton *et al.* (2017)	Analytical Methods	A Novel, density-independent and FTIR-compatible approach for the rapid extraction of microplastics from aquatic sediments	Oil extraction protocol with Canola oil	Sediment	Oil, Saline Solution	Polyester, PA, PVC, ABS, PS	Fibres, Fragments	MP, LMP	76.9-98.5%
Dawson *et al.* (2020)	Frontiers in Environmental Science	Solving a sticky situation: Microplastic analysis of Lipid-Rich Tissue	Four experiments with differing temperatures and different KOH and ethanol concentrations	Tissue of Organism	Alcohol, Base	PS, PE, Rayon, Polyester	Fragments, Fibres	LMP	93.3-100%
Enders *et al.* (2020a)	MethodsX	When every particle matters: A QuEChERS approach to extract microplastics from environmental samples	QuECHhERS method (Quick, Easy, Cheap, Effective, Rugged, Safe)	Sediment	Saline Solution	PA, PE	Fragments, Beads	MP	78-95.6%
Enders *et al.* (2020b)	Frontiers in Environmental Science	Evaluation of Electrostatic separation of microplastics form mineral rich environmental samples	Small scale version of the Korona-Walzen-Scheider system	Sediment	N/A	PA66, HDPE, PE, PA6	Pellets, Fibres, Beads	MP, LMP	36.89-99.67%

Gonzalez-Jauregui *et al.* (2019)	MethodsX	Stomach Flushing technique applied to quanitfy microplastics in Crocodilians	Stomach flushing	Gastrointestinal Tract	N/A	PE, LDPE, PA6, PA66	Fragments	LMP	75.3%
Hengstmann *et al.* (2018)	Marine Pollution Bulletin	Microplastic in beach sediment of the Isle of Rugen (baltic Sea) - implementing a novel glass elutriation column	Density separation using a glass elutriation column	Sediment	Oxidising Agent, Saline Solution	PET, PVC	Fragments, Film	MP, LMP	70.28-87.78%
Hernández-Arenas *et al.* (2021)	Environmental Pollution	The effect of sewage sludge containing microplastics on growth and fruit development of tomato plants	Digestion with acid and a density separation	Waste Water Treatment/Sludge	Acid, Saline Solution, Oxidising Agent	HDPE, PP, LDPE, PET, Copolymer	Fragments, Films, Beads	MP, LMP	83-91%
Imhof *et al.* (2012)	Limnology and Oceanography: Methods	A novel, highly efficient method for the separation and quantification of plastic particles in sediments of aquatic environments	Test density separation against froth floatation and improved on the munich plastic sediment separator (MPSS)	Sediment	Water, Saline Solution	PA, PC, HDPE, PE, PET, PP, PS, PVC, PA66, POM	Fragments	MP, LMP	39.8-100%
Jaafar *et al.* (2020)	Chemosphere	Improving the efficiency of post-digestion method in extracting microplastics from gastrointestinal tract and gills of fish	Sieving, density separation with zinc chloride and oil extraction protocol	Gastrointestinal Tract Tissue of Organism	Base, Saline Solution, Oil	PET, PVC, HDPE, LDPE, PP	Fragments	MP	70.7-84.33%
Konechnaya *et al.* (2020)	Water Science and Technology	Optimized microplastic analysis based on size fractionation, density separation and uFTIR	Grain size fractionation and density separation	Sediment	Saline Solution	PE, PP, PVC, PET, PA, PS, PU, PMMA	Fragments	MP, LMP	93-104%

Lares *et al.* (2019)	Environmental Science and Pollution Research	Intercomparison study on commonly used methods to determine microplastics in wastewater and sludge samples	Six methods tested: filtration, wet peroxide oxidation, KOH degradation, Oil extraction protocol, density separation and drying.	Waste Water Treatment/Sludge	Water, Oxidising Agent, Base, Oil, Saline Solution	PS, PE, PVC, SBR, PET, PA, PP	Fragments, Fibres	MP, LMP	6.7-100%
Lechthaler *et al.* (2020)	Analytical Methods	Canola oil extraction in conjuction with a plastic free separation unit optimises microplastics monitoring in water and sediment	Canola oil extraction and zinc chloride separation	Sediment Water	Saline Solution, Oil	PS, PA, PET, Carbon, SBR, PVC, PE, PP	Beads, Fibres, Pellets, Fragments, Films, Foams	MP, LMP	40-100%
Loder *et al.* (2017)	Environmental Science and Technology	Enzymatic purification of microplastics in environmental samples	Several technical grade enzymes used (Basic enzymatic purification protocol (BEPP))	Water	Oxidising Agent, Saline Solution, Enzyme	PE	Beads	MP	84.5%
Mani *et al.* (2019)	Analytical Methods	Using Castor oil to separate microplastics from four different matrices	Castor oil separation	Water Sediment Soil	Oil	PP, PS, PMMA, PET	Fragments	MP	94.25-100%
Nakajima *et al.* (2019)	Peer J	A New small device made of glass for separating microplastics from marine and freshwater sediments	Small glass separator (JAMSTEC microplastic-sediment separators)	Sediment	Saline Solution	PE, PP, PET, PS, PVC,	Particle	MP, LMP	95-100%
Nel *et al.* (2019)	MethodsX	Simple yet effective modifications to the operation of the SMI unit to avoid PVC contamination	Modification of the sediment microplastic isolation unit	Sediment	Saline Solution	PET, PE, PP	Fragment	N/A	83-87%

Palermo *et al.* (2020)	Global Journal of Environmental Science and Management	Susceptibility of Sardinella lemuru to emerging marine microplastic pollution	Rose Bengal Dye used for identification of microplastics	Gastrointestinal Tract	Alcohol	N/A	N/A	MP LMP	100
Peez *et al.* (2019)	Analytical and Bioanalytical chemistry	Quantitative analysis of PET microplastics in environmental model samples using quantitiative H-NMR spectroscopy: validation of an optimized and consistent sample clean up method	Samples were spiked with PET microplastics before digestion, after digestion or after filtration	Water Biological Material Whole Organism Sediment	Acid, Solvent, Oxidising agent	PET	Fibres	MP	72.1-104.5%
Prata *et al.* (2020a)	Science of the Total Environment	Major factors influencing the quantification of nile red stained microplastics and improved automatic quantification	Nile Red Staining	Sediment Water	Oxidising Agent, Alcohol, Base, Dye	PE	Fragments	MP	85.4-95.23
Prata *et al.* (2020b)	MethodsX	An easy method for processing and identification of natural and synthetic microfibres and microplastics in indoor and outdoor air	Hydrogen peroxide digestion followed by density separation	Air	Oxidising Agent, Saline Solution	PS, PE	Fibres, Fragments	N/A	94.4%
Prata *et al.* (2020c)	Water	What is the minimum volume of sample to find small microplastics: laboratory experiments and sampling of Aveiro Lagoon and Vouga River, Portugal	Nile Red Staining	Water	Oxidising Agent, Alcohol, Dye	PE	N/A	LMP	112.15%

Prata *et al.* (2019)	Science of the Total Environment	A new approach for routine quantification of microplastics using Nile Red and automated software (MP-VAT)	Nile Red Staining	N/A	Dye	LDPE, PP, PS, HDPE, PET, CA, PVC, Cotton, Linen, Polyester, PA, Rayon	Fragments	N/A	102.75%
Roch and Brinker (2017)	Environmental Science and Technology	Rapid and efficient method for the detection of microplastics in the gastrointestinal tract of fishes	Sodium hydroxide, nitric acid and sodium iodide used	Gastrointestinal Tract	Acid, Base, Saline Solution	PS	Particles	MP	97.5%
Scopetani *et al.* (2020)	Science of the Total Environment	Olive oil based method for the extraction, quantification and identification of microplastics in soil and compost	Using Olive oil lto extract microplastics from soil and compost	Soil Waste Water Treatment/Sludge	Oil, Solvent	PE, PU, PS, PC, PVC, PET	N/A	MP, LMP	89.1-95.9%
Thiele *et al.* (2021)	Scientific Reports	Microplastics in fish and fishmeal: and emerging environmental challenge?	NaCl Density Separation and Sediment microplastic isolation unit	Fishmeal	Saline Solution	PS, PP, PET, PA, Rayon	Fragments, Fibres	MP	27-88.3%
Tsangaris *et al.* (2021)	Marine Pollution Bulletin	Interlaboratory comparison of microplastic extraction methods from marine biota tissues: A harmonisation exercise of the plastic busters MPAs project	15% Hydrogen peroxide digestion anf 10% Potassium hydroxide digestion	Gastrointestinal Tract	Oxidising Agent, Base	PE, PP, PET	Fragments	MP, LMP	86.25- 98.75%

Vermeiren *et al.* (2020)	Environmental Pollution	Microplastic identification and quantification from organic rich sediments: a validated laboratory protocol	Fentons digestion and Zinc chloride density separation and nile red staining for identification	Sediment	Oxidising Agent Saline Solution, Dye	PE, PP, PS, PA, PET, PVC	N/A	MP, LMP	81.5-117.6%
Weber *et al.* (2021)	Science of the Total Environment	Investigation of microplastics in drinking water of a German city	Hydrochloric acid used for digestion	Water	Acid	PE	Beads	MP	66.75%
Wu *et al.* (2020)	Environmental Science and Pollution Research	A novel method for extraction of polyprophylene microplastics in swine manure	Centrifugation method and fractional distilling method	Excrement	Oxidising Agent, Base, Saline Solution	PP	Fragments, Fibres	MP, LMP	60-72.75%
Xu *et al.* (2020)	Journal of Hazardous Materials	Investigation of the microplastics profile in sludge from Chinas largest water reclamation plant using a deasible isolation device	Sodium Chloride and Zinc Chloride density separations compared	Waste Water Treatment/Sludge	Saline Solution	PP, PC, PET, Polyester	Pellets, Fibres	MP, LMP	51.11-99.45%
Yan *et al.* (2020)	Journal of Hazardous Materials	An efficient method for extracting microplastics from feces of different species	Used Fentons reagent and nitric acid. Absolute ethyl alcohol to dissolve organic matter	Excrement	Acid, Alcohol, Oxidising Agent	PS, PE, PVC	N/A	MP	95-100%
Studies found by 'Citation Chasing'									
Avio *et al.* (2015)	Marine Environmental Research	Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: First observation in commercial species from Adriatic Sea	Testing 6 protocols	Gastrointestinal Tract	Acid, Saline Solution, Oxidising Agent	PS, PE	Particle	MP, LMP	37-95%

Bianchi *et al.* (2020)	Marine Pollution Bulletin	Food preferences determines the best suitable digestion protocol for analysing microplastic ingestion by fish	10% Potassium hydroxide and 15% Hydrogen peroxide, 5% Nitric acid and 15% Hydrogen peroxide	Gastrointestinal Tract	Acid, Oxidising Agent	PA, PVC, PP, PE, PS, PET	N/A	MP	98%
Budimir *et al.* (2018)	Marine Pollution Bulletin	Effective and easy to use extraction method shows low numbers of microplastics in offshore planktivorous fish from the northern Baltic Sea	Sodium hydroxide, Hydrochloric acid, Sodium hypochlorite, Hydrogen peroxide, nitric acid	Gastrointestinal Tract	Acid, Base, Saline Solution, Oxidising Agent	HDPE	Flakes	MP	84%
Caron *et al.* (2018)	MethodsX	Validation of an optimised protocol for quantification of microplastics in heterogenous samples: A case study using green turtle chyme	Acid digestion and density separation	Gastrointestinal Tract	Acid, Saline Solution	HDPE	Beads	MP	100%
Catarino *et al.* (2017)	Environmental Toxicology and Chemistry	Development and optimization of a standard method for extraction of microplastics in mussels by enzyme digestion of soft tissues	Sodium Hydroxide and Nitric acid and Protease enzyme	Whole Organism	Base, Enzyme	PET, HDPE, PA	N/A	MP	93-94%
Claessens *et al.* (2013)	Marine Pollution Bulletin	New techniques for the detection of microplastics in sediments and field collected organisms	Extractions with Sodium Iodide	Sediment Tissue of Organism	Saline Solution	PA, PS, PVC	Fibres, Granules, Particles	MP	49.15-95.75%

Corradini *et al.* (2019)	Science of the Total Environment	Evidence of microplastic accumulation in agricultral soils from sewage sludge disposal	Density separations with Sodium chloride and zinc chloride	Soil	Saline Solution	PMMA, LDPE	Fibres, Fragments	MP, LMP	49-98%
Dehaut *et al.* (2016)	Environmental Pollution	Microplastics in seafood: Benchmark protocol for their extraction and characterisation	Compared six existing methods	Tissue of Organism	Base	PA6	Particle	MP	100%
Di and Wang (2018)	Science of the Total Environment	Microplastics in surface waters and sediments of the three gorges reservoir, China	Double density separation and digestion	Sediment	Oxidising Agent, Saline Solution	PE, PS, PP, PVC	Particle	MP, LMP	60-95%
Digka *et al.* (2018)	Marine Polluion Bulletin	Microplastics in mussels and fish from the Northern Ionian Sea	Hydrogen peroxide digestion	Tissue of Organism	Oxidising Agent	PE, PP, PVC, PS, PET	Particle	MP	60-100%
Dyachenko *et al.* (2017)	Analytical Methods	Extraction and identification of microplastic particles from secondary wastewater treatment plant (WWTP) effluent	Wet peroxide digestion	Waste Water Treatment/Sludge	Oxidising Agent	PS	Beads	MP	87%
Felsing *et al.* (2018)	Environmental Pollution	A new approach in separating microplastics from environmental samples based on their electrostatic behaviour	Electrostatic separator	Sediment	N/A	HDPE, LDPE, PET, PP, PS, PVC, PMMA, PLA, PE, Tyre	Fragments, Fibres	MP, LMP	97.5-98.75%
Fuller and Gautam (2016)	Environmental Science and Technology	Aprocedure for measuring microplastics using pressurised fluid extraction	Pressurised fluid extraction	Waste Water Treatment/Sludge	Solvent	HDPE, PP, PVC, PS, PET	N/A	MP	101-111%

Funck *et al.* (2020)	MethodsX	Identification of microplastics in wastewater after cascade filtration using Pyrolysis-GC-MS	Cascading filtration	Water	N/A	PE	Particles	MP	86.67%
Grbic *et al.* (2019)	Environmental Science and Technology	Magnetic Extraction of microplastics from environmental samples	Magnetic extraction	Water Sediment	N/A	PP, PVC, PU, PS, HDPE, PET, PE	Fragments, Fibres, Beads	MP, LMP	48.7-104.8%
Herrera *et al.* (2018)	Marine Pollution Bulletin	Novel methodology to isolate microplastics from vegetal-rich samples	Tested five existing digestion protocols a novel density separation	Plant Material	Acid, Base, Alcohol, Oxidising Agent	PE, PP, PS, PA, Polyester	Pellets, Fibres	MP, LMP, MAC	0-100%
Hildebrandt *et al.* (2019)	Marine Environmental Research	Evaluation of contiuous flow centrifugation as an alternative technique to sample microplastic from water bodies	Volume reduced sampling and continous flow centrifugation	Water	Alcohol, Saline Solution	PET, PE, PS, PVDC, PP	N/A	MP	76.61-99.1%
Hurley *et al.* (2018)	Environmental Science and Technology	validation of a method for extracting microplastics from complex, organic-rich, environmental matrices	Hydrogren peroxide, Fentons reagent, Sodium hydroxide and potassium hydroxide	Soil Waste Water Treatment/Sludge	Oxidising Agent, Base, Saline Solution	PET, PE	Fibres, Beads	MP	80.99-98.08%
Karlsson *et al.* (2017)	Marine Pollution Bulletin	Screening for microplastics in sediment, water, marine invertebrates and fish: Method development and microplastic accumulation	Density separation for sediment, enzymatic digestion for biota	Sediment Whole Organism	Saline Solution, Oil, Oxidising Agent, Enzyme	LDPE, HDPE, PP, PS, PE, PA, PET	N/A	MP, LMP	82-97%
Li *et al.* (2018)	Water Research	Microplastics in sewage sludge from wastewater treatment plants in China	Sodium chloride density separation and hydrogen peroxide digestion	Waste Water Treatment/Sludge	Oxidising Agent, Saline Solution	PS, PE, PP	N/A	MP	74.5-83.2

Liu *et al.* (2018)	Environmental Pollution	Microplastic and mesoplastic pollution in farmland soils in suburbs of Shanghai China	Density separation and 30% hydrogen peroxide	Soil	Oxidising Agent, Saline Solution	PP, PE, PA, PET, PVC, PC, ABS, PMMA, PS	N/A	LMP	0-100%
Mahon *et al.* (2017)	Environmental Science and Technology	Microplastics in sewage sludge: effects of treatment	Elutriation column	Sediment	Water	HDPE, PVC	N/A	LMP	80-91.67%
Mohamed Nor and Obbard (2014)	Marine Pollution bulletin	Microplastics in Singapore's coastal mangrove ecosystems	Floatation in saline solution	Sediment	Saline Solution	PE	Beads	MP	63.35%
Munno *et al.* (2018)	Environmental Toxicology and Chemistry	Impacts of temperature and selected chemical digestion methods on microplastic particles	Comparision of Alkaline and wet peroxide oxidation digestions	Gastrointestinal Tract	Water, Base, Oxidising Agent	PE, PS, PA	Beads, Foams, Fibres	MP	80.32-103.3%
Nuelle *et al.* (2014)	Environmental Pollution	A new analytical approach for monitoring microplastics in marine sediments	Air induced overflow in sodium chloride followed by a sodium iodide floatation	Sediment	Oxidising Agent, Saline Solution	PE, PP, PVC, PET, PS, PU	Pellets, Particles	LMP	80-100%
Pagter *et al.* (2018)	Marine Pollution bulletin	Microplastics in Galway Bay: A comparison of sampling and separation methods	Compairing density separation methods (elutriation column, sodium chloride, sodium tungstate dihydrate)	Sediment	Saline Solution	LDPE	Fragments	MP	92.5%
Scheurer and Bigalke (2018)	Environmental Science and Technology	Microplastics in Swiss floodplain soils	Density separation and oxidation of organic matter	Sediment	Saline Solution	PP	Particle	MP	96.25%
Stolte *et al.* (2015)	Marine Pollution bulletin	Microplastic concentrations in beach sediments along the German Baltic coast	Density separation in saline solution	Sediment	Oxidising Agent, Saline Solution	PE	N/A	MP	55.5%

Sujathan *et al.* (2017)	Archives of Environmental Contamination and Toxicology	Heat and Bleach: A cost-efficient method for extracting microplastics from Return activated sludge	6% hydrogen peroxide at 70°C followed by density separation with sodium nitrate/ sodium thiosulfate solution	Waste Water Treatment/Sludge	Oxidising Agent, Saline Solution	PE	Beads	MP	78%
Tagg *et al.* (2015)	Analytical Chemistry	Identification and Quantification of microplastics in wastewater using focal plane array-based Reflectance Micro-FT-IR Imaging	30% hydrogen peroxide used	Waste Water Treatment/Sludge	Oxidising Agent	PE, PP, PVC, PS	Fragments	N/A	96.67-100%
Thiele *et al.* (2019)	Marine Pollution Bulletin	Evaluation of existing methods to extract microplastics from bivalve tissue: Adapted KOH digestion protoccol improves filtration at single-digit pore size	KOH digestion	Whole Organism	Base	PA, PP, PMMA, PVC, LDPE	Fragments	MP	86.2%
Tsangaris *et al.* (2020)	Marine Pollution Bulletin	Using boops boops (osteichthyes) to assess microplastic ingestion in the Mediterranean Sea	Digestion with hydrogen peroxide	Gastrointestinal Tract	Oxidising Agent	PP, PE, PET, PVC, PS	N/A	MP	83-95%
Wiggin and Holland (2019)	Marine pollution Bulletin	Validation and application of cost and time effective methods for the detection of 3-500um sized microplastics in the urban marine and esturaine environments surrounding Long Beach, California	Hydrogen peroxide digestion and nile red for identification	Water	Oxidising Agent	PE, PS	Beads	MP	78.5%

Yu *et al.* (2019)	Environmental Toxicology and Chemistry	Development and validation of an efficient method for processing microplastics in Biota samples	Hydrogen peroxide/nitric acid digestion and sodium chloride density separation if needed	Whole Organism	Acid, Oxidising Agent, Saline Solution	LDPE, PS, PTFE, PET, PVC, PE	Beads	MP	93-100%
				Gastrointestinal Tract					
Zhou *et al.* (2018)	Geoderma	The distribution and morphology of microplastics in coastal soils adjacent to the Bohai Sea and the Yellow Sea	Continuous flow and floating separation	Sediment	Saline Solution	PP, PE	Particles	MP, LMP	97%
Zobkov and Esiukova (2017)	Limnology and Oceanography: Methods	Evaluation of the Munich Plastic sediment separator efficiency in extraction of microplastics from natural marine bottom sediments	Munich plastic sediment separator	Sediment	Saline Solution	PET	Fragments	MP	97.1%

Appendix. A.2 Number of studies in the meta-analysis using each reagent

Appendix.A.2. Table containing total number of studies in the meta-analysis using each saline solution, oxidising agent, acid, base, oil, solvent, enzyme and alcohol.

Reagent	Number of Studies
Saline Solutions	
Sodium Chloride (NaCl)	16
Sodium Iodide (NaI)	10
Zinc Chloride (ZnCl2)	10
Calcium Chloride ($CaCl_2$)	4
Sodium Dodecyl Sulphate (SDS)	1
Sodium Hypochlorite (NaClO)	2
Sodium Polytungstate ($Na_6O_{39}W_{12}$)	2
Sodium Bicarbonate ($NaHCO_3$)	1
Sodium Thiosulphate ($Na_2S_2O_3$)	1
Potassium Formate (HCO_2K)	1
Oxidising agents	
Hydrogen Peroxide (H_2O_2)	27
Wet Peroxide ($FeSO_4$)	9
Acids	
Nitric Acid (HNO_3)	7
Hydrochloric Acid (HCl)	3
Sulfuric Acid (H_2SO_4)	2
Bases	
Potassium Hydroxide (KOH)	10
Sodium Hydroxide (NaOH)	6
Oils	
Canola Oil	4
Castor Oil	1
Olive Oil	2
Solvents	
Hexane	2
Dichloromethane	1
Chloroform	1
Acetone	1
Enzymes	
Protease	2
Cellulase	1
Chitinase	1
Coralase	1
Proteinase K	1
Alcohols	
Ethanol	4
Ethyl Alcohol	1

Appendix. A.3 Meta-analysis assessment criteria

Appendix.A.3. Assessment criteria for recovery rate studies included in the meta-analysis. Assessment is based on the quality of the methods and results. Criteria adapted from Fidai et al. (2020) and Porter et al. (2014).

Rating	**Assessment**	**Example**
5	**Methods**: The method for the recovery rate study is very clearly displayed and outlined. There is clear evidence of media and reagents used in the study. The type, size, shape and amount of spiking plastics used is very clearly shown. A wide array of spiking plastics are used, at an environmentally relevant concentration. The number of replicates is sufficient and displayed. The method is detailed enough to allow for replication. **Results**: Results are clearly displayed in tabulate or graphical form, also displaying variance if applicable.	Birkenhead *et al.* (2020)
4	**Methods:** The method for the recovery rate study is clearly displayed and outlined. There is some evidence of media and reagents used in the study. There is some information on the type, size, shape and amount of spiking plastics used. A small variety of spiking plastics are used, but at an environmentally relevant concentration. The number of replicates used is displayed. **Results:** Results are displayed in either tabulate or graphical form, with the variance displayed if applicable.	Yu *et al.* (2019)
3	**Methods:** The method for the recovery rate study is present but lacking detail. There is little evidence of media, reagents, and type, shape, size and amounts of spiking plastics used. Only 1-2 types of spiking plastic are used, not an environmentally relevant concentration. The number of replicates used is not clearly evident. **Results:** Results are displayed in either tabulate or graphical form, with no evidence of variance.	Wiggin and Holland (2019)
2	**Methods:** The method for the recovery rate study is mentioned briefly, with limited detail. Only 1 type of spiking polymer is used. **Results:** Results are present in the text with an explanation, but not displayed in either tabulate or graphical form.	Budimir *et al.* (2018)
1	**Methods:** The recovery rate method is not displayed. Key information on spiking polymers used is missing. **Results:** Results are displayed in the text with no further explanation.	Dyachenko *et al.* (2017)

Appendix. A.4 Weighted mean equations

Appendix.A.4. Equations to calculate a weighted mean underestimation of microplastics in the environment. Equations gathered from Gurnsey (2017)

As we are looking to calculate a weighted sum, we first need to work out the weights (w_i^*) (This is the value given to the means due to the different sample sizes). As this is a random effects meta-analysis, w_i^* considers variability attributable to the population means and variability within the population. To calculate w_i^*, we first need to calculate S_{Meta}^2, which also needs values for Q and c, as follows:

1) First compute the weighted sum of squared deviations of the sample means (m_i) from M using weights of the fixed-effects model:

$$Q = \sum_{i-1}^{k} w_i m_i^2 - \frac{(\sum_{i=1}^{k} w_i m_i)^2}{\sum_{i=1}^{k} w_i}$$

$$Q = 1{,}734{,}062 - \frac{(19{,}402.109)^2}{224.724}$$

$$\boldsymbol{Q = 58{,}931.529}$$

2) To compute c :

$$c = \sum_{i=1}^{k} w_i - \frac{\sum_{i=1}^{k} w_i^2}{\sum_{i=1}^{k} w_i}$$

$$c = 224.724 - \frac{2179.219}{224.724}$$

$$\boldsymbol{c = 215.0266}$$

3) To compute S_{Meta}^2

$$S_{Meta}^2 = \frac{Q - df}{c}$$

$$S_{Meta}^2 = \frac{58{,}931.529 - 70}{215.0266}$$

$$\boldsymbol{S_{Meta}^2 = 273.7407}$$

4) Now S_{Meta}^2 is computed, the random effect weights (w_i^*) can be computed:

$$w_i^* = \frac{1}{\frac{s_i^2}{n_i} + S_{Meta}^2}$$

$$\boldsymbol{w_i^* = 19{,}660.32}$$

5) From this, we can now calculate the weighted mean (M^*) and its variance (S_M^*)

$$M^* = \frac{\sum_{i=1}^{k} w_i^* m_i}{\sum_{i=1}^{k} w_i^*}$$

$$\boldsymbol{M^* = 86.185}$$

$$S_M^* = \sqrt{\frac{1}{\sum_{i=1}^{k} w_i^*}}$$

$$\boldsymbol{S_M^* = 0.0071}$$

From these calculations, this meta-analysis shows an overall average recovery rate of microplastics of **86.185 ± 0.0071%.** Therefore, we estimate that microplastic research could be underestimating how many microplastics are found in the environment by approximately **14%.**

Appendix. A.5 Publication of Chapter 2

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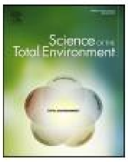

Review

Evidence of underestimation in microplastic research: A meta-analysis of recovery rate studies

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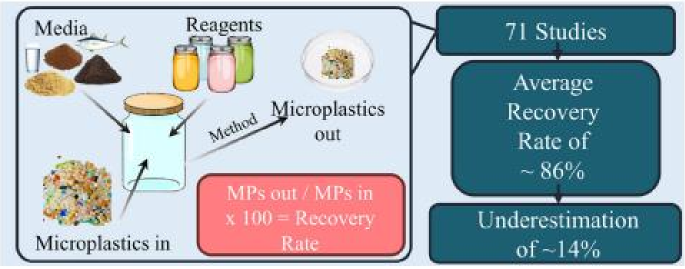
HIGHLIGHTS

- Meta-analysis shows recovery rates rarely used to validate microplastic methods.
- Sediment is the most studied medium and saline solutions the most used reagent.
- All reagents but water recovered over 80% of spiked microplastics.
- Based on average recoveries, microplastics could be underestimated by approx. 14%.
- Quality of recovery studies should be improved to help with standardisation.

GRAPHICAL ABSTRACT



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ABSTRACT

Research on microplastics in the environment is of high interest to many scientists and industries globally. Key to the success of this research is the accuracy, efficiency, reliability, robustness and repeatability of the method (s) used to isolate the microplastics from environmental media. However, with microplastics now being found in new complex media, many multifaceted methods have been developed to research the quantities of these pollutants. To validate new methods, recovery studies can be undertaken by spiking the test medium with known quantities of plastics. The method is typically run as normal, and the recovered plastics counted to give a recovery rate. A current issue in this field is that methods are rarely or poorly validated in this way. Here, we conducted a meta-analysis on 71 recovery rate studies. We found sediment was the most studied medium and saline solutions were the most used reagents. Polyethylene and polystyrene were the most used spiking polymers, which is relevant to the most common polymers in the environment. We found that recovery rates were highest from plant material, whole organisms and excrement (>88%), and lowest from fishmeal, water and soil (58–71%). Moreover, all reagents but water were able to recover more than 80% of the spiked plastics. We believe we are the first (to our knowledge) to provide an overarching indication for the underestimation of microplastics in the environment of approximately 14% across the studies we reviewed, varying with the methods used. Furthermore, we recommend that the quality, use and reporting of recovery rate studies should be improved to aid the standardisation and replication of microplastic research.

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typically as a percentage recovery rate. When implemented effectively, this could provide an insight in to how well a method could perform compared to others. Further to this, a recovery below 100% could suggest how using a certain method may underestimate the amount of microplastics in a matrix, and a recovery over 100% could show a potential for overestimation. Under and/or overestimating the number of microplastics in the environment could have further implications. For example, underestimating the number provides the chance of underplaying the severity of this pollution, whereas overestimating may allow for scaremongering of an issue which is not that severe. Both of these scenarios can have a negative impact if used in the media, particularly to those readers in the wider non-scientific population.

This meta-analysis aims to identify the recovery rates from multiple studies, and critically review how they vary when using different methods to extract microplastics from a wide range of matrices. The analysis is the first (to our knowledge) to provide an estimate of how much microplastic research may be under or over-estimating current levels of microplastics based on the methods utilised and the recovery rates found. Finally, recommended reporting criteria are provided for future recovery rate studies to allow for improved validation and simpler replication.

2. Method

2.1. Methodology for literature search – identification

The methodological approach of this meta-analysis was carried out by following the guidance of the PRISMA 2020 flow diagram (Page et al., 2021) (Fig. 1). The PRISMA flow diagram allows for a visual depiction of the different stages of the systematic literature search; including how sources were identified and how many were found, how many

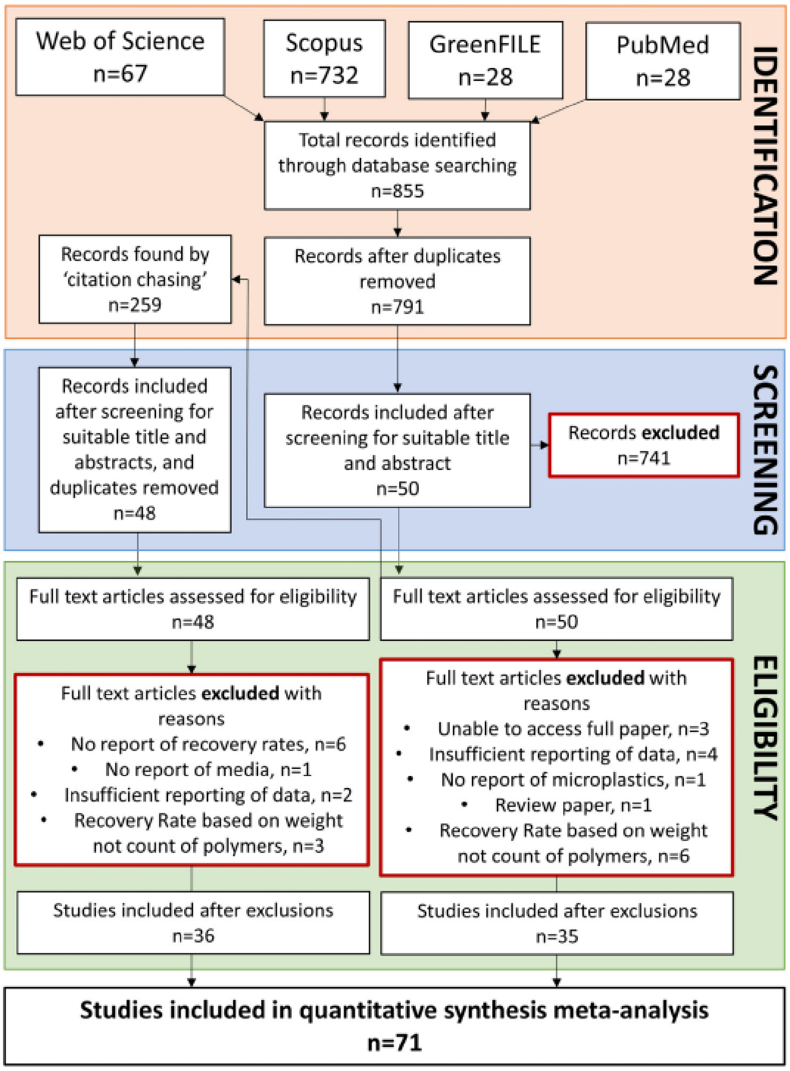

Fig. 1. Literature search flowchart. Including literature identification, screening and eligibility process. Including number of articles found and/or excluded at each stage. Adapted from PRISMA flow diagram (Page et al., 2021).

were screened for suitability, how many were included in the analysis and reasons for those excluded. Sections of the PRISMA 2020 checklist (Page et al., 2021) were also complied and followed, with the inclusion of the eligibility criteria, information sources, a full search strategy, study selection, the data collections process and the data extracted. The PRISMA approach has been widely applied to optimise methodologies and reporting quality in research studies (Liu et al., 2019).

During January 2021, a database search was undertaken using Web of Science, Scopus, GreenFILE and PubMed search engines. The search was conducted using the following search terms: "recovery rate" OR "recovery efficiency" AND microplastic OR plastic OR nanoplastic AND extraction OR identification OR validation.

The search was filtered further to only include peer-reviewed articles; however, no limit was put on date of publication. Following from the database searches, 855 records were found, and a reference manager (Endnote) was used to organise the articles. Duplicates were removed, leaving 791 papers to be screened for suitable titles and abstracts (Fig. 1).

2.2. Criteria for inclusion – screening and eligibility

During the screening for suitable titles and abstracts, certain inclusion criteria were applied. Articles incorporated must include plastics that are either below 5 mm (large microplastics) or plastics between 1 μm–1 mm (microplastics) (International Organization for Standardization, 2020). The titles and/or abstract must also indicate that the method used was validated in some way, either by including a recovery rate or using another term such as efficiency. The media tested in the studies were not limited.

Following on from screening for suitable title and abstracts, 50 full text articles were assessed for eligibility. Articles were excluded for the following reasons: No access to the full paper and data, insufficient reporting of data (recovery rates were reported in graphs/figures, but an accurate average and/or variation could not be extracted and used), no report of using microplastics in the study or spiking trial, no report of recovery rates, any review papers and recovery rates calculated by weight difference, not count.

Due to recovery rate studies being often undertaken as a side project alongside microplastic extraction/identification studies, many recovery rate studies may have not been identified during screening titles and abstracts. Therefore, "citation chasing" (Barrett, 2005) was carried out to counterbalance this. When reading the full text articles, suitable references were identified and pooled. 259 potentially suitable articles were identified and managed within the reference manager. After duplicates were removed and abstracts and titles were screened for the same inclusions mentioned previously, 48 articles were selected to be checked for full paper eligibility.

After all articles were assessed for eligibility, including those found by citation chasing, 71 papers were included for the meta-analysis.

2.3. Data extracted

Data extracted from the articles included basic information such as the authors' names, the journal name and date of publication. Other material extracted included a short detail on the method used, the test media, the types of reagent used, the spiking microplastic polymer types, the spiking microplastic shapes, the spiking microplastic sizes and the recovery rates found.

The quantitative analysis was further conducted in Microsoft Excel and RStudio (version 3.6.1). The microplastic size category was further subdivided into MP (microplastic) (any microplastics between 1 μm and 1 mm) and LMP (large microplastic) (any microplastics between 1 mm and 5 mm) (International Organization for Standardization, 2020). Similarly, the test reagents were categorised into oils, alcohols, dyes, acids, oxidising agents, bases, saline solutions, water, enzymes and solvents. The test media were categorised into plant material (vegetal plant material), air, fishmeal, biofilms, excrement, whole organisms, tissues of organisms, soil (horticultural/agricultural soil, farmland soil, compost), wastewater effluent/sludge, water and sediment (marine and freshwater sediment/beach and river sediment). All information on studies included is provided in the supplementary material (Table S1).

Due to the lack of control samples used in recovery rate studies, and lack of reported sample sizes for the recovery rate part of a study, a sample effect size was not able to be calculated. However, this limitation will be examined in the discussion.

2.4. Quality of selected studies

The quality of the selected studies in this analysis is assessed by ranking each study subjectively from 1 to 5 (1 being low quality, 5 being high quality). The criteria (Table S3) are adapted from Porter et al. (2014) and Fidai et al. (2020), and are based on the quality of the recovery rate method, comprising of the inclusion of the test media, the reagent used and information on the spiking plastics used. Furthermore the criteria included whether the studies have potential for replication and the clarity and presentation of results.

3. Results

3.1. Summaries of studies included in meta-analysis

3.1.1. Quality of selected studies

The purpose of reviewing the quality of included studies is to highlight the areas of recovery rate studies which need improvement. The mode score for the 71 studies included in this meta-analysis is 4. With only 14 studies achieving the rank of 5, it shows there are many limitations of recovery rate studies to be discussed.

3.1.2. Media and reagent used

A total of 12 different types of media were studied, including fishmeal (Thiele et al., 2021), plant material (Herrera et al., 2018), air (Prata et al., 2020a), biofilms (Peez et al., 2019), excrement (Wu et al., 2020; Yan et al., 2020), whole organisms (Catarino et al., 2017; Karlsson et al., 2017; Peez et al., 2019; Thiele et al., 2019; Yu et al., 2019), tissues of organisms (Claessens et al., 2013; Dawson et al., 2020; Dehaut et al., 2016; Digka et al., 2018; Jaafar et al., 2020), soil (Büks et al., 2021; Corradini et al., 2019; Scopetani et al., 2020), wastewater/sludge (Dyachenko et al., 2017; Scopetani et al., 2020; Xu et al., 2020), gastrointestinal tracts (Munno et al., 2018; Tsangaris et al., 2020; Yu et al., 2019), water (Birkenhead et al., 2020; Hildebrandt et al., 2019; Wiggin and Holland, 2019) and sediment (Mahon et al., 2017; Mohamed Nor and Obbard, 2014; Pagter et al., 2018) (for a breakdown of these media categories see Section 2.3 Data Extracted). One study did not report the medium used (N/A in Fig. 2). The most tested medium is sediment (n = 26), followed by water (n = 14) and gastrointestinal tracts (n = 12) (Fig. 2).

Several different reagents were used in the studies when performing recovery rate trials. These include solvents (Peez et al., 2019; Scopetani et al., 2020), enzymes (Catarino et al., 2017; Karlsson et al., 2017), dyes (Prata et al., 2020b; Vermeiren et al., 2020), bases (Dawson et al., 2020; Jaafar et al., 2020), acids (Hernández-Arenas et al., 2021; Weber et al., 2021), oxidising agents (Nuelle et al., 2014; Stolte et al., 2015), water (Lares et al., 2019; Mahon et al., 2017), alcohol (Hildebrandt et al., 2019; Palermo et al., 2020), oil (Karlsson et al., 2017; Scopetani et al., 2020) and saline solutions (Büks et al., 2021; Crichton et al., 2017). The most frequently used reagents were saline solutions (n = 39), followed by oxidising agents (n = 31), oxidising agents combined with saline solutions (n = 17) and bases (n = 14) (Fig. 2). The most commonly used saline solutions include sodium chloride (n = 15), sodium iodide (n = 10) and zinc chloride (n = 10) (Table S2). Moreover,

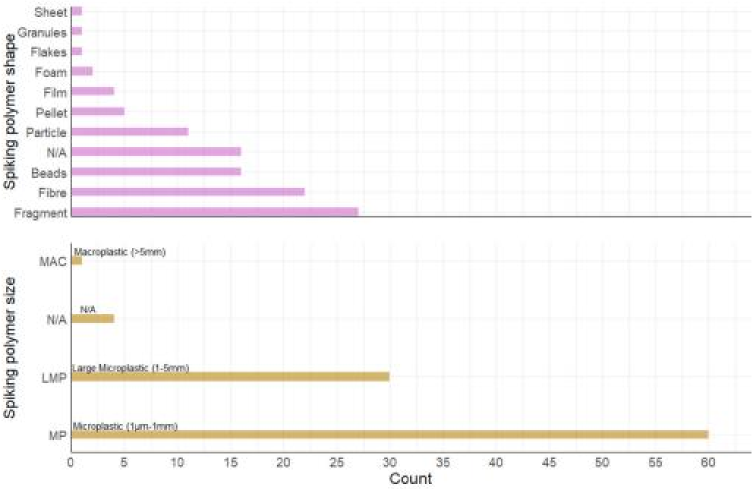

Fig. 4. The count of different shape and size of spiking plastics used in the studies selected for this meta-analysis. Large microplastics are those classed between 1 mm–5 mm, microplastics are those classed between 1 μm–1 mm (International Organization for Standardization, 2020). N/A represents the number of studies not reporting the spiking polymer shape or size. Many studies used more than one different shape and size of spiking plastic.

3.2.2. Recovery rates of polymers from different media

The majority of the lower recovery rates in each media type came from the high-density polymers (PVC, PET and PA). This is the case for fishmeal, water, wastewater/sludge, tissues of organisms and whole organisms (Fig. 6). However, in the studies that have used gastrointestinal tracts and excrement as the study medium, the opposite is found, with lower recovery rates of low-density polymers (PS, PP, PE, LDPE, HDPE). Overall, polymers were recovered more effectively from plant material (all 100%), biofilms (96%), whole organisms (91–95%) and excrement (88–95%); and recovered least from fishmeal (58–70%), water (67–82%) and wastewater effluent/sludge (76–89%) (Fig. 6). The difference in recovery rates between high and low-density polymers is much larger in some media compared to others. For example, 22% more low-density polymer was recovered from soil than high-density polymers. However, from tissues of organisms only 3% more low-density polymers were recovered than high-density polymers (Fig. 6).

3.2.3. Recovery rates of polymers using different reagents

Similarly to the trend found in the recovery of polymers in different media, most reagents recovered more low-density polymers than high density polymers, which is the case for water, saline solutions, oxidising agents, bases and dyes. However, the opposite is found when studies used solvents, alcohols, acids and oils, which removed more high-density polymers. Moreover, all but one reagent (water) recovered more than 80% of spiking polymers on average. However, the studies that used water as a reagent to recover the polymers showed the lowest recovery rates (averages 53% for high-density polymers, 65% for low-density polymers) (Fig. 7).

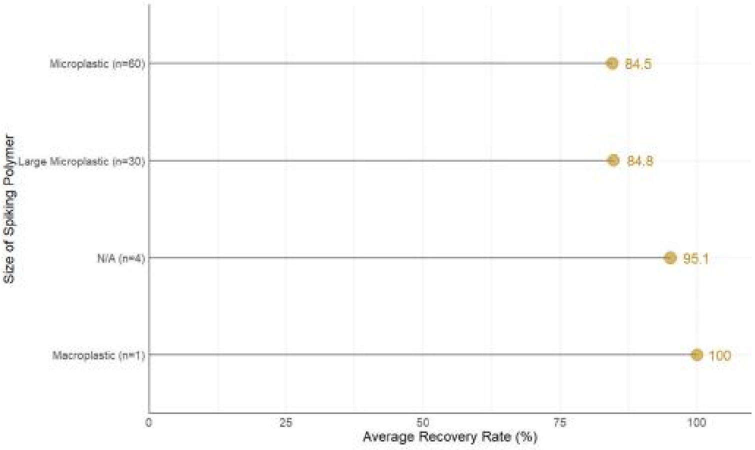

Fig. 5. Average recovery rates across studies of different sized spiking plastics. Macroplastics are those plastics above 5 mm, large microplastics are those classed between 1 mm–5 mm, microplastics are those classed between 1 μm–1 mm (International Organization for Standardization, 2020). N/A represents the studies which did not classify the size of the spiking plastics used.

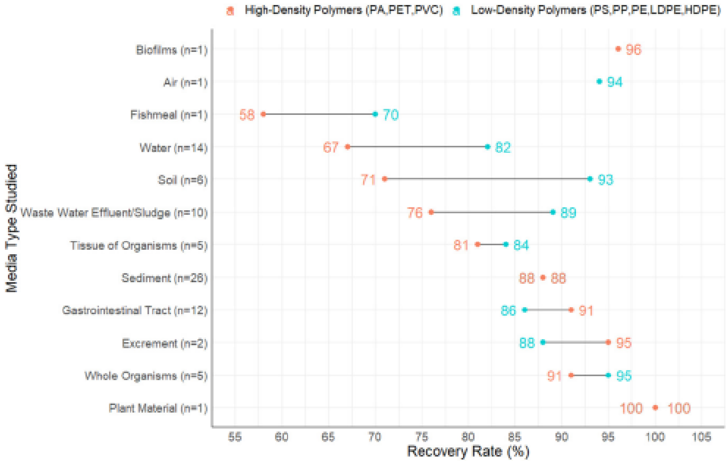

Fig. 6. Average recovery rates across studies of high and low-density polymers when extracted from different media. Numbers against the media represent number of studies in this meta-analysis using each medium.

3.2.4. *Combination of different reagents and media on the recovery rates of polymers*

Individually, reagents and type of media have an effect on recovery of microplastic polymers (Figs. 6 & 7), however they can also have an effect on recovery when combined (Fig. 8). For example, the use of an acid as a reagent results in higher recovery than other reagents when used in the same media. This is the case for excrement, sediment and whole organisms. However, when an acid is used to recover polymers from wastewater/sludge and water, lower recovery rates are found (Fig. 8). The use of oxidising reagents recovered the most polymers from air, excrement, gastrointestinal tracts and plant material; however, these reagents resulted in very low recoveries of high-density polymers from soil (Fig. 8).

Similarly, saline solutions recover high amounts of polymers from air and whole organisms, but lower amounts from media such as excrement, fishmeal, soil, tissues of organisms and wastewater/sludge (Fig. 8).

Moreover, the use of oil as reagent to recover plastics produced high recovery rates in soil. However, much lower recovery rates were found when using the same reagent to extract polymers from gastrointestinal tracts and tissues of organisms.

3.3. *Assessment of underestimation*

As seen in Figs. 6–8, very few combinations of reagents and media tested result in 100% recovery of spiking microplastics, meaning there is a level of underestimation when using these methods to extract polymers. Due to the lack of consistent information reported and the low importance given to recovery experiments in much microplastic research, an effect size could not be calculated for this meta-analysis. Therefore, we have counterbalanced this by calculating a weighted mean based on equations provided by Gurnsey (2017). Here we estimate that microplastic research could be underestimating how many microplastics are found by approximately 14% (calculation in supplementary material (Eq. S1)), based on the type of reagents and medium used. We recommend taking any underestimations found by method validation into account when concluding how many microplastics are found in environmental samples.

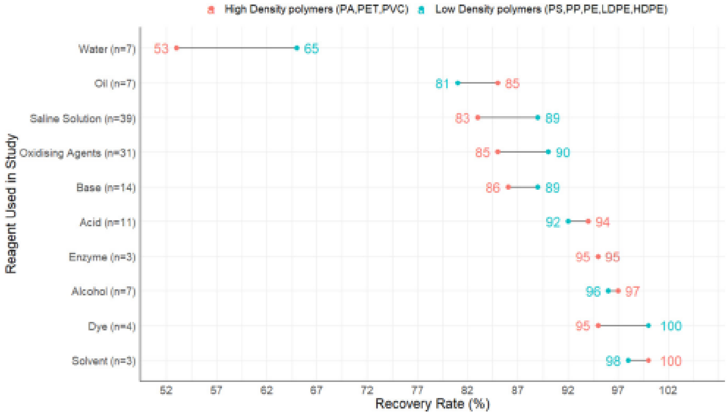

Fig. 7. Average recovery rates across studies of high and low polymers when extracted using different reagents. Numbers against the reagents represent number of studies in this meta-analysis using each reagent.

may be more abundant in the environment but have yet to be studied in depth due to technological limitations. An example of this limitation is the ability to identify and quantify such small particles. Even if nanoplastics are in high abundance, their mass could be so low that it is difficult to detect with current technology and methods, or nanoplastics may be found aggregated to other particles due to their size, making them difficult to isolate (Jakubowicz et al., 2021). This provides further evidence that smaller microplastics are more difficult to isolate, with this analysis showing that on average, macroplastics and larger microplastics were recovered at a higher rate than the smaller microplastics (Fig. 5).

The environmental relevance of the types of plastics used as spiking polymers is crucial as it must represent as close to a true environmental sample as possible. Microplastics in the environment may vary in bioavailability and toxicity depending on many factors including the aforementioned type, shape and size, but also due to their colour, crystallinity and stability (Ma et al., 2020). These properties will not only affect the organisms in the environment but will also affect the way in which the plastics can be extracted from the environmental medium. Furthermore, these types of spiking recovery studies typically use new or 'virgin' plastic to spike the sample. However, true extractions from environmental media will usually involve isolating material that has been subjected to some ageing and weathering thus will behave differently from the virgin spiking material. Routine spiking studies with weathered microplastics would be challenging to deliver but is an area that could reward some further study.

When looking at the recovery of microplastics from different media types, microplastics were recovered at higher rates from some types over others. For example, plant material, biofilms, air, whole organisms and excrement had recovery rates over 95%, whereas fishmeal, water, soil and wastewater effluent/sludge had recovery rates below 80% (Fig. 6). This could be due to some of the properties of those media types. For example, there would be less organic material to breakdown in air than in fishmeal and soil. Radford et al. (2021) found organic material was one of the key factors in hindering the recovery of microplastics. Similarly, Wang et al. (2018) found that particle size influences the ability to extract microplastics from soil and biosolids, as some nano and micro-sized plastics take longer to float than larger sized plastics. Moreover, the range of recovery between low and high-density microplastics varies considerably between the different media types. For example, there is 22% difference between low and high-density microplastics recovered from soil (71–93%) (Fig. 6), but only 3% different from those recovered from tissues of organisms (81–84%). This could be due to the complexity of the test media. For example, the soil used in the different studies may vary considerably in regards to particle size distribution and organic matter which depending on the quantity of each, may benefit the lower-density plastics, but hinder the high-density plastics.

Similarly, this meta-analysis has revealed that using different reagents can yield different recovery rates. The majority of the reagents (oil, saline solutions, bases, acids, oxidising agents, enzymes, alcohols, dyes and solvents) recovered more than 80% of the spiking plastics (Fig. 7). However, in the studies which used water, recovery rates were below 65% (53–65%) (Fig. 7). This is not surprising as the density of water is approximately 0.99 g/cm^3 (Tanaka et al., 2001), which is lower than many plastics (PET: 1.37 g/cm^3, PVC: 1.38 g/cm^3 (British Plastics Federation, 2020)). However, what is surprising is that in some cases when using oils, alcohols and solvents, more high-density polymers were recovered than low density polymers (Fig. 7). A reason for this could be due to the density of these reagents. Chloroform has a density of 1.49 g/cm^3 but is corrosive enough to attack plastics (National Center for Biotechnology Information, 2021). The high density of chloroform will allow for higher density plastics to float, however, depending on the concentration of chloroform and length of exposure, certain types and sizes of microplastics may corrode.

What is overwhelmingly clear from the results of this meta-analysis is that it is rare for all spiking plastics to be recovered, thus a 100% recovery rate is seldom achieved. This meta-analysis found that on average-across all studies, microplastics could be underestimated by approximately 14% (see Supplementary information for calculation). More so, studies rarely account for any underestimation brought about by the methods used. If underestimations are accounted for, the amounts of microplastics in the environment could be a lot larger than estimated to date.

Overall, this meta-analysis has highlighted many issues within recovery rate studies and microplastic research. Firstly, recovery rate studies are rarely used to validate methods in published studies. For example, the 71 studies found and used in this analysis is a minute size compared to the large number of microplastic research papers and methods that have been published over time (Provencher et al., 2020). Furthermore, those papers that are published with a recovery rate study are often poorly executed with key information missing, such as sample size and the type, shape and size of the spiking plastic used. With this missing information, it is difficult to make further inferences regarding the effect size and publication bias, also this makes it problematic for others to replicate the method used. Often recovery rate results are poorly displayed and are seen as unimportant compared to the main results of a study. A standardisation needs to be agreed on in several aspects of these studies. Firstly, it should be agreed on whether recoveries are calculated by weight difference or difference by count; and secondly, the terms used to describe the shapes of the spiking polymers, often the term 'particle' is used, which can be interpreted in many ways. Due to the aforementioned limitations we have assembled recommended reporting criteria specifically for recovery rate studies, with the intention of making validation of microplastic extraction methods clearer to others.

5. Conclusions and recommendations

The varying range of recovery rates found in the studies included in this meta-analysis was dependent on the media types and reagents used. However, very rarely were 100% of the spiking plastics recovered, and overall an underestimation of 14% was discovered, meaning the amount of microplastics in the environment could be higher than estimated from research studies to date. From this meta-analysis it is clear that recovery studies are not utilised enough and, on the occasion, when they are, they are often poorly executed. It could be argued, that with a more holistic approach to validating methods, by studying the properties of the test medium, and clearly and concisely reporting the recoveries, it could help with the ever-growing issue of standardisation in microplastics research. This meta-analysis flagged several limitations within recovery rate studies, which we recommend improvements:

Report all raw or average recovery rates with variance in both tabulate AND graphical form. Include this in supplementary material if needed. Many studies either reported a single percentage in the text or displayed recovery rates in graphical form, often making it difficult to extract an exact percentage, thus making it difficult for others to accurately assess the effectiveness of the method.

Calculate the recovery rate by count of recovered plastics. Few studies calculated the recovery rate by change in weight; these studies were removed from this meta-analysis as they were not comparable to the majority which use counts. If this is adopted by all, it allows for standardisation.

Use triangulation: have multiple researchers count recovered plastics in a study. If counted by eye, counts of recovered microplastics could be different depending on the observer's experience carrying out this task.

Report the number of samples used in the recovery rate study. Many studies did not report the sample size, making it difficult for further analysis.

Report the shape, size, type and size of spiking plastics used. The reporting style of the spiking plastics across the studies varied considerably. For example, one study did not state the type of polymer used, ten studies did not state the shape of the polymer used, eleven studies used the word 'particle' to describe the shape, which could be interpreted differently by others, and four studies did not report the size of the polymer used. We recommend reporting these properties clear enough for replication and to use environmentally relevant quantities which are reported in the literature for each test medium.

Do the recovery rate study on the same media which is to be tested for the main experiment. Methods will work differently on media with different properties, thus different recovery rates will be found.

The aim of this meta-analysis is to highlight the importance to researchers of using a recovery rate study/trial to validate their methods, with the proposal that in the future this becomes a "new normal" during method development, and the quality of these types of studies are up to a standard that can be replicated. Furthermore, if the amount of underestimation, brought about by the methods used is accounted for in each study, the amounts of microplastics reported will probably be higher but more realistic, which can offer more robust evidence for policy makers.

Data availability

Data supporting this study are openly available from the University of Southampton repository at: https://doi.org/10.5258/SOTON/D1954.

CRediT authorship contribution statement

Chloe Way: Conceptualisation, methodology, validation, formal analysis, investigation, data curation, writing-original draft, visualisation. **Malcolm Hudson:** Supervision, project administration, funding acquisition, writing-review and editing. **Ian Williams:** Supervision, writing-review and editing. **John Langley:** Supervision, writing-review and editing.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.150227.

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Appendix B Chapter 3 Supplementary material

Appendix. B.1 Publication of Chapter 3

Analytical Methods

ROYAL SOCIETY OF CHEMISTRY

PAPER

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Assessing the effectiveness of microplastic extraction methods on fishmeal with different properties

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and Robert Marsh[d]

Microplastic presence in fishmeal is an emerging research area because of its potential to enter food chains, and the importance of fishmeal within global food security. However, fishmeal is a complex medium dependant on fish composition. This study measured properties (organics, carbonates, protein and density) of five fishmeal types (trimmings, sardine and anchovy, krill, tuna and salmon), sourced from locations worldwide (Norway, South America, Antarctica, Spain and Scotland). Microplastic recovery rates were compared for existing methodologies using sodium chloride overflows and potassium hydroxide digestions and then compared to newly developed methods. These methods included dispersants and calcium chloride density separations which were developed and designed to be environmentally conscious and affordable, which we argue should become an international standard approach for researchers. A calcium chloride overflow with dispersant and potassium hydroxide digestion provided the highest recovery rate in sardine and anchovy fishmeal (66.3%). Positive correlations with recovery rate were found with protein content, and negative correlations with organic content. Low recovery rates found here suggest microplastics in fishmeal reported in the literature are underestimated. With complex media such as fishmeal, attention must be paid to variation between types and composition when choosing methods and interpreting results.

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rsc.li/methods

Introduction

Plastic pollution is a concern worldwide. Tides, rivers and currents such as the North Atlantic current,[1] the Norwegian Coastal current (NCC),[1] the Humboldt current,[2] the Canary current,[3] and the melting of sea ice around the Antarctic peninsula[4] provide pathways for plastics to enter the marine environment. It is thought than an estimated 1.15–2.41 million tonnes of plastic enter the ocean from rivers alone.[5] Once in the marine environment, plastic debris is subject to fragmentation into secondary microplastics by ultraviolet radiation, and mechanical and microbial degradation.[6] Other forms of microplastics include primary microplastics that enter the marine environment as a small size, such as those in toiletries, cosmetics, tyre wear particles and synthetic fibres from washing clothes.[7] A definition of microplastics which includes their physiochemical properties was proposed by Frias and Nash (2019):[8] "Microplastics are any synthetic solid particle or polymeric matrix, with regular or irregular shape and with size ranging from 1 µm to 5 mm, of either primary or secondary manufacturing origin, which are insoluble in water". However, others believe large microplastics are between 1–5 mm.[9]

Due to the widespread nature of marine microplastics, there is a high potential for them to infiltrate the human food chain. Many studies have identified microplastics in the gastrointestinal tract[10–12] and gills[13,14] of marine life; however, few have studied either the whole fish or the tissue used as food for humans. Ribeiro *et al.* (2020)[15] investigated the edible sections of commonly eaten seafood such as oysters, prawns, squid, crabs and sardines, and found sardines had the highest amount of microplastic in mass (0.3 mg g^{-1} tissue). Similarly, Karami *et al.* (2017)[16] found more MP in the flesh of dried fish than the organs. There are many avenues microplastics may enter this pathway. For example, in areas where microplastics concentrations are high, it is more likely that some will be ingested by organisms (non-selective feeding).[17] Moreover, some marine organisms have shown an ability to selectively ingest microplastics of certain sizes.[18] Many marine organisms exposed to microplastics are harvested for fishmeal production, which indicates the potential for microplastic-contaminated fishmeal to get into the human food chain.

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Table 1 Different types of saline solution commonly used in the literature. With the common densities in solution, its effect on the environment and approximate costs as a salt and in solution. Environmental friendliness based on whether an aquatic toxicity hazard is listed on the safety data sheets of Fisher Scientific[50][d]

Separating solution	Density of salt in solution[e] (g cm^{-3})	Solution density in literature[c] (g cm^{-3})	Environmentally friendly?[b]	Approx. cost[a] (USD per kg)	Approx. cost per litre[e] (USD per L)
Seawater	1.02	N/A	Y	N/A	N/A
Sodium chloride (NaCl)	1.19 (26 wt% @ 25 °C)	1.2^{a}	Y	~\$60.54	~\$15.74
Calcium chloride ($CaCl_2$)	1.39 (40 wt% @ 20 °C)	1.46^{b}, 1.4^{c}	Y	~\$60.69	~\$24.27
Sodium bromide (NaBr)	1.41 (40 wt% @ 20 °C)	1.37^{d}, 1.55^{e}	N	~\$96.14	~\$38.45
Zinc chloride ($ZnCl_2$)	1.7 (60 wt% @ 20 °C)	1.5^{f}	N	~\$87.31	~\$52.38
Sodium iodide (NaI)	1.8 (60 wt% @ 20 °C)	1.566^{d}, 1.8^{g}	N	~\$533.98	~\$320
Sodium polytungstate	3.1 (85 wt% @ 20 °C)	1.5^{h}	N	~\$623.42	~\$497.94

[a] Cost per kg listed on Fisher scientific,[50] in US Dollars (USD). [b] Sodium iodide hazards includes aquatic toxicity. Zinc chloride hazards include chronic aquatic toxicity. Sodium bromide should not be released into the environment. Sodium polytungstate may cause long term adverse effects in the aquatic environment. [c] Literature: a (ref. 46), b (ref. 32), c (ref. 51), d (ref. 52), e (ref. 53), f (ref. 54), g (ref. 55), h (ref. 56). [d] N/A (not applicable). [e] Approximate cost per litre of salt solution at specific density.

(Table 1). These methods are detailed in Table 3. The effectiveness of each method on each fishmeal was assessed by determining the recovery of spiked microplastics. Polymers were not assessed for signs of degradation: KOH at a temperature of 40 °C was the only digestion solution used and has already been tested for its ability to degrade polymers at this temperature, with no effect found.[49]

Spiking microplastics

Microplastic polymer types, sizes and amounts used for spiking were based on the methods used by Radford *et al.* (2021).[70] Materials used to create the spiking plastics were from common consumer products and consisted of the main six plastic resin codes[57] (Table 2). Each polymer was either sorted into fibres and fragments (PET and PP) or sorted into two size categories (0.25–0.5 mm and 0.5–1 mm) (HDPE, PVC, LDPE and PS). Plastic fragments were sized using a household coffee grinder and sized metal sieves (1 mm, 0.5 mm, 0.25 mm), and fibres were manually cut. The spiking plastics were chosen due their specific characteristic and/or colours to aid straightforward identification when mixed with a sample, and included polymers that could be broadly categorised as high (>1 g cm^{-3}: PET, PVC) and low (<1 g cm^{-3}: HDPE, LDPE, PP, PS) density. The spiking plastic polymer types were confirmed with high matches (>85% for all polymers) using Attenuated Total Reflectance Fourier-Transform Infrared spectroscopy (ATR FTIR) (Frontier, PerkinElmer). Each fishmeal sample was spiked with a total of 60 microplastic particles (five of each type of spiking plastic created).

Fishmeal

Commercial fishmeal samples were bought from online UK suppliers, with focus on collecting fishmeal made from various fish caught from different locations around the world. Fishmeal collected included Norwegian LT94 fishmeal, South American sardine and anchovy fishmeal, Antarctic krill meal, Spanish tuna fishmeal and Scottish salmon fishmeal. Properties of the fishmeal are detailed in Table 4. Protein and oil content of fishmeal was listed on their product specification sheets. The organic matter content was calculated using loss-on-ignition (LOI) at 550 °C and carbonate content was calculated using

Table 2 Spiking plastics used in this method with corresponding resin code, shape (fibre/fragment), size, colour, origin product and density (g/cm3)

Resin code	Abbreviation	Shape	Size (mm)	Colour	Original product	Density[a] (g cm^{-3})
1	PET	Fragment	0.5–1	Blue	Drinks bottle	1.37
		Fibre	1–5	Green	Craft ribbon	
2	HDPE	Fragment	0.25–0.5	Pink	Cleaning product bottle	0.944–0.965
		Fragment	0.5–1			
3	PVC	Fragment	0.25–0.5	Red	Tablecloth	1.38
		Fragment	0.5–1			
4	LDPE	Fragment	0.25–0.5	Purple	Carrier bag	0.917–0.930
		Fragment	0.5–1			
5	PP	Fragment	0.5–1	White	Storage bottle	0.905
		Fibre	1–5	Purple	Carpet	
6	PS	Fragment	0.25–0.5	White	Packaging	0.028–0.045
		Fragment	0.5–1			

[a] Densities of plastics gathered from British Plastics Federation (2020).[58]

Table 3 Summary of five methods used in this study, consisting of two from existing literature[45,46] and three newly developed

Thiele *et al.* (2021)[46] (method 1)	- 40 g fishmeal to 550 ml glass jar - Add NaCl (1.2 g cm^{-3}) (99.5%, Acros Organics) to sample up to a cm (50 ml) from top of 550 ml jar - Add lid and agitate for 30 seconds - Leave for a minimum of 30 minutes Overflow method - Place jar in larger container and remove lid - Slowly pour NaCl into jar to allow supernatant to overflow into container - Rinse outside of jar and inside of lid with pure water into overflow liquid - Repeat overflow three times for each sample, filtering each overflow separately - Filter supernatant through 20–25 µm filter paper and place in Petri dish
NaCl density separation and KOH digestion (method 2)	- 40 g fishmeal to 550 ml glass jar - Add NaCl (1.2 g cm^{-3}) to sample up to a cm (50 ml) from top of 550 ml jar - Add lid and agitate for 30 seconds - Leave for a minimum of 30 minutes - Follow overflow method - Filter supernatant onto 25 µm metal mesh - Place metal mesh in 200 ml 10% KOH (>85%, Fisher Scientific) and heat to 40 °C at 100 rpm for 1 hour - Filter over 20–25 µm filter paper
Dispersant, NaCl density separation and KOH digestion (method 3)	- 40 g fishmeal to glass 550 ml jar - Add NaCl (1.2 g cm^{-3}) and 50 ml dispersant (5% sodium hexametaphosphate) (general purpose grade, Fisher Scientific) to sample up to a cm (50 ml) from top of jar - Add lid and agitate for 30 seconds - Leave for a minimum of 30 minutes - Follow overflow method - Filter supernatant onto 25 µm metal mesh - Place metal mesh in 200 ml 10% KOH and heat to 40 °C at 100 rpm for 1 hour - Filter over 20–25 µm filter paper
Karbalaei *et al.* (2020)[45] (method 4)	- Place 20 g fishmeal sample into 250 ml DURAN glass bottle - Add 200 ml KOH to each sample - Incubate sample at 40 °C for 72 hours - Filter sample over 149 µm filter paper - Place 149 µm filter paper in 10–15 ml NaI (≥99.5%, Sigma-Aldrich) and sonicate for 5 min at 50 Hz by ultrasonic bath - Remove filter papers and repeat sonication process - Centrifuge solution at 500 × *g* for 2 min at room temperature - Filter the supernatant though 8 µm filter paper and place in Petri dish
Dispersant, $CaCl_2$ density separation and KOH digestion (method 5)	- 40 g fishmeal to 550 ml glass jar - Add $CaCl_2$ (1.4 g cm^{-3}) (93%, Fisher Scientific) and 50 ml dispersant (5% sodium hexametaphosphate) to sample up to a cm (50 ml) from top of jar - Add lid and agitate for 30 seconds - Leave for a minimum of 30 minutes - Follow overflow method - Filter supernatant onto 149 µm metal mesh - Place metal mesh in 200 ml 10% KOH and heat to 40 °C at 100 rpm for 1 hour - Filter over 20–25 µm filter paper

LOI at 950 °C. Bulk density of the fishmeal was calculated by weighing 1 cm^3 of dried fishmeal.

Each fishmeal sample was weighed in triplicate according to the amount needed for each method (Table 3). Methods used include those from existing literature[45,46] and new methods based on steps commonly used for other media (density separation (NaCl) with digestion and two density separations (NaCl and $CaCl_2$) with dispersant and digestion), which use environmentally friendly chemicals and solutions, with minimal steps to avoid loss of microplastics.

Method by Thiele *et al.* (2021)[46] (method 1)

Glass jars (550 ml) were used to accurately weigh 40 g of fishmeal in triplicate. NaCl (1.2 g cm^{-3}) was added to the fishmeal

Table 4 Properties of five fishmeal types (Norwegian LT94, South American sardine and anchovy, Antarctic krill, Spanish tuna and Scottish salmon), including organic content (%), carbonate content (%), bulk density (g cm^{-3}), protein content (%) and oil content (%). Protein and oil contents were provided by the respective product specification sheets

Fishmeal	Type of fish used	Organic content (%)	Carbonate content (%)	Bulk density (g cm^{-3})	Protein (%)	Oil[a] (%)
Norwegian LT94	Species unknown, mix of whole fish and trimmings	81.75 ± 0.04	5.47 ± 0.03	0.74 ± 0.01	71	12
S American sardine & anchovy	Whole sardines and anchovies	74.69 ± 0.05	3.419 ± 0.006	0.827 ± 0.007	68	N/A
Antarctic krill	Antarctic krill	87.49 ± 0.01	3.554 ± 0.004	0.47 ± 0.01	56	N/A
Spanish tuna	Whole tuna	77.89 ± 0.23	3.46 ± 0.04	0.69 ± 0.01	60	12
Scottish salmon	Whole salmon	76.49 ± 3.41	5.38 ± 0.58	0.752 ± 0.009	66	9

[a] N/A: not available in fishmeal specification sheet.

in 550 ml jars up to approximately 1 cm (50 ml) from the top, the lid was added, and the jar was shaken for 30 seconds. Thiele *et al.* (2021)[46] stated jars must be left to stand to settle for a minimum of 30 minutes, in the case of this study, samples were left for 24 hours. Once settled, the jar was placed in a larger beaker and lid was removed. NaCl was slowly poured into the jar to allow the supernatant to overflow into beaker. The outside of the jar and the lid was rinsed with pure water into the overflow liquid. This "overflow method" was repeated three times for each sample, filtering each overflow separately. The supernatant was vacuum filtered through 20–25 µm filter paper and stored in a Petri dish for analysis.

NaCl density separation with KOH digestion – (method 2)

This method was created with similarities to the steps used by Thiele *et al.* (2021),[46] to maintain levels of standardisation. 40 g of fishmeal was placed in 550 ml jars in triplicate and NaCl was added up to 1 cm (50 ml) from the top, before being shaken for 30 seconds and left to settle for 24 hours. The overflow method was applied; however, supernatant was filtered on to 25 µm metal filters. The metal filter was placed in glass jars with 200 ml 10% KOH and heated to 40 °C and agitated at 100 rpm for 1 hour. The sample was then vacuum filtered through a 25 µm filter paper and stored in a Petri dish for analysis.

NaCl density separation with dispersant and KOH digestion – (method 3)

This method was followed the same as the density separation with KOH digestion (method 2), with one difference. Before NaCl is added to the sample, 50 ml dispersant (5% sodium hexametaphosphate) was added.

Method by Karbalaei *et al.* (2020)[45] (method 4)

This method was followed as closely as possible to the method reported. Glass jars were used to accurately weigh out 20 g of each fishmeal, in triplicate. Following this, 200 ml of 10% KOH was added to the glass jars, which were then incubated at 40 °C for 72 hours. The contents of the jar were then vacuum filtered through 149 µm metal filters. This metal filter was then placed in 10 ml of 4.4 M sodium iodide (NaI) and sonicated at 50 Hz for 5 minutes, before the filter was removed, and the sonication step was repeated once more. The mixture was centrifuged at 500 × *g* for two minutes before allowing the supernatant to be filtered through an 8 µm filter membrane.

$CaCl_2$ density separation with dispersant and KOH digestion – (method 5)

This method was followed the same as the density separation with dispersant and KOH digestion (method 3), with one difference; the saline solution was changed to a higher density (1.4 g cm^{-3}) solution of calcium chloride. Note the solution was filtered through a larger pore size filter (149 µm) due to the viscosity of the calcium chloride solution.

Calculating spiked plastic recovery rates

Recovered microplastic particles were manually counted under a Nikon SMZ100 microscope (×40 magnification) and percentage of microplastics recovered (recovery rate) was calculated.

Statistics

Statistical analysis was undertaken *via* RStudio (1.3.1093). Distribution of data were shown using histograms and Shapiro–Wilks normality tests. Non-normal distributions were observed in all data sets. Therefore, Kruskal–Wallis tests were used for the recovery rates of microplastics using different methods, and Dunn's test to look for pairwise comparisons between fishmeal types. Kruskal–Wallis tests were used to analyse recovery rates of specific polymers between methods, and to analyse the recovery rates of different size and shape microplastics between methods used, followed by *post hoc* analysis with Dunn's test. Correlations between recovery rate and all four fishmeal properties were estimated using Spearman's rank.

Results

Fishmeal properties

Fishmeal properties measured include organic content (%), carbonate content (%), bulk density (g cm^{-3}), protein (%) and oil (%) (Table 4). Antarctic krill meal had the highest organic

0.05, Kruskal Wallis). Significantly more microplastics were extracted from the sardine and anchovy fishmeal (66.3% (11.6 IQR) RR) than the Norwegian LT94 fishmeal (13.33% (5 IQR) RR) and the Antarctic krill meal (10% (4.2 IQR) RR) ($p < 0.05$, Dunn's Test). Also significantly more microplastics were recovered from the Scottish salmon fishmeal (30% (10.8 IQR) RR) than the Antarctic krill meal using this method ($p < 0.05$, Dunn's Test).

Effect of fishmeal properties on recovery rates

All methods but the method by Karbalaei *et al.* (2020)[45] (method 4) produced strong significant positive correlations between spiked microplastic recovery rates and bulk density ($r_s = 0.71$ (method 1), $r_s = 0.73$ (method 2), $r_s = 0.63$ (method 3), $r_s = 0.75$ (method 5), $p < 0.05$, Spearman's rank) (Fig. 2). The NaCl density separation with added KOH digestion method (method 2), the density separation with dispersant and KOH digestion method (method 3) and the method by Karbalaei *et al.* (2020)[45] (method 4) all had the strongest significant positive correlation between spiked microplastic recovery rate and protein content ($r_s = 0.76$, 0.71, 0.59 (respectively), $p < 0.05$ Spearman's rank) (Fig. 2). These three methods and the method with $CaCl_2$ used as a saline solution (method 5) shared the strongest significant negative correlation between recovery rate and organic content (r_s = -0.52, −0.38, −0.41, −0.89 (respectively), $p < 0.05$ Spearman's rank). Moreover, there was no significant correlation between spiked microplastic recovery rate and organic content when using the NaCl density separation (method 1) ($r_s = -0.46$, $p > 0.05$, Spearman's rank) (Fig. 2).

Recovery of individual polymers

All five methods used recovered significantly different amounts of spiked microplastic polymer types ($p < 0.05$ for all, Kruskal Wallis) (Fig. 3). The NaCl density separation method (method 1) extracted significantly more low-density polymers such as HDPE (48% RR), LDPE (56.7% RR) and PS (42.7% RR) than high-density polymers such as PET (4.7% RR) and PVC (0.7% RR) ($p < 0.05$ for all, Dunn's test). This method also extracted significantly more LDPE than PP (28.7% RR) (<0.05, Dunn's test).

The methods with added KOH digestion (method 2) and added dispersant (method 3) recovered significantly more low-density polymers [such as HDPE (KOH: 57.3% RR, dispersant: 70.7% RR), LDPE (KOH: 60% RR, dispersant: 75.3% RR), PP (KOH: 32% RR, dispersant: 44.7% RR) and PS (KOH: 41.3%, dispersant: 50.7% RR)] than high-density PET [(KOH: 4% RR, dispersant: 6% RR) and PVC (KOH: 2.7% RR, dispersant: 2% RR)] ($p < 0.05$, Dunn's test).

The method by Karbalaei *et al.* (2020)[45] (method 4) recovered significantly more low-density polymers [such as HDPE (14.7% RR), LDPE (32.7% RR), PP (7.3% RR) and PS (10.7% RR)] than high-density PET (0.7% RR) ($p < 0.05$ Dunn's test). However, this method only found significantly more low-density HDPE and LDPE than high-density PVC (4% RR) (<0.05, Dunn's test). This method also recovered significantly more LDPE polymers than any other polymer ($p < 0.05$, Dunn's test).

The method with an increased density saline solution of calcium chloride, a dispersant and a KOH digestion (method 5) also recovered significantly more low-density polymers of HDPE

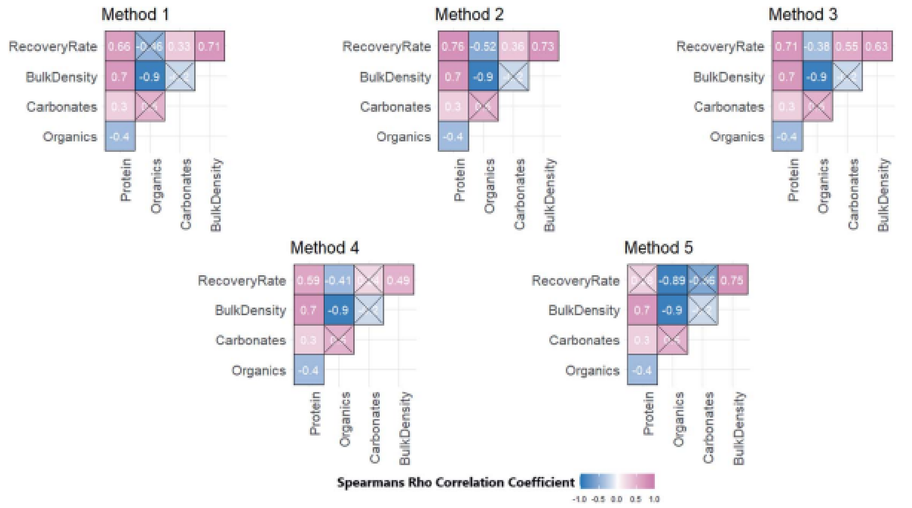

Fig. 2 Correlogram showing Spearman Rho correlation coefficients between fishmeal properties (organic content, carbonate content, protein content and bulk density) and spiked microplastic recovery rate. −1 indicates strong negative correlation, +1 indicates strong positive correlation. Squares including a black cross represent those correlations with no significance ($p > 0.05$). The five methods include: NaCl density separation (method 1), NaCl density separation followed by a KOH digestion (method 2), NaCl density separation with sodium hexametaphosphate dispersant followed by KOH digestion (method 3), a previously published method by Karbalaei *et al.* (2020)[45] (method 4) and a calcium chloride density separation with sodium hexametaphosphate dispersant followed by KOH digestion (method 5).

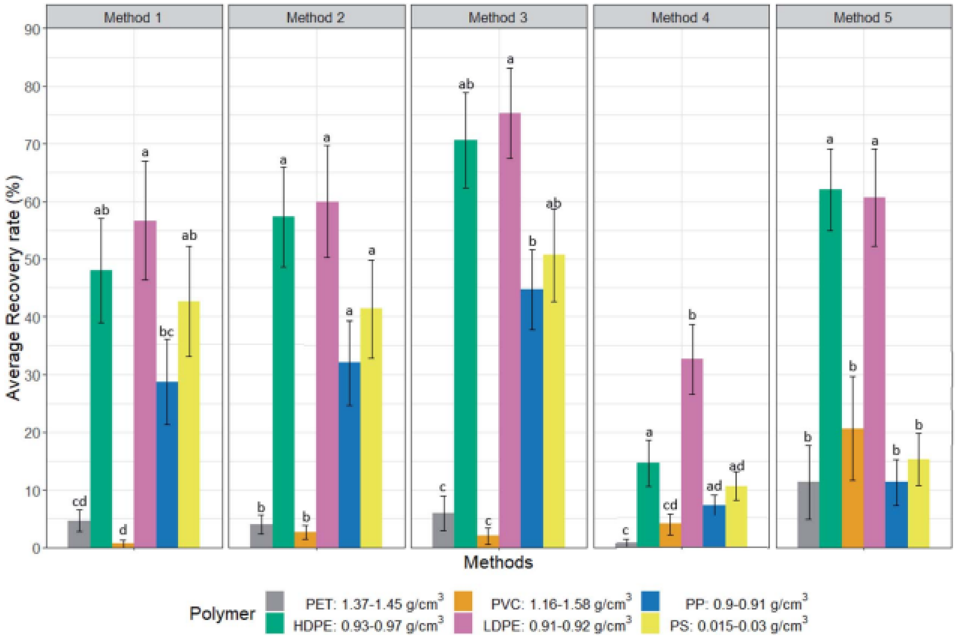

Fig. 3 Average recovery rates (%) of 6 common microplastic polymers (first six plastic resin codes), extracted from fishmeal, using five separation/digestion methods used in existing literature (NaCl density separation (method 1), NaCl separation with a KOH digestion (method 2), NaCl separation with sodium hexametaphosphate dispersant followed by KOH digestion (method 3), a previously published method by Karbalaei *et al.* (2020)[45] (method 4) and a calcium chloride density separation with sodium hexametaphosphate dispersant followed by KOH digestion (method 5)). Error bars represent standard error of the mean. Bars with different letter notations within each method are significantly different (Dunn's test, $p < 0.05$).

(62% RR) and LDPE (60.6% RR) than the higher density polymers of PET (11.3% RR) and PVC (20.6% RR) ($p < 0.05$, Dunn's test). However, polystyrene (15.3% RR), which has the lowest density, was recovered significantly less than the other low-density polymers of LDPE and HDPE ($p < 0.05$, Dunn's test). This method also recovered the highest amount of the high-density polymers such as PET and PVC compared to the other four methods, with recovery rates of 11.3% and 20.6% respectively (Fig. 3).

Individual polymer properties

All methods that include a NaCl (methods 1, 2 and 3) or a $CaCl_2$ density separation (method 5) recovered significantly more big (0.5–1 mm) microplastics (41.7%, 42%, 51.3%, 47% RR respectively) than the method by Karbalaei *et al.* (2020)[45] (14.3% RR) ($p < 0.05$, Dunn's test) (Fig. 4A). These four methods also recovered significantly more fragments (RR = method 1: 32%, method 2: 34.8%, method 3: 43%, method 5: 31.3%) than the method by Karbalaei *et al.* (2020)[45] (method 4) (RR = 10.7%) ($p < 0.05$, Dunn's test for both) (Fig. 4B).

However, method 4 (Karbalaei *et al.* 2020) recovered on average more small (0.25–0.5 mm) microplastics (16.7% RR) than big microplastics (14.3% RR) which is an opposite trends to all other methods which recovered more big microplastics than small.

Discussion

When investigating microplastics in a new medium, it is paramount to understand the properties of the medium and whether these will have an effect on extraction of plastic particles. Here, we measured four properties of five commercially available types of fishmeal and subjected them to five different methods to establish recovery rate of spiked microplastics. We found the method of $CaCl_2$ density separation with dispersant and KOH digestion recovered the most microplastics in the sardine and anchovy fishmeal. However, the NaCl density separation with dispersant and a KOH digestion stage recovered the most microplastics from the four other fishmeal types. Moreover, the organic content of fishmeal was found to be negatively correlated with microplastic recovery rate. Overall, recovery rates varied across fishmeal types when using the same method (Fig. 1), suggesting that the properties of the fishmeal could influence the amount of microplastics recovered. In addition, recovery rates were also low (0–66.3%), suggesting a potential for general underestimation of microplastics reported in fishmeal literature.

Sodium chloride density separation has been used as a method to separate microplastics from a matrix for a long time.[59] More recently, it has been utilised to recover microplastics from fishmeal. Thiele *et al.* (2021)[46] used a NaCl density

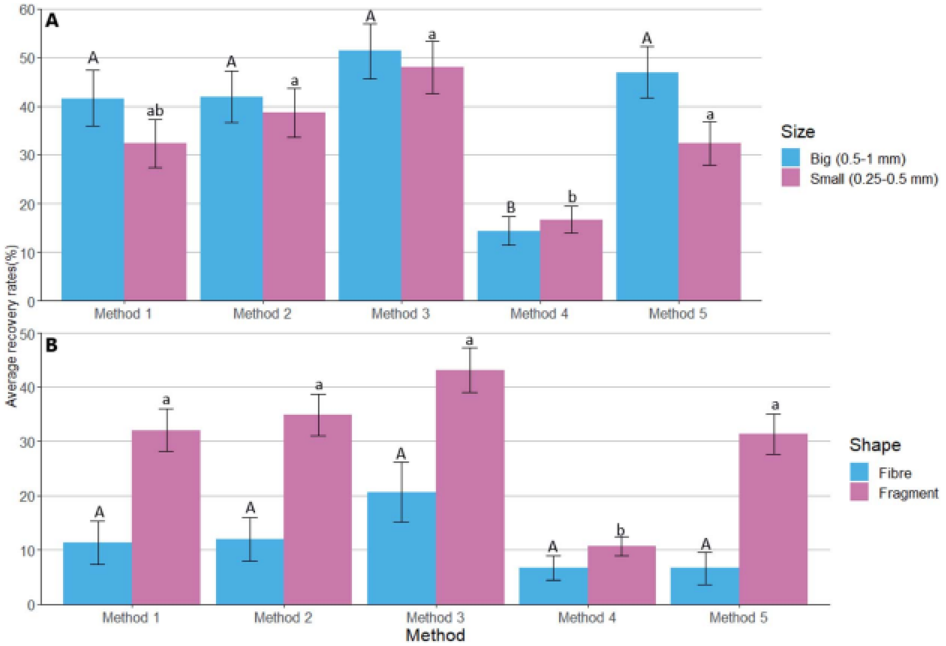

Fig. 4 Average recovery rate (%) of big (0.5–1 mm) (A), small (0.25–0.5 mm) (A), fibres(B) and fragments (B) spiked microplastics extracted from fishmeal, using five different methods (NaCl density separation (method 1), NaCl separation with a KOH digestion (method 2), NaCl separation with sodium hexametaphosphate dispersant followed by KOH digestion (method 3), a previously published method by Karbalaei *et al.* (2020)[45] (method 4) and a calcium chloride density separation with sodium hexametaphosphate dispersant followed by KOH digestion (method 5)). Bars with different letter notations are significantly different (Dunn's test, $p < 0.05$), different case of letters represents different tests in each plot.

separation 'Overflow' method (Table 3) to extract microplastics from two fishmeal types. They found a recovery rate of 49.3 ± 1.2% in sardine and anchovy fishmeal, whereas this study found 33.3% recovery rate with the same fishmeal type (but obtained from a different source). This difference in recovery rate suggests there is a variability in the same fishmeal when manufactured in different places, or that the fish is sourced from different locations. This in turn may influence the effectiveness of the method. The study by Thiele *et al.* (2021)[46] used different spiking polymers consisting of PS, PP, PET, PA and rayon, which have different densities than the polymers used in this study (PET, HDPE, LDPE, PVC, PS and PP), making it difficult to compare recovery rates. Sodium chloride is frequently used when studying microplastics. For example, Hanvey *et al.* (2017)[60] compared studies looking into microplastics in sediments, and almost half (19/43) used NaCl as a saline solution. Similarly, a meta-analysis looking into recovery rate studies by Way *et al.* (2022)[61] found that 16 out of the 71 studies included used NaCl, which was the most frequently used reagent in the analysis. Using NaCl as a density separation is also recommended by the Marine Strategy Framework Directive (MSFD).[62] There are several reasons as to why this method is widely used and accepted: ease of use, affordability, and its non-toxic properties (Table 1). Although the studies which use zinc chloride ($ZnCl_2$)[63] and NaI[64] have found high recovery rates (95.5–100% and >98% respectively), the use of the more expensive and hazardous saline solutions involve multiple steps to reduce sample mass, allowing for less of the solution to be used.[40] Moreover, many studies do not use these higher-density, expensive saline solutions at the highest density the salt can reach at 20 °C (Table 1), suggesting that it is much more economically viable to use the lower-density, lower expense saline solutions. For these reasons, this study used and developed methods with NaCl over other more expensive and toxic reagents such as $ZnCl_2$ and NaI, in order to encourage replication and standardisation from others.

This study combined NaCl with KOH to facilitate digestion and found recovery rates of between 5% and 48.3%, depending on the fishmeal type. Many studies have reported KOH an effective digestion reagent, which depending on the incubation temperature, it can have little effect on the polymer properties. For example, Karami *et al.* (2017)[16] found that using KOH at 40 °C had no effect on the polymers and was effective at digesting fish tissues. Thiele *et al.* (2021)[46] trialled the use of KOH in recovering microplastics and found fishmeal that was digested in 10% KOH was not filterable through 25 µm filter papers. This study used KOH to digest residual fishmeal after density separation with 5% sodium hexametaphosphate as a dispersant, allowing for easier filtration. This proved to be an effective method in extracting the spiked microplastics with recovery rates between 15% and 60%. Other studies have used a pollutant themselves, this balance is something all microplastic researchers should consider when developing a method they hope to be universally accepted.

Conclusions

Fishmeal is a globally important feed in aquaculture and agriculture. Consequently, microplastic presence in fishmeal is concerning and analytical methodologies are emerging. This study highlights the variability of fishmeal media, the complexity this brings when attempting to extract microplastics, and the importance of using environmentally conscious and affordable methods.

We recommend using a dispersant with NaCl density separation and a KOH digestion; and analysing the fishmeal properties: lower recoveries may be anticipated from fishmeal types with higher organic and lower protein content. This method is of low cost and is environmentally friendly, which is a balance we argue should become an international standard approach for researchers to allow for a method that is widely accepted (philosophically and scientifically) and easy to replicate. The low recovery rates found in this study highlight the possibility of variable underestimation of microplastics being reported in fishmeal. This is an issue that probably applies to other complex media and must also be accounted for if the method is used for microplastic extraction in the future.

Conflicts of interest

The authors receive no third-party funding for this related work and have no affiliation to the fishmeal or food industry.

Credit author statement

Chloe Way: conceptualisation, methodology, validation, formal analysis, investigation, data curation, writing-original draft, visualisation. Malcolm Hudson: supervision, project administration, funding acquisition, writing-review and editing. Ian Williams: supervision, writing-review and editing. John Langley: supervision, writing-review and editing. Robert Marsh: supervision, writing-review and editing.

Data availability

Data supporting this study are openly available from the University of Southampton repository at: https://doi.org/10.5258/SOTON/D2102

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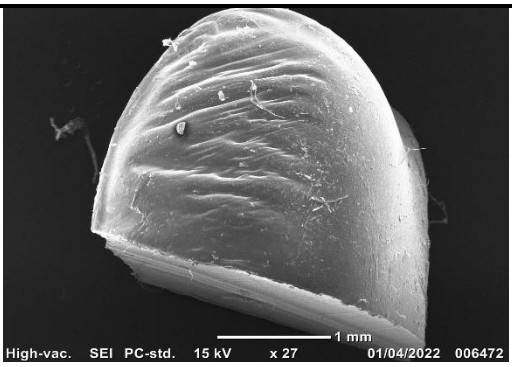
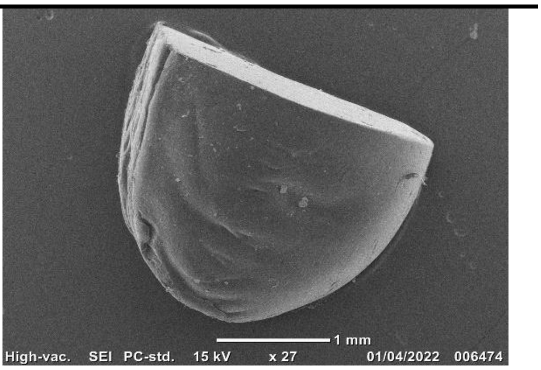
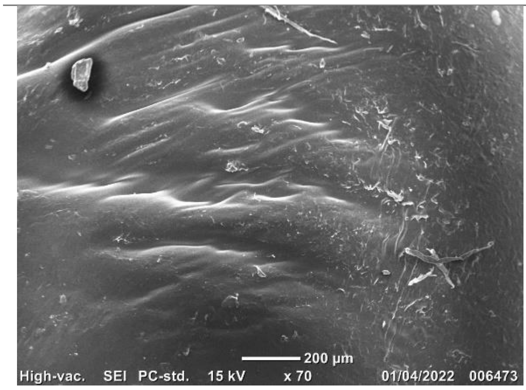
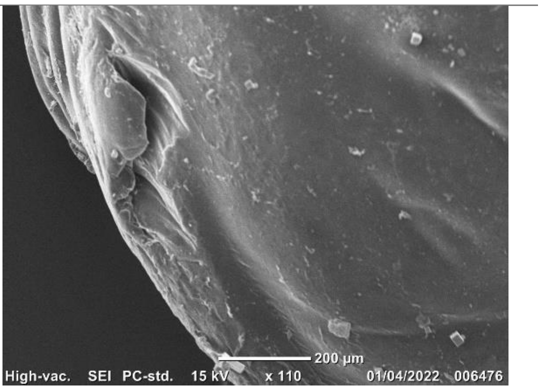
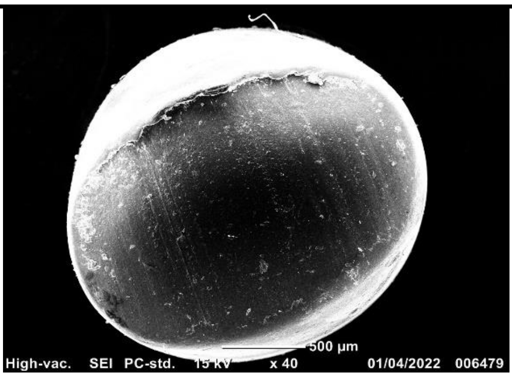
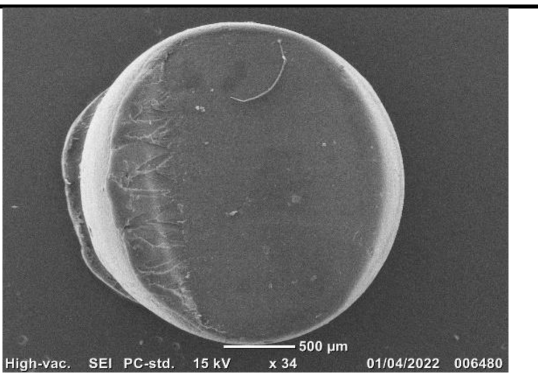
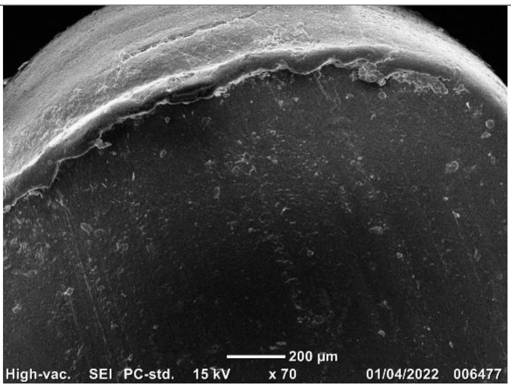
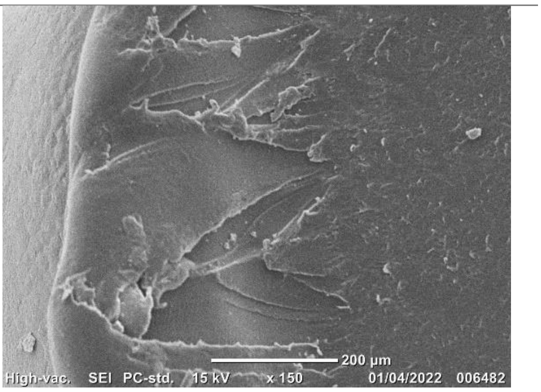
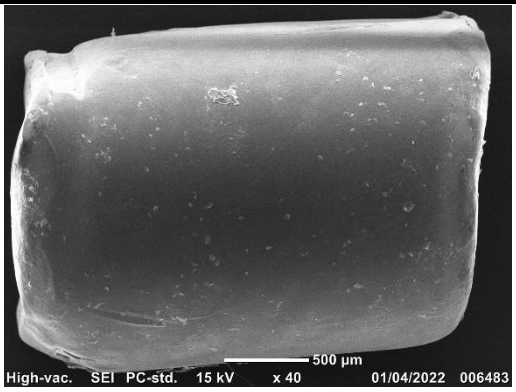
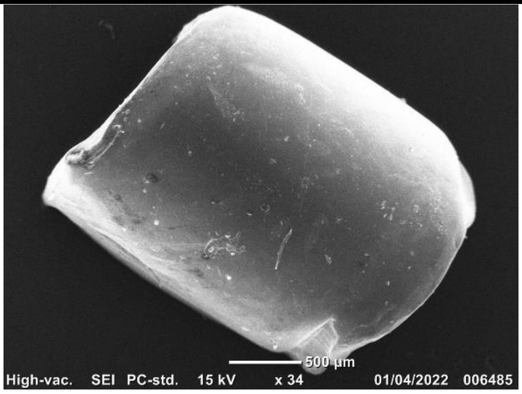
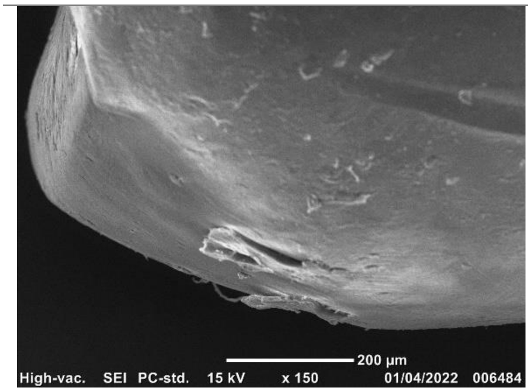
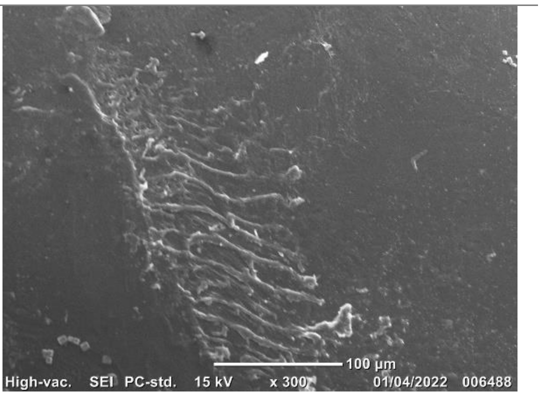
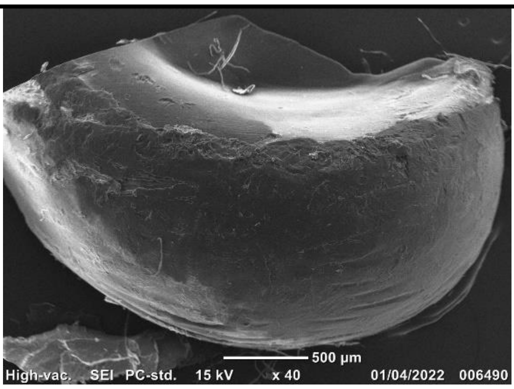
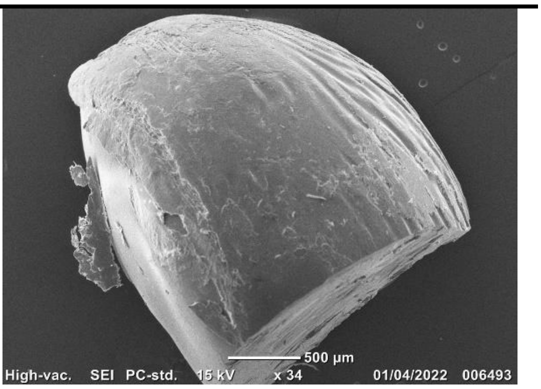
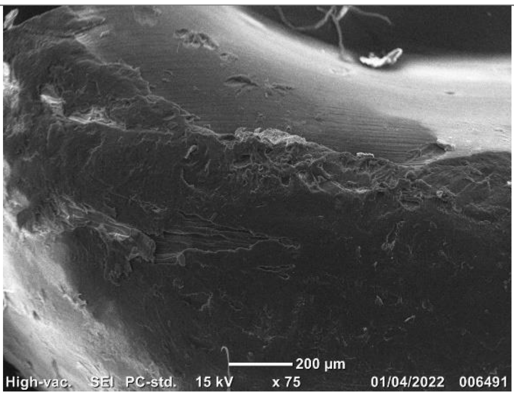
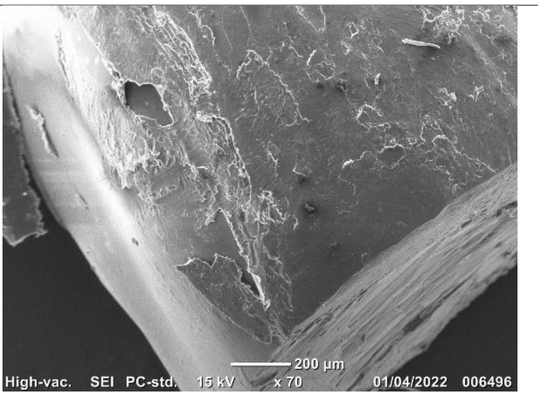
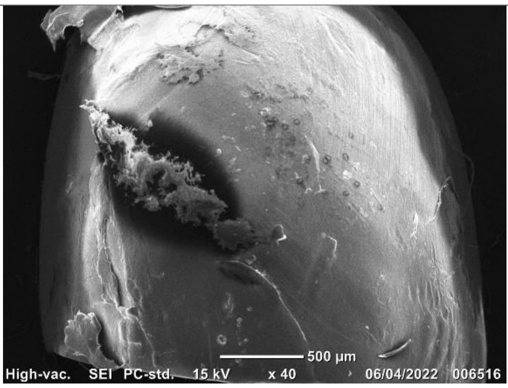
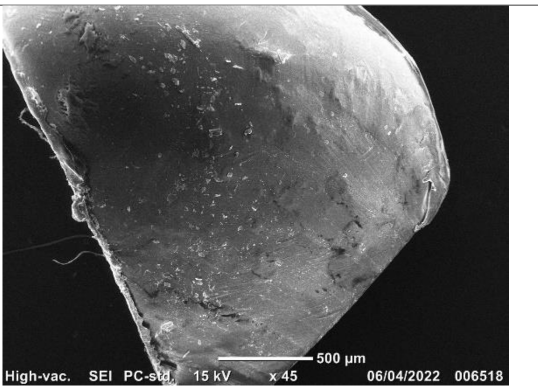
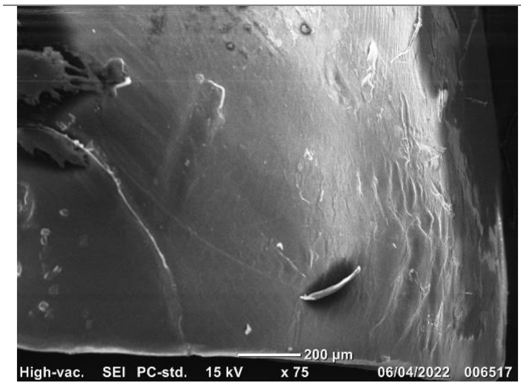
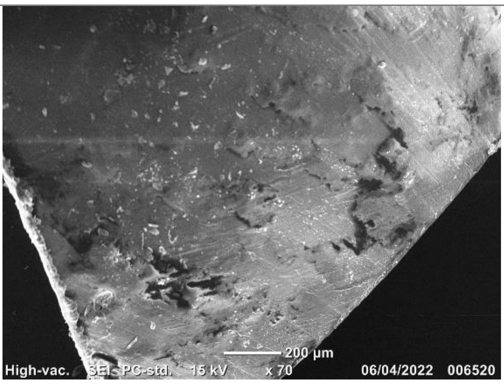
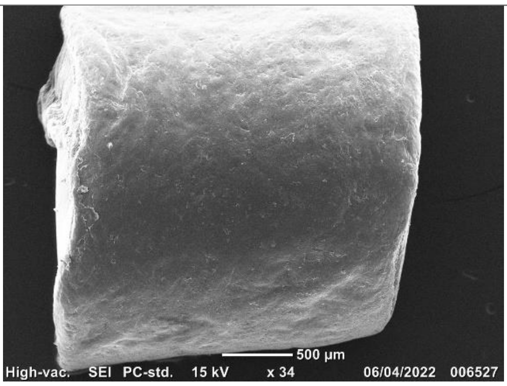
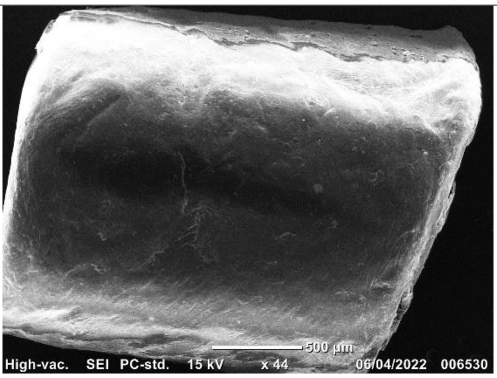
Appendix C Chapter 4 Supplementary material

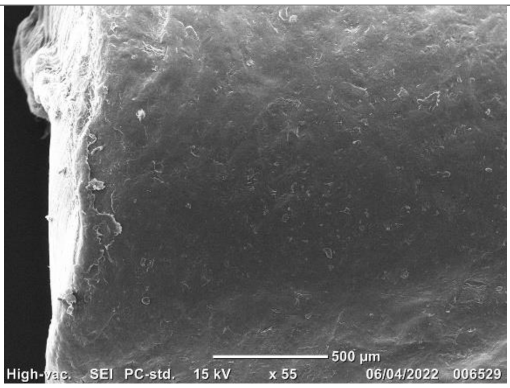
Appendix. C.1 SEM images of microplastics

Appendix. C.1 SEM images of the standard virgin microplastics before and after being artificially weathered for 1 month.

Polymer	Standard	Weathered
LDPE		
		
PA		

	200 µm High-vac. SEI PC-std. 15 kV x 70 01/04/2022 006477	200 µm High-vac. SEI PC-std. 15 kV x 150 01/04/2022 006482
PC	500 µm High-vac. SEI PC-std. 15 kV x 40 01/04/2022 006483	500 µm High-vac. SEI PC-std. 15 kV x 34 01/04/2022 006485
	200 µm High-vac. SEI PC-std. 15 kV x 150 01/04/2022 006484	100 µm High-vac. SEI PC-std. 15 kV x 300 01/04/2022 006488
PS	500 µm High-vac. SEI PC-std. 15 kV x 40 01/04/2022 006490	500 µm High-vac. SEI PC-std. 15 kV x 34 01/04/2022 006493

	200 µm High-vac. SEI PC-std. 15 kV x 75 01/04/2022 006491	200 µm High-vac. SEI PC-std. 15 kV x 70 01/04/2022 006496
HDPE	500 µm High-vac. SEI PC-std. 15 kV x 40 06/04/2022 006516	500 µm High-vac. SEI PC-std. 15 kV x 45 06/04/2022 006518
	200 µm High-vac. SEI PC-std. 15 kV x 75 06/04/2022 006517	200 µm High-vac. SEI PC-std. 15 kV x 70 06/04/2022 006520
PET	500 µm High-vac. SEI PC-std. 15 kV x 34 06/04/2022 006527	500 µm High-vac. SEI PC-std. 15 kV x 44 06/04/2022 006530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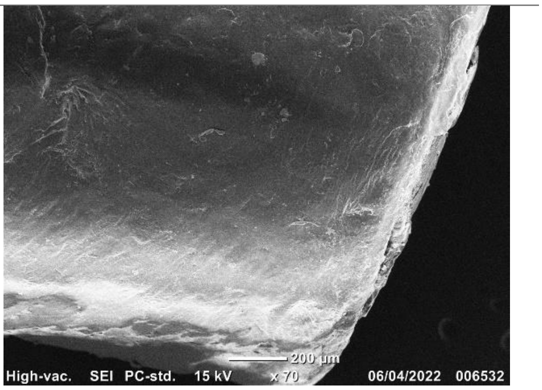
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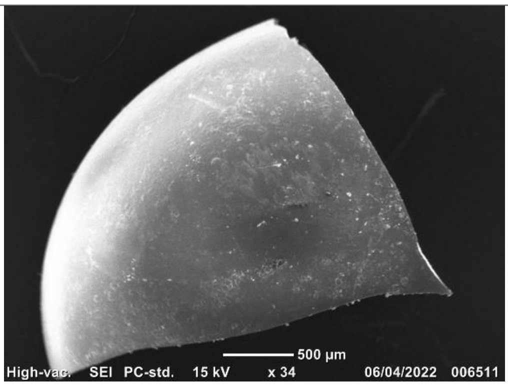
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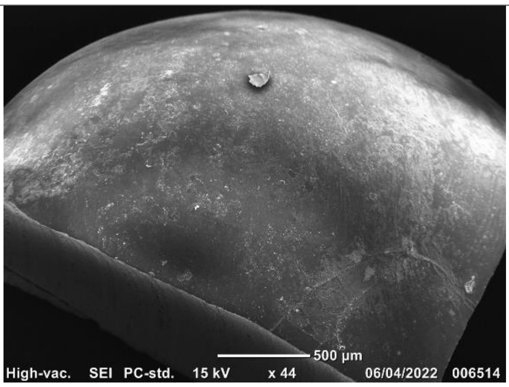
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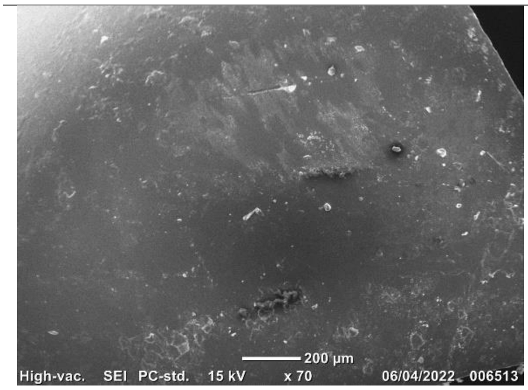
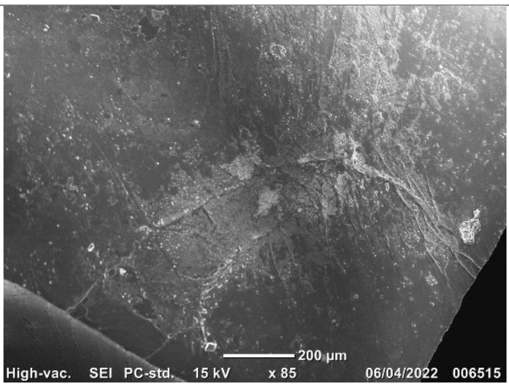
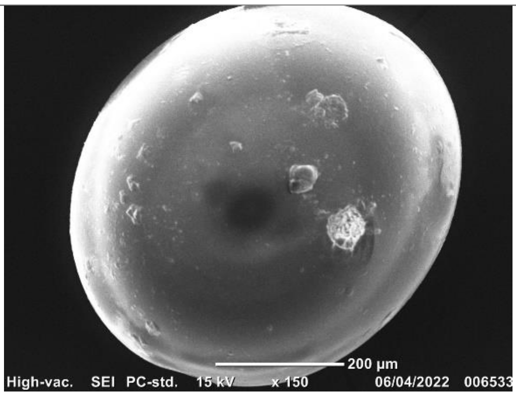
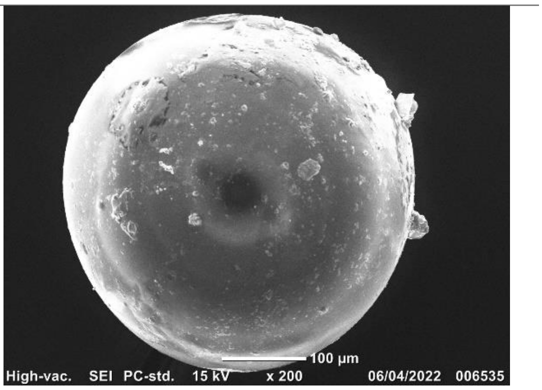
High-vac. SEI PC-std. 15 kV x 44 06/04/2022 006514

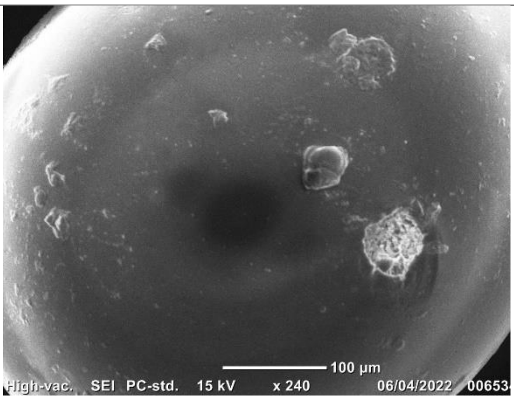
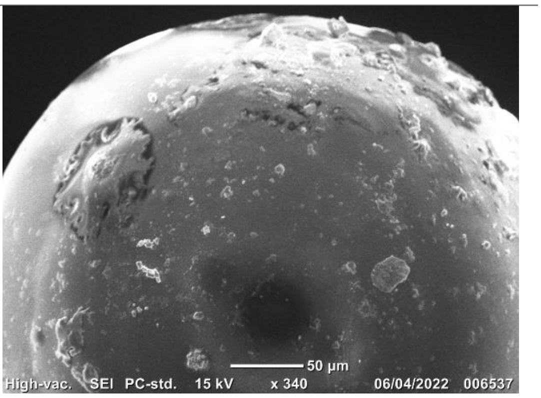
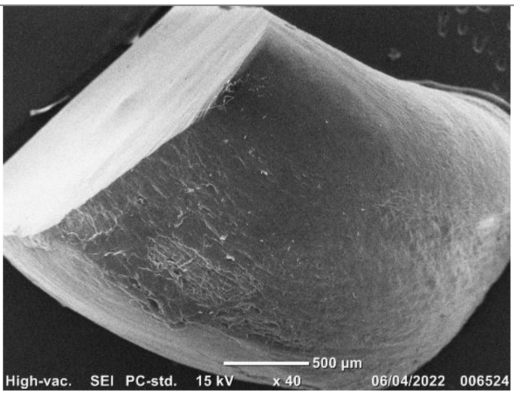
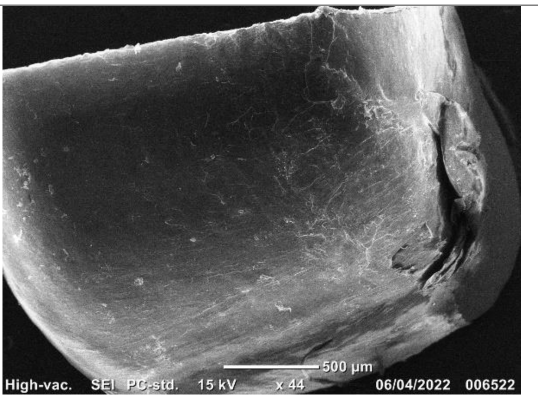
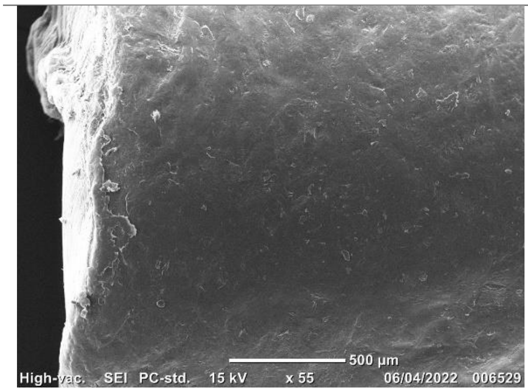
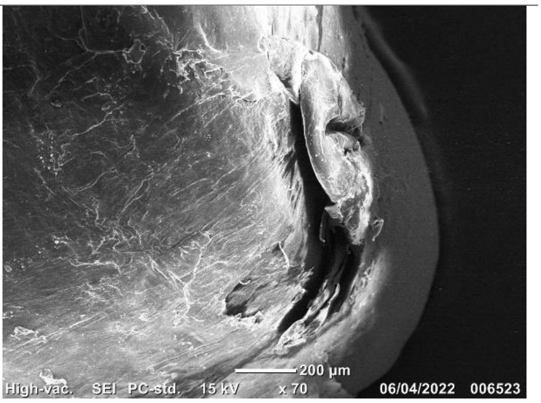

200 µm
High-vac. SEI PC-std. 15 kV x 70 06/04/2022 006513

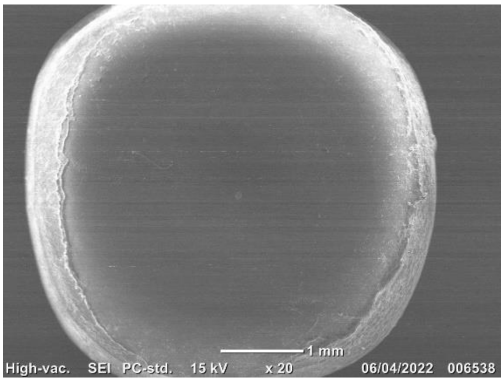
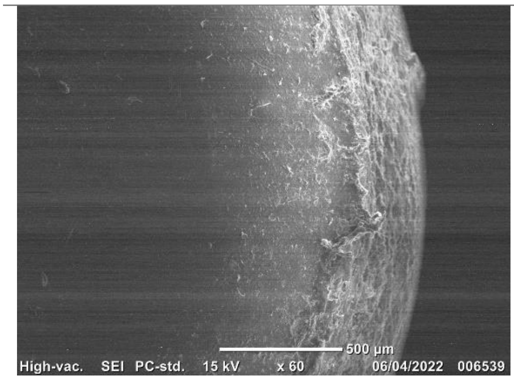

200 µm
High-vac. SEI PC-std. 15 kV x 85 06/04/2022 006515

PMMA


200 µm
High-vac. SEI PC-std. 15 kV x 150 06/04/2022 006533


100 µm
High-vac. SEI PC-std. 15 kV x 200 06/04/2022 006535

	100 µm High-vac. SEI PC-std. 15 kV x 240 06/04/2022 00653	50 µm High-vac. SEI PC-std. 15 kV x 340 06/04/2022 006537
PP	500 µm High-vac. SEI PC-std. 15 kV x 40 06/04/2022 006524	500 µm High-vac. SEI PC-std. 15 kV x 44 06/04/2022 006522
	500 µm High-vac. SEI PC-std. 15 kV x 55 06/04/2022 006529	200 µm High-vac. SEI PC-std. 15 kV x 70 06/04/2022 006523
PVDC	1 mm	

	 1 mm	 1 mm
Nurdle		 1 mm High-vac. SEI PC-std. 15 kV x 20 06/04/2022 006538  500 µm High-vac. SEI PC-std. 15 kV x 60 06/04/2022 006539

Appendix. C.2 Chemical indicator markers

Appendix.C.2. Chemical indicator markers found from each sample preparation followed by TD GC-MS of each microplastic (virgin, artificially weathered and naturally weathered) and their corresponding m/z ions, similarity (%) and retention time. Greyed out boxes indicate no chemical markers found. Chemical markers in bold are those recommended to be used to identify the plastic.

Virgin Microplastics				
Liquid Extraction				
Polymer	**Chemical indicator marker**	***m/z***	**Similarity (%)**	**Retention time**
PS	**Styrene**	**104, 78, 51**	**91**	**5:27.61**
PP				
LDPE				
HDPE				
PET				
PU	Butylated hydroxytoluene Methyl methacrylate	57, 145, 205, 220 41, 69, 100	88 96	10: 18.77 3:41.11
PA				
PC				
PVDC				
PMMA	**Methyl methacrylate** Propanenitrile, 2,2’-azobis [2-methyl-	**100, 69, 41** 41, 54, 69	**96.3** 94.6	**3:41.71** 6:46.29
Liquid Extraction with Sonication				
PS	**Styrene** Methylethyl benzene Cyclotetrasiloxane	**104, 78, 51** 51,77,105,120 133,207,281	**97** 95 88	**5:20.39** 5:40.89 6:18.39

	Nitroxide	29,41,57	75	9:11.08
	Hexadecanoic acid	29,43,56,69,73,83,29	75	14:33.86
	Cyclohexane	91,117,194,207	91	15:02.15
	(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans-	91,115,129,207	83	15:30.65
PP	Decane	43,57,71,84	84	8:00.98
	Tetrahydrofuran	27, 42, 72	79	14:33.26
LDPE	Dodecane	29, 43, 57, 71, 85	92	8:00.58
	Dioxolane	42, 55, 71	80	19:30.14
HDPE	Decane	43, 57, 71, 84	68	8:01.48
	Tridecene	29,41,55,69,83,97	71	10:38.27
PET	Dodecane	29,43,57,71,85	92	8:00.68
PU	Tetrahydrofuran	27, 42, 72	81	15:24.55
	Butylated hydroxytoluene	57,145,205,220	80	10:15.17
	Octene	29,41,55,70,83,112	72	14:38.06
PA	Dodecane	29, 43, 57, 71, 85	92	8:00.78
PC	Isopropylidenediphenol	119	84	13:50.36
	Chlorobenzene	51, 77, 112	92	4:52.49
PVDC	Methyl methacrylate	100, 69, 41	87	3:44.90
	Hexachlorocyclohexane	38, 51, 61, 75, 85, 96, 111, 121, 145, 156, 181, 219, 254	61	9:31.87
	Chloroundecane	29,41,55,69,83,91	86	9:55.27
	Trifluoroacetoxypentadecane	41,51,55,69,83,97,111	78	10:21.77
PMMA	**Methyl methacrylate**	**41, 59,69,100**	**78**	**3:23.70**
	Propanenitrile	27,41,54,69	93	6:40.79
No Sample Preparation – Direct Thermal Desorption				

PS	**Styrene**	**51, 78, 104**	**97**	**4:13.90**
	Benzene, 1,1'- (1,2-cyclobutanediyl) bis-, trans-	78, 104	91	10:51.57
PP	Dodecane	29,43,57,71,85	74	5:32.49
	Heptadecane	43, 57, 71, 85	76	7:13.29
	2,4,6, - trimethyl decane	43, 57, 71, 85	79	5:52.89
	hexadecanol	31,41,55,69,83,97,111	93	13:00.46
LDPE	1-Decanol, 2-hexyl-	29,43,57,69,83,97,111	84	10:09.07
	Carbamic acid	43,91,134,220	53?	5:59.39
	Decane, 6-ethyl-2-methyl-	43,57,71,85	65	8:12.18
	Phenol, 2,4,6-tris(1-methylethyl)-	205,220	84	8:40,28
	Octadecane	28,43,57,71,85,98	88	9:47.18
HDPE	Dodecene	29,43,55,69,83,97	95	6:36.09
	Tetradecene	29,41,55,69,83,97,111	94	8:12.18
	Octadecene	29,43,55,69,83,97,111	96	9:48.28
	Eicosene	**29,43,55,69,83,97,111**	**97**	**12:26.87**
PET	Nonanal	29,41,57,70,82,98	88	5:51.69
	Decanal	29,43,57,70,82,95,112	92	6:39.69
	Propenoic acid	**43,55,69,73,83,97**	**76**	**10:26.77**
PU	Butylated hydroxytoluene	57, 145, 205, 220	94	9:10.78
	Eicosene	29,43,55,69,83,97,111	94	10:10.17
PA	**Caprolactam**	**30, 42, 55, 84, 113**	**93**	**7:16.58**
PC	Chlorobenzene	51, 77, 112	89	4:03.60
	Diphenyl carbonate	**39, 51, 65, 77, 94, 141, 170, 214**	**80**	**10:34.97**
PVDC	Chloroundecane	29, 41, 55, 69, 83, 91, 105	87	8:49.68
	2-Ethyl hexanol	29,41,57,70,83,98	90	5:14.79
PMMA	Tetramethyl Butane Dinitrile	41, 54, 69	97	5:18.79
	Methyl 4-(methylthio)butyrate	15,27,41, 45, 59,69, 74, 87, 101, 117, 148	62	6:52.49

Artificially Weathered				
Solvent Extraction				
Polymer	**Chemical indicator/Marker**	***m/z***	**Similarity (%)**	**Retention time**
PS	Cyclotrisiloxane, hexamethyl-	96, 207	90	4:44.39
	Styrene	**51,78,104**	**97**	**5:16.09**
	Cyclotetrasiloxane, octamethyl-	133,207,281	86	6:18.89
	Dichlorobenzene	75, 111, 146	96	6:33.39
	Cyclopentasiloxane, decamethyl-	73,267,355	88	7:36.58
	Decanal	29,43,57,70,82,95,112	72	8:08.18
	Benzene, 1,1'- (1,2-cyclobutanediyl) bis-, trans-	78,104	88	12:15.97
	(2,3-Diphenylcyclopropyl) methyl phenyl sulfoxide, trans-	91,105,129,206	72	16:18.85
PP	Cyclotrisiloxane, hexamethyl-	96, 207	93	4:42.39
	Cyclotetrasiloxane, octamethyl-	133,207,281	88	6:19.09
	Cyclopentasiloxane, decamethyl-	73,267,355	91	7:36.68
	Decanal	29,43,57,70,82,95,112	76	8:08.38
LDPE	Cyclotrisiloxane, hexamethyl-	96, 207	91	4:44.99
	Cyclotetrasiloxane, octamethyl-	133,207,281	87	6:18.79
HDPE	Cyclotrisiloxane, hexamethyl-	96, 207	88	4:44.39
	Cyclotetrasiloxane, octamethyl-	133,207,281	88	6:19.69
	Cyclopentasiloxane, decamethyl-	73,267,355	91	7:36.88
	Decanal	29,43,57,70,82,95,112	73	8:08.68
PET	Cyclotrisiloxane, hexamethyl-	96, 207	89	4:44.19
	Cyclotetrasiloxane, octamethyl-	133,207,281	85	6:19.29
	Nonanal	29,41,57,70,82,98	89	7:19.38
	Cyclopentasiloxane, decamethyl-	73,267,355	91	7:36.68
	Decanal	29,43,57,70,82,95,112	78	8:07.78

PU	Cyclotrisiloxane, hexamethyl- 2H-1,2,3,4-Tetrazole-2-ethanol, α-(chloromethyl)-5-phenyl- **1,4-Butanediol** Cyclotetrasiloxane, octamethyl- Nonanal Decanal	96, 207 39,43,51,63,77,104,131 27,31,42,57,71 **133,207,281** 29,41,57,70,82,98 29,43,57,70,82,95,112	86 66 93 **64** 96 91	4:44.79 5:28.29 5:56.09 **6:17.09** 7:17.58 8:05.78
PA	Cyclotrisiloxane, hexamethyl- Cyclotetrasiloxane, octamethyl- Nonanal Cyclopentasiloxane, decamethyl- Decanal	96, 207 133,207,281 29,41,57,70,82 73,267,355 29,43,57,70,82,95,112	87 86 92 90 87	4:45.39 6:18.19 7:18.58 7:36.68 8:08.78
PC	Cyclotrisiloxane, hexamethyl- Cyclotetrasiloxane, octamethyl- Nonanal Cyclopentasiloxane, decamethyl- Decanal	96, 207 133,207,281 29,41,57,70,82,98 73,267,355 29,43,57,70,82,95,112	74 85 924 91 94	4:45.29 6:19.29 7:19.18 7:36.47 8:06.78
PVDC	Cyclotrisiloxane, hexamethyl- Cyclotetrasiloxane, octamethyl- Nonanal Cyclopentasiloxane, decamethyl- Decanal	96, 207 133,207,281 29,41,57,70,82,98 73,267,355 29,43,57,70,82,95,112	90 87 89 91 79	4:43.89 6:19.29 7:18.68 7:36.58 8:08.28
PMMA	**Methyl methacrylate** Cyclotrisiloxane, hexamethyl- Cyclotetrasiloxane, octamethyl- Tetramethyl butanedinitrile Decanal Propionic acid	**41,59,69,100** 96, 207 133,207,281 41,54,69 29,43,57,70,82,95,112 43,55,59,74,89,102,131,176,253	**79** 93 86 93 93 59	**3:46.40** 4:44.59 6:19.69 6:40.59 8:06.08 8:37.68
Solvent Extraction with Sonication				

PS	Cyclotrisiloxane, hexamethyl-	96, 207	93	4:42.49
	Styrene	**51,78,104**	**96**	**5:15.69**
	Cyclotetrasiloxane, octamethyl-	133,207,281	86	6:19.19
	Dichlorobenzene	50,75,111,146	95	6:33.19
	Cyclopentasiloxane, decamethyl-	73,267,355	88	7:35.98
	Thiocarbamic acid, N, N-dimethyl, S-1,3-diphenyl-2-butenyl ester	42,51,72,77,91,105,115,129,178,191,207	61	16:16.65
PP	Cyclotrisiloxane, hexamethyl-	96, 207	89	4:41.79
	Cyclotetrasiloxane, octamethyl-	133,207,281	89	6:18.69
	Cyclopentasiloxane, decamethyl-	73,267,355	92	7:36.08
LDPE	Cyclotrisiloxane, hexamethyl-	96, 207	88	4:44.69
	Cyclotetrasiloxane, octamethyl-	133,207,281	87	6:18.69
	Cyclopentasiloxane, decamethyl-	73,267,355	90	7:36.58
HDPE	Cyclotrisiloxane, hexamethyl-	96, 207	86	4:42.79
	Cyclotetrasiloxane, octamethyl-	133,207,281	89	6:18.79
	Cyclopentasiloxane, decamethyl-	73,267,355	91	7:36.58
PET	Cyclotrisiloxane, hexamethyl-	96, 207	88	4:43.89
	Cyclotetrasiloxane, octamethyl-	133,207,281	87	6:18.99
	Cyclopentasiloxane, decamethyl-	73,267,355	88	7:36.78
PU	**1,4-Butanediol**	**27,31,42,57,71**	**93**	**5:54.19**
	Phenol	39,66,94	93	6:15.29
	Ethylenediamine	30	82	7:35.28
PA	Cyclotrisiloxane, hexamethyl-	96, 207	69	4:41.99
	Cyclotetrasiloxane, octamethyl-	133,207,281	88	6:18.89
	Cyclopentasiloxane, decamethyl-	73,267,355	91	7:36.38
PC	Cyclotrisiloxane, hexamethyl-	96, 207	65	4:45.19
	Chlorobenzene	51,77,112	80	4:56.49
	Cyclotetrasiloxane, octamethyl-	133,207,281	88	6:18.99
	Cyclopentasiloxane, decamethyl-	73,267,355	92	7:36.78

PVDC	Cyclotrisiloxane, hexamethyl-	96, 207	90	4:43.09
	Cyclotetrasiloxane, octamethyl-	133,207,281	89	6:18.69
	Cyclopentasiloxane, decamethyl-	73,267,355	92	7:36.58
	Cyclopropane,1-methyl-2-octyl	29,41,56,69,83,97	95	7:58.68
	Hexasiloxane, tetradecamethyl	73,147,221,267,281,355	70	11:44.37
PMMA	**Methyl methacrylate**	**41,59,69,100**	**79**	**3:47.70**
	Cyclotrisiloxane, hexamethyl-	96, 207	88	4:43.79
	Cyclotetrasiloxane, octamethyl-	133,207,281	87	6:18.99
	Tetramethyl butanedinitrile	41,54,69	92	6:40.59
	Cyclopentasiloxane, decamethyl-	73,267,355	91	7:36.38
No Sample Preparation – Direct Thermal Desorption				
PS	Ethylbenzene	51,65,91,106	80	4:01.10
	Styrene	**51,78,104**	**97**	**4:14.70**
	Benzaldehyde	51,77,106	81	4:47.49
	Octanal	29,43,56,69,84,100	84	5:01.59
	Nonanal	29,41,57,70,82,98	95	5:50.79
	Decanal	29,43,57,70,82,95,112	95	6:39.19
	Diphenyl ether	51,77,141,170	72	8:17.08
	Naphthalene, 1,2,3,4-tetrahydro-1-phenyl-	39,51,65,76,91,104,115,130,165,180,208	87	10:58.07
PP	Hexanoic acid	27,41,45,55,60,73,87	87	4:51.09
	Nonanal	29,41,57,70,82,98	95	5:51.29
	Decanal	29,43,57,70,82,95,112	88	6:38.89
	Nonanoic acid	29,41,60,69,73,87,98,115,129	93	8:18.98
LDPE	1-Pentanol, 3,4-dimethyl-	27,31,43,55,70,83	74	3:26.90
	Butanoic acid	27,41,60,73	80	4:01.40
	Pyrrolidine, 1-[2-(4-bromophenoxy) ethyl]-	84	90	4:26.40
	Hexanoic acid	27,41,45,55,60,73,87	85	4:51.39
	Heptanoic acid	27,41,55,60,73,87	79	5:39.89
	Nonanal	**29,41,57,70,82,98**	**86**	**5:51.59**
	Decanal	**29,43,57,70,82,95,112**	**71**	**6:39.19**

	Nonanoic acid	29,41,60,69,73,87,98,115,129	70	8:27.08
	Phthalic acid, di(oct-3-yl) ester	149,167	78	13:15.36
HDPE	Hexanoic acid	27,41,45,55,60,73,87	96	4:49.89
	Nonanal	29,41,57,70,82,98	83	5:51.59
	Decanal	29,43,57,70,82,95,112	94	6:38.49
	3-Tetradecene	29,41,55,69,83,97,111	83	8:09.08
	5-Octadecene	29,43,55,69,83,97,111	95	9:43.58
	E-15-Heptadecanal	43,55,69,83,97,111,125	96	11:04.87
	Cyclohexane, 1,3,5-triphenyl-	91,117,194,207	911	11:38.47
PET	Heptanol	29,41,55,70,81,86,96	80	4:12.30
	Hexanoic acid	27,41,45,55,60,73,87	97	4:49.09
	Octanal	29,43,56,69,84,100	86	5:00.79
	Nonanal	29,41,57,70,82,98	96	5:49.99
	Decanal	29,43,57,70,82,95,112	87	6:38.49
	2-Propenoic acid, tridecyl ester	**43,55,69,73,83,97**	**76**	**7:08.89**
	Nonanoic acid	29,41,60,69,73,87,98,115,129	94	8:12.98
PU	Heptanol	29,41,55,70,81,86,96	80	4:13.10
	Hexanoic acid	27,41,45,55,60,73,87	90	4:48.29
	Octanal	29,43,56,69,84,100	96	5:01.59
	1-Hexanol,2-ethyl-	29,41,57,70,83,98	94	5:14.89
	Nonanal	29,41,57,70,82,98	96	5:50.79
	Decanal	29,43,57,70,82,95,112	84	6:37.69
	Nonanoic acid	29,41,60,69,73,87,98,115,129	94	8:12.18
	Butylated Hydroxytoluene	57,145,205,220	70	11:45.47
	Cyclohexane, 1,3,5-triphenyl-	91,117,194,207	69	11:43.97
	Carbonic acid, octadecyl vinyl ester	43,57,71,85,97,111	87	12:22.17
PA	Butanoic acid	27,41,60,73	80	4:00.60
	Pyrrolidine, 1-[2-(4-bromophenoxy) ethyl]-	84	90	4:26.40
	Heptanoic acid	27,41,55,60,73,87	78	5:39.09
	Nonanal	29,41,57,70,82,98	90	5:50.79

	Decanal	29,43,57,70,82,95,112	71	6:38.49
	Tetradecanoic acid	29,43,55,60,69,73,83,87,97,115,129,143,171,185,228	88	11:29.57
	Hexadecanoic acid	29,43,55,60,69,73,83,87,97,129,256	92	13:12.16
	Pentacosane	43,57,71,85,99,113	87	13:39.56
PC	Chlorobenzene	51,77,112	89	3:56.70
	Octanal	29,43,56,69,84,100	74	5:01.59
	1-Hexanol, 2-ethyl-	29,41,57,70,83,98	86	5:14.09
	Heptanoic acid	27,41,55,60,73,87	77	5:35.89
	Nonanal	29,41,57,70,82,98	95	5:49.99
	Decanal	29,43,57,70,82,95,112	97	6:39.19
	Vinyl lauryl ether	29,43,57,69,83,97	70	10:20.87
	Diphenyl carbonate	**39,51,65,77,94,141,170,214**	**81**	**10:28.97**
PVDC	Hexanoic acid	27,41,45,55,60,73,87	96	4:49.29
	Octanal	29,43,56,69,84,100	95	5:00.99
	Dichlorobenzene	**50,75,111,146**	**97**	**5:11.09**
	1-Hexanol,2-ethyl-	29,41,57,70,83,98	77	5:15.09
	Nonanal	29,41,57,70,82,98	96	5:50.99
	Decanal	29,43,57,70,82,95,112	93	6:39.39
	Nonanoic acid	29,41,60,69,73,87,98,115,129	76	8:18.18
	Decane, 1-chloro-	29,43,57,69,83,91,105	90	8:49.38
	Hexadecanoic acid	29,43,55,60,69,73,83,87,97,129,256	87	13:15.86
	Hydrogen chloride	**36**	**69**	**11:58.47**
PMMA	Heptanol	29,41,55,70,81,86,96	84	4:13.90
	Hexanoic acid	27,41,45,55,60,73,87	97	4:49.89
	Octanal	29,43,56,69,84,100	94	5:02.39
	Tetramethyl butanedinitrile	41,54,69	95	5:17.99
	Nonanal	29,41,57,70,82,98	96	5:50.79
	Propionic acid, 3-(butylthio)-, methyl ester	29,41,45,55,61,77,88,103,116,120,133,145,176	56	6:52.49
	Nonanoic acid	29,41,60,69,73,87,98,115,129	94	8:21.58

	Butyric acid, 4-phenyl-, tetradecyl ester	43,57,71,83,91,97,104,117,146,164	61	10:38.27
	Tetra decanoic acid	29,43,55,60,73,83,87,97,115,129,143,171,185,228	83	11:26.77

Naturally Weathered				
Liquid Extraction				
Polymer	**Chemical indicator/Marker**	***m/z***	**Similarity (%)**	**Retention time**
HDPE	Cyclotrisiloxane, hexamethyl-	96, 207	88	4:41.49
	Cyclotetrasiloxane, octamethyl-	133,207,281	86	6:19.19
	Cyclopentasiloxane, decamethyl-	73,267,355	91	7:36.28
Liquid Extraction with Sonication				
HDPE	Butane, 2,3-dimethyl-2-nitro-	18,30,41,53,69,84	63	3:47.90
	Cyclotrisiloxane, hexamethyl-	96, 207	93	4:43.59
	Cyclotetrasiloxane, octamethyl-	133,207,281	87	6:18.69
	Cyclopentasiloxane, decamethyl-	73,267,355	91	7:36.58
No Sample Preparation – Direct Thermal Desorption				
HDPE	Octanal	29,43,56,69,84,100	87	5:02.09
	1-Hexanol,2-ethyl-	29,41,57,70,83,98	88	5:15.59
	Nonanal	29,41,57,70,82,98	93	5:50.59
	Decanol	29,43,57,70,82,95,112	96	6:38.39
	Pentadecane	29,43,57,71,85	96	8:11.28
	Heptadecane	29,43,57,71,85,99	92	12:19.67
	Eicosane,1-iodo	43,57,71,85,99,113	84	13:58.06

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