The Distribution of Microplastics in Salt Marshes

by

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Abstract

Microplastics are becoming widely recognized as an increasing pollutant, found in all studied ecosystems with potential impacts to fauna, flora, and the general environment. However, salt marsh ecosystems remain largely understudied. This thesis presents a series of projects designed to measure microplastic abundance in two European salt marshes, whilst studying the spatial and temporal distribution of microplastics, and the factors affecting these patterns.

Firstly, method development was carried out to determine the most effective procedures for salt marsh sediments. The consequent methodology incorporates extraction using sediment-microplastic isolation (SMI) units, digestion using KOH: NaClO, identification using Nile Red staining, and analysis using micro-FTIR spectroscopy.

Secondly, sediment samples from Blakeney Point salt marsh were collected to study abundance and spatial distribution of microplastics. Microplastics were present in all samples from the marsh, averaging 21,216.68 ± 2,259.54 particles/m^2 (3,429.94 ± 387.82 particles/kg d.w.). Vegetation zonation was observed to impact distribution, with samples in the Lower/Mid zone having over twice as many microplastics as any other zone. However, when considering the factors vegetation height, elevation and distance from the salt marsh edge, only vegetation height was found to have a significant correlation with microplastic abundance, and none were found to have any observable trends with the distribution of microplastics.

Lastly, sediment samples from a salt marsh in the Wadden Sea were used to determine temporal variability of microplastics over the past 60 years. Values found ranged from 8,486.56 – 74,257.43 particles/m^2 (472.44 – 9,615.39 particles/kg d.w.), and 1,414.43 – 20,509.19 particles/m^2 (160.64 – 1,836.16 particles/kg d.w.), with a general trend of microplastics decreasing with depth and time. However, unexpected values were found at certain depths in each core, perhaps relating to changes in vegetation coverage. A significant relationship for microplastics and sediments was found, based on their shared size fractions. Despite this, end-member determined sediment depositional processes could not be used to explain microplastic abundance.

Overall, microplastics were present in all samples from both salt marshes. Vegetation was found to have a key role in microplastic distribution, with vegetation zonation having impacts both spatially and temporally.

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Chapter 1 - Introduction

The discovery of plastics began back in the late 1800s, with natural polymers being incorporated into various works. However, in 1907 Leo Baekeland invented Bakelite, the first fully synthetic plastic as he then dubbed it (American Chemical Society National Historic Chemical Landmarks, 2024; Edgar D, 2009). Following the mass industrialisation of plastics in the 1950s (Thompson *et al.,* 2004), numerous different plastics now exist, each with its own unique properties that allow for a widespread series of applications such as packaging, construction and textiles (British Plastics Federation, 2024). Plastic production is only continuing to increase, with the estimated 400 million tons of plastic produced in 2020 (UN Environment Programme, 2021) expected to almost double again by 2040 (Napper and Thompson, 2020). Due to their durability, versatility and low cost to produce, plastics are in widespread demand around the world. However, these very same properties are allowing plastics to persist long after their usage, and so plastic pollution is now recognised as a long-term global issue (Chamas *et al.,* 2020). Plastics have the potential to inflict various hazards, including both geophysical and biological impacts (MacLeod *et al.,* 2021), thus long-term exposure to plastics can lead to numerous negative impacts for both organisms and ecosystems. Whilst plastic pollution monitoring and clean-up processes are being implements around the world, these typically focus on larger plastics that are more visible and easier to handle. It is only recently that attention has begun to shift towards the smaller fraction of plastics and the potential threats that they may pose (Napper and Thompson, 2020).

1.1 Microplastics

An emerging global pollutant, microplastics are broadly defined as synthetic polymer pieces which are in the size range of 1 μm – 5 mm, though the lower limit is still often discussed (*Proceedings of the International Research Workshop on the Occurrence, Effects, and Fate of Microplastic Marine Debris*, 2009; Thompson *et al.,* 2004). The presence of plastics on this scale was first observed back in the 1970s (Carpenter and Smith, 1972), however it was not till the early 2000s that microplastics were classified as a hazard themselves. Since then,

microplastics have rapidly garnered interest over the past two decades and have become a major environmental issue that is now the focus of intense research (Galloway *et al.,* 2017).

Microplastics can be split into two main classifications based on their source: primary and secondary microplastics (Figure 1.1).

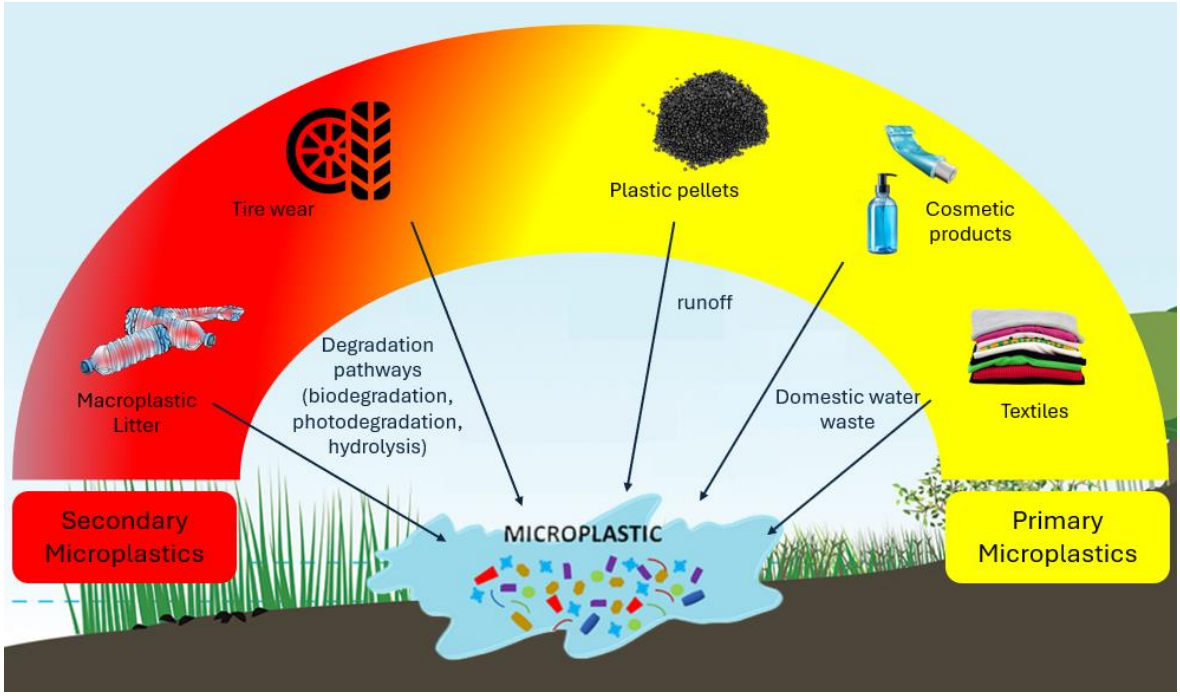

Figure 1.1 - Primary and secondary microplastics with examples of sources and pathways to the environment, adapted from Borah et al. (2023).

Primary microplastics are those which are synthetically manufactured already at that scale. Commonly produced through grinding or extrusion, they are used either in the manufacturing of other products (Turner and Holmes, 2015), or directly as products themselves (Bergmann *et al.,* 2015). Some of the most widely occurring primary microplastics include fibres from polymer-based clothes and other textiles (De Falco *et al.,* 2019), and beads and fragments used in cosmetic and cleaning products (Browne, 2015).

Secondary microplastics occur when larger plastic pieces (macro and mesoplastics) breakdown through either use or natural degradation (Hale *et al.,* 2020). This is a constant process which results in the formation of ever smaller microplastics and even nanoplastics (Lambert and Wagner, 2016). There are numerous types of degradation pathways which plastics can undergo: biodegradation, photodegradation, thermooxidative degradation,

thermal degradation and hydrolysis, as well as physical degradation process such as abrasion and shearing. Furthermore, tire wear has recently become recognised as a significant source of microplastics (Knight *et al.,* 2020), formed from the friction between tire tread and road surface. Tire particle emissions were estimated around 6 million tonnes a year, comprising 5-10% of all microplastics that end up in marine environments (Jan Kole *et al.,* 2017). With an estimated 117.3 million tonnes of macroplastic reaching the ocean since plastic production began (García Rellán *et al.,* 2023), this is a huge source of potential microplastics. Their ability to form from any plastic litter makes secondary microplastics the most prominent form of plastic pollution in marine environments (Strand and Bioscience, n.d.).

Microplastics can also be classified by their polymer type and morphology. The most common microplastics reflect their parent polymers, and are polyethylene (PE), polypropylene (PP) and polystyrene (PS) (Andrady, 2011). However, due to the breakdown of larger fragments, microplastics have been found for nearly all chemical polymers, including polyethylene terephthalate (PET), polyacrylamide (PA), polyvinyl chloride (PVC), polyurethane (PU) and many others (Burns and Boxall, 2018).

Considering microplastic morphologies, they can be generally separated into five different shape categories (Table 1.1) (Joint Research Centre (European Commission), 2023). Microplastic fibres/filaments are released from synthetic clothes during washing machine cycles (Frost *et al.,* 2022) or are shed from degraded rope and fishing gear (Napper *et al.,* 2022; Wright *et al.,* 2021). Fibres are the most reported microplastic morphology in both marine and sedimentary based studies (Salvador Cesa *et al.,* 2017; Uddin *et al.,* 2021; Welden and Cowie 2017). Fragments are irregularly shaped microplastics occurring from the abrasion of larger plastics (Mohamed Nor and Obbard 2014), and are found in high amounts in both water (29%) and sediment (35%) studies (Burns and Boxall 2018). The other three main morphologies are films; occurring from thinner plastic items such as poly bags (Tziourrou *et al.,* 2021), pellets/beads; mostly manufactured for various cosmetic products (Napper *et al.,* 2015) and foams (Burns and Boxall 2018). However, several classifications are given in different studies, including, filaments, granules (Hidalgo-Ruz *et al.,* 2012), and rods (Hartmann *et al.,* 2019). This variation in terminology makes comparing different studies challenging (Rosal, 2021), however these five classifications are now being

more widely accepted and incorporated into ongoing studies (Joint Research Centre (European Commission), 2023).

Table 1.1 Morphology classifications of Microplastics (Joint Research Centre (European Commission), 2023)

Morphology class	Definition
Filaments	Slender thread-like microlitter particles, that is, it also covers fibres and threads
Fragments	Irregularly shaped hard microlitter particles with broken-off edges that may be rounded or angular
Films	Microlitter particles derived from sheets or thin films
Foams	Flexible microlitter particles in which material cells are all o partly intercommunicating (a), including expanded polystyrene (EPS) and extruded polystyrene (XPS) foams
Pellets/granules/beads	Microlitter particles that are spherical, flat on one side or cylindrical in shape

Microplastics are now recognised as a global pollutant, found in marine, freshwater, terrestrial and even aerial systems around the world (Shahul Hamid *et al.,* 2018). Studies have shown microplastics present on every continent, as well as every ocean studied (see Figure 1.2). Whilst there is a clear bias towards certain countries conducting more studies, microplastics have nonetheless been found in every location which has tested for them including remote locations such as the Antarctic (Aves *et al.,* 2022; Waller *et al.,* 2017), Arctic Sea ice (Peeken *et al.,* 2018), the peak of Mount Everest (Napper *et al.,* 2020), and at the bottom of ocean trenches (Abel *et al.,* 2021; Jamieson *et al.,* 2019). While many studies only report trace amounts, it is predicted that microplastic contamination will only continue to expand in the future (Isobe *et al.,* 2019).

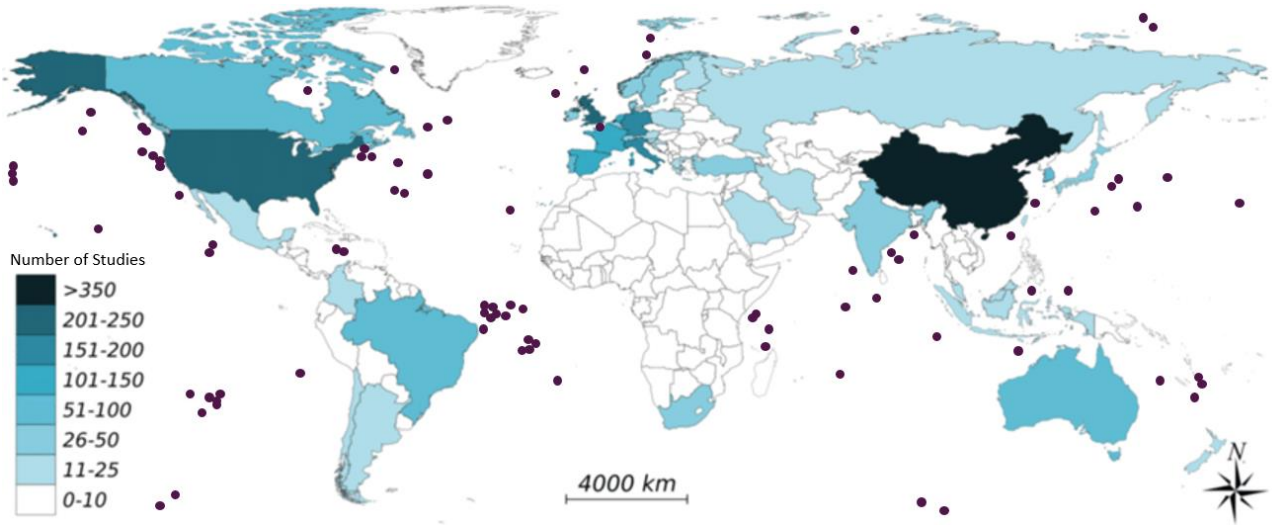

Figure 1.2 - Global distribution of microplastic studies, showing geographical differences, dots show individual ocean-based studies (adapted from Can-Güven, 2021; Mutuku *et al.,* 2024).

Microplastics have various entry points into the environment, including agricultural and urban runoff, domestic and industrial wastewater, litter, sewage treatment outputs and degradation of already present plastic (Park and Park, 2021). Water systems provide excellent transport pathways, with rivers carrying an estimated 80% of the microplastics that reach the sea (Lebreton *et al.,* 2017; Ockelford *et al.,* 2020), where they can then be further distributed around the globe (Rochman, 2018). Whilst waterways lead to the horizontal transport of microplastics around the world, they are also vertically distributed within aqueous systems, found throughout the water column from the surface waters to the deepest benthic layers (Van Cauwenberghe *et al.,* 2013). Whilst much plastic is buoyant, both the physical mechanisms of degradation and biological mechanisms such as biofouling (the accumulation of a layer/film of organic matter on the surface of a particle (Kaiser *et al.,* 2017) can alter these properties and lead to the sinking of microplastics (Kowalski *et al.,* 2016). These particles will eventually deposit in the sediment, where they may become permanently trapped (Rochman, 2018). Despite aqueous ecosystems being a key focus of microplastic research, much about the occurrence and distribution of microplastics in these environments remains unknown and so further research is needed to fully understand the impact microplastics are having (Bhatt *et al.,* 2021; Park and Park, 2021).

1.2 Microplastics in Sediments

The water column is estimated to hold only 8% of environmental microplastics, with over 90% predicted to be found within sediments (Booth *et al.,* 2019). Although different polymers have varying densities (Table 1.2), most have higher densities than freshwater (1 g/cm^3) and saltwater (1.06 g/cm^3) and so will settle under non-turbulent conditions (Yuan *et al.,* 2023). Whilst some particles will resuspend (Xia *et al.,* 2021), and others are mobile within the soil through processes such as bioturbation (Näkki *et al.,* 2017), most microplastics within the sediment are trapped and so accumulate over time. Bottom sediments are therefore potential sinks for microplastics and represent a significant portion of the global microplastic population (Uddin *et al.,* 2021).

Table 1.2 Densities of microplastics and microfibres (synthetic and natural) (Preston-Whyte *et al.,* 2021)

Microplastic and Microfibres	Density (g/cm^3)	Behaviour
Polypropylene (PP)	0.9	Float
Low density polyethylene (LDPE)	0.95	
High density polyethylene (HDPE)	0.95	
Freshwater	1	
Polystyrene (PS)	1.05	
Seawater (salinity 35g/kg, 25°C, pressure = 1000)	1.06	
Nylon 66, nylon 6	1.14	Sink
Polyamide (PA)	1.16	
Acrylic	1.19	
Polycarbonate (PC)	1.2	
Wool	1.3	
Polyvinyl chloride (PVC)	1.3-1.45	
Polyester	1.39	
Cellophane	1.42	
Regenerated cellulose	1.44	
Natural cellulose	1.5	
Viscose rayon	1.52	
Cotton	1.55	

Once in the sediment plastics will breakdown at varying rates, depending on their polymer structure but also the environmental conditions they are exposed to. Exact lifetimes for plastics in sediments can be hard to determine, however Chamas et al. (2020) estimated half-lives ranging from 58 – 1200 years for HDPE in the natural environment. With such longevity, microplastics could remain in the sediment for several decades. Microplastics therefore have a geological record and could perhaps be used as indicators and tracers by which to study the effects of the Anthropocene within sediments (Stubbins *et al.,* 2021; Zalasiewicz *et al.,* 2016). However, for this a proper understanding of the depth profiles of microplastics in different sediments is required as much remains unknown (Uddin *et al.,* 2021).

1.3 The Effects of Microplastics

1.3.1 Microplastic Effects on Fauna

The widespread occurrence and high residence time of microplastics within the environment, coupled with their small size, makes them widely available to marine organisms at numerous different trophic levels (Moore, 2008). Since microplastics can be similar in size to plankton or marine litter, they are often mistaken for food particles and ingested by a variety of species. This was first noticed by (Carpenter *et al.,* 1972) who reported the ingestion of polystyrene spheres in eight different fish species. Since then, microplastics have been found in the digestive tracks of a whole host of species, including birds (Hoang and Mitten, 2022; Mallory, 2008), fish and crustaceans (Bakir *et al.,* 2020), turtles (Mascarenhas *et al.,* 2004), plankton (Lin, 2016) and even large marine mammals (Besseling *et al.,* 2015; Lusher *et al.,* 2015a) and urban pets (Prata *et al.,* 2022).

Since microplastics cannot be broken down by digestive enzymes (Andrady, 2011), they are either excreted or collect within the digestive tract of the organism (Wright *et al.,* 2013). The process of ingestion and accumulation can lead to various negative effects for different species. In smaller species, the main danger is microplastics blocking the digestive tract or filling the stomach leading to false satiation. This leads to reduced energy levels within the organism and can even result in starvation (Cole *et al.,* 2011; Derraik, 2002; Galloway *et al.,* 2017). This can also lead to reduced feeding rates, which then further causes lower growth

rates and malnutrition (Welden and Cowie, 2017), and even reduced reproduction rates (Cole *et al.,* 2015). In larger species such as shellfish and mussels, severe inflammation of glands, gills and liver has been observed (Lu *et al.,* 2016; von Moos *et al.,* 2012; P. Yu *et al.,* 2018), whilst in cetaceans microplastics have been linked to acute injuries and increased pollutant loads resulting in higher risk of mortality (Baulch and Perry, 2014). Whilst the majority of microplastic studies show predominantly non-lethal effects (Galloway *et al.,* 2017), increasing levels of microplastics around the world may see these impacts continue to grow. Furthermore, microplastics represent an additional stressor for many organisms, which when combined with other environmental pressures may have enhanced negative impacts (Ferreira *et al.,* 2016; Silva *et al.,* 2022).

Another potential concern surrounding microplastics, is their indirect ingestion through trophic transfer (Farrell and Nelson, 2013). When prey organisms are consumed, microplastics within their digestive tracts are then transferred to the predator, where they begin to accumulate. This has been shown to occur in small organisms like crabs (Watts *et al.,* 2014), and is further suggested for larger fish and mammals (Nelms *et al.,* 2018). This transfer has also been identified as a potential pathway to humans via trophic consumption (Van Cauwenberghe and Janssen, 2014). There are several other pathways by which people may consume microplastics, including particles on food from plastic packaging, microplastics in drinking water, and the direct inhalation of particles from the air (Walker *et al.,* 2022). Whilst the expected amount of ingested microplastics is low, research is still ongoing around this and so the potential effects on people are currently not fully understood.

The direct effects of microplastic consumption on organisms is a relatively new area of study, however there are prominent concerns about effects caused by toxic components adhered to the surface of the plastic. Microplastics have been shown to be vectors for persistent organic pollutants (POPs) (IOC, 2010) which have demonstrated harmful effects in organisms. Another potentially toxic component of microplastics are chemical additives. These are mixed in with the plastic matrix during synthesis or processing and can provide a variety of chemical properties such as colour, luminescence, fire retardation and increased durability. Often these are not directly bonded to the polymer matrix, and so when degradation occurs, these additives can leach out into the environment where they can

pose a potential threat (Hermabessiere *et al.,* 2017). Some additives are chemically inert; however, the most common additives are phthalates, bisphenol A, and brominated flame retardants, all of which have been labelled as hazardous to the environment. Combining these direct and indirect harmful effects could lead to widespread ecological disruptions, which along with their irreversible contamination and global ubiquity, would qualify microplastics as a chemical pollution planetary boundary threat (Hale *et al.,* 2020).

Finally, microplastics have the capacity to act as vectors for viruses and pathogens. Several studies have recorded the presence of viruses on plastics, including the bacterial fish pathogen *Aeromonas salmonicida* (Viršek *et al.,* 2017), *Vibrio spp.* and *Escherichia coli* (Rodrigues *et al.,* 2019). Various pathogens have been found in microplastics around the world, demonstrating this vector effect. This raised concerns around microplastics being able to transport harmful pathogens between different environments and potentially bring non-native viruses to new habitats (Walker *et al.,* 2022).

1.3.2 Microplastic Effects on Soil and Flora

The effects of microplastics are not just limited to fauna but have been shown to impact plants and sediments as well. Microplastics have been found in the canopy and root systems of various plants (J. Li *et al.,* 2022; Zhang and Liu, 2018), and are well known to accumulate in sediments across many different environments (Büks and Kaupenjohann, 2020).

As pollutants, microplastics are often considered to be harmful to the environments they end up in. However, some plant studies show that the presence of microplastics had positive effects (J. Li *et al.,* 2022). Studies in wheat found that plant growth and chlorophyll content were greatly increased when plants were exposed to polystyrene microplastics (Lian *et al.,* 2020; Liao, 2019). Other benefits have also been observed, including increased colonisation of soil root microbes after PES exposure (de Souza Machado *et al.,* 2019), and increased plant biomass when PET fibres and fragments are mixed into the sediment (Lozano *et al.,* 2021).

However, in most studies, microplastics were found to have negative, inhibitory effects on plants (J. Li *et al.,* 2022). Impacts include inhibiting seed germination (Boots *et al.,* 2019), reduced root growth and activity (de Souza Machado *et al.,* 2019; Y. Dong *et al.,* 2020), disrupting photosynthetic efficiency (Zeb *et al.,* 2022), increasing oxidative stress (Yu *et al.,*

2021), and reducing overall biomass (Boots *et al.,* 2019; Lozano and Rillig, 2020). These are caused by a variety of direct and indirect mechanisms.

Many of these mechanisms are caused by direct microplastic contact. Particles smaller than 3 μm can be taken up directly through the root system (L. Li *et al.,* 2020; Lian *et al.,* 2020), where they may damage cell membranes (Navarro *et al.,* 2008). Even if microplastics do not pass into the plant, they can block pores in the roots (Sun *et al.,* 2021) and seed capsules (Bosker *et al.,* 2019). This can affect the uptake of nutrients by the roots (Jiang *et al.,* 2019), or inhibit water uptake and reduce germination (Zhang *et al.,* 2021). Whilst larger microplastics cannot be taken up by the roots, they can still accumulate on and around them. This can result in mechanical damage to the root system, reducing root activity and root growth (Rozman *et al.,* 2021).

Microplastics can also have cytotoxic effects on plants, resulting in the altering of different genes and gene expression (J. Li *et al.,* 2022). Studies have observed exposure to microplastics causing inhibition of disease resistance genes (Maity *et al.,* 2020), and reduced stimulation of genes involved in antioxidant enzyme activity (Zhang *et al.,* 2021).

Finally, microplastics will readily leach additives such as plasticizers, pigments and stabilisers (Hahladakis *et al.,* 2018), which then have their own toxicities (Rozman *et al.,* 2021). Leachates such as lactic acid (degradation production of PLA) have been shown to impact root length (Lee *et al.,* 2022), whilst Pflugmacher et al. (2021) found that compounds released from PC reduced germination by up to 60% in seeds.

As plant growth is directly influenced by sediment properties (J. Li *et al.,* 2022), microplastic impacts on sediments also indirectly affects plants as well. Several studies report the effect that microplastics have on soil properties, including soil structure, soil density, porosity and water retention (De Souza MacHado *et al.,* 2018; Lozano *et al.,* 2021). These effects are not always negative, such as microplastic films creating space for increased water movement (Wan *et al.,* 2019), thus leading to increased soil aeration and thus better conditions for root growth. The impacts can be very localised however, as depending on the ecosystem and plant community, increased aeration could also lead to drought. Other effects include changes to soil pH (Boots *et al.,* 2019) and decreased soil aggregation (Lozano *et al.,* 2021).

Any change to sediment properties is also likely to affect microbial communities present (J. Li *et al.,* 2022). However, microplastics can also directly affect soil microbes. Small microplastics were found to inhibit the abundance of Proteobacteria in rhizosphere soils (Xu *et al.,* 2021), whilst both PE and PVC microplastics have been linked to reduced growth of soil bacteria (Fajardo *et al.,* 2022; J. Zhou *et al.,* 2020). Leachates can also affect microbes, particularly carbon released during the degradation of microplastics (Rillig, 2018). An influx of large amounts of carbon can disrupt the nutrient balance within soil and impact its microbial communities (Lowery and Ursell, 2019).

Microplastics may also increase the bioavailability of other pollutants within sediments. These include heavy metals such as Cd, Cu and Pb, which adsorb to the surface of microplastics and then are transported into the sediment along with the microplastic (Abbasi *et al.,* 2020; Jia *et al.,* 2022). Microplastics have also been found to enhance the accumulation of other toxins however, including phenanthrene (Xu *et al.,* 2021) and PCBs (Tumwesigye *et al.,* 2023).

Having so many potential responses caused by just microplastics, it is likely that different plant and sediment communities will be affected to different degrees. With certain species being potentially more vulnerable, this means that microplastics could lead to changes in community composition and diversity. While there is evidence to support all these threats, the extent to which they will have any impact is tied to the amount of microplastic within the ecosystem (Rillig *et al.,* 2019). While this is most obvious in sites near agriculture or urbanisation, there is also growing concern around coastal ecosystems and their potential to act as microplastic sinks.

1.4 Microplastics in Coastal Ecosystems

Coastal ecosystems are a growing area of global concern, with almost 40% of the global population distributed within 100 km of them (Small and Nicholls, 2003). As the interface between terrestrial and marine ecosystems, they are particularly vulnerable to the accumulated input of microplastic from both sides. It is not surprising then that microplastics have been reported in all coastal environments, including beaches (Piperagkas *et al.,* 2019), shores (Kazour *et al.,* 2019), estuaries (Willis *et al.,* 2017), mangroves

(Mohamed Nor and Obbard, 2014), lagoons (Vianello *et al.,* 2013) and marshes (J. Li *et al.,* 2020). Coastal wetlands have already been shown to be very effective in trapping marine litter such as macroplastics (Martin *et al.,* 2019), and their dynamic conditions provide the right environment for this plastic litter to degrade into microplastics very quickly (Weinstein *et al.,* 2016). These habitats therefore represent areas of microplastic accumulation and generation, leading to potentially high concentrations when compared to other ecosystems (Kumar *et al.,* 2021). However, research into microplastics in coastal wetlands is still a newly growing area, and so more studies are needed to understand microplastic behaviour in these ecosystems. Within coastal wetlands, salt marshes remain understudied. However, the combination of regular inundation and their varied topography makes them very interesting habitats when considering how microplastics may be deposited and trapped.

1.5 Salt Marshes

Found at the transition zone between land and sea, salt marshes are coastal ecosystems formed by the gradual build-up of sediment from slow moving, low energy water systems (Bakker, 2014). They can be characterized by salt resistant plant species (Dobben and Slim, 2012), which are regularly inundated by the tide. Salt marshes can be further described using their physical characteristics, flora species, and their local and global location (Bakker, 2014; Baugh *et al.,* 1990; Yando *et al.,* 2023). Salt marshes are found around the world; however, they cover less than 5% of the global coastlines (Murray *et al.,* 2022). They mostly occur in temperate climates or regions outside of the tropics, predominately in Europe, America, Australia and parts of East Asia (Figure 1.3) (Mcowen *et al.,* 2017).

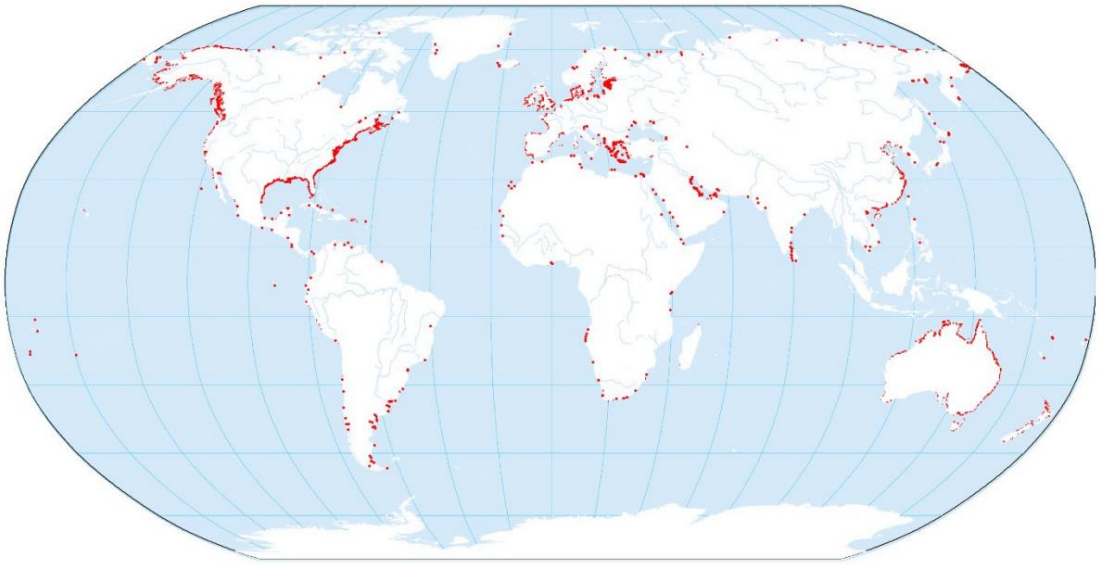

Figure 1.3 - Global distribution of coastal salt marshes, red dots represent individual salt marshes (Mcowen *et al.,* 2017).

In North-West Europe, salt marsh formation begins with pioneer vegetation, which can establish itself upon the bare tidal flats. These pioneer species can endure long inundation times by the tide, during which they help to slow down the water flow velocity and encourage the sedimentation of particles in the water column (van Hulzen *et al.,* 2007). This sediment deposition gradually leads to an increase in the surface elevation relative to sea level, allowing for the establishment of further plant species (Rupprecht *et al.,* 2015). This zone, the low marsh, receives less inundation than the tidal flats and pioneer zone, and so a more diverse plant species community can thrive (Esselink *et al.,* 2000; Suchrow and Jensen, 2010). The continual vertical growth of the marsh is termed accretion and eventually leads to the formation of a high marsh zone. The high marsh is only inundated during storm surge events, and therefore is dominated by larger, less resilient grasses, shrubs and bushes (Andresen *et al.,* 1990; Suchrow and Jensen, 2010; Wanner *et al.,* 2014). Finally, many marshes also feature a Spartina zone. The various species in the grass family *Spartina* are particularly hardy, able to thrive in tough conditions therefore will often be found in large patches from the edge of mudflats up to the low marsh. So dominant is this species in some marshes, it forms its own zone where it outcompetes other salt tolerant plants (Strong and Ayres, 2013). In summary, the processes of accretion and vegetation succession lead to the formation of many different vegetation zones (Figure 1.4) across the

elevation and inundation time gradients, a characteristic feature of many salt marshes (Bakker, 2014).

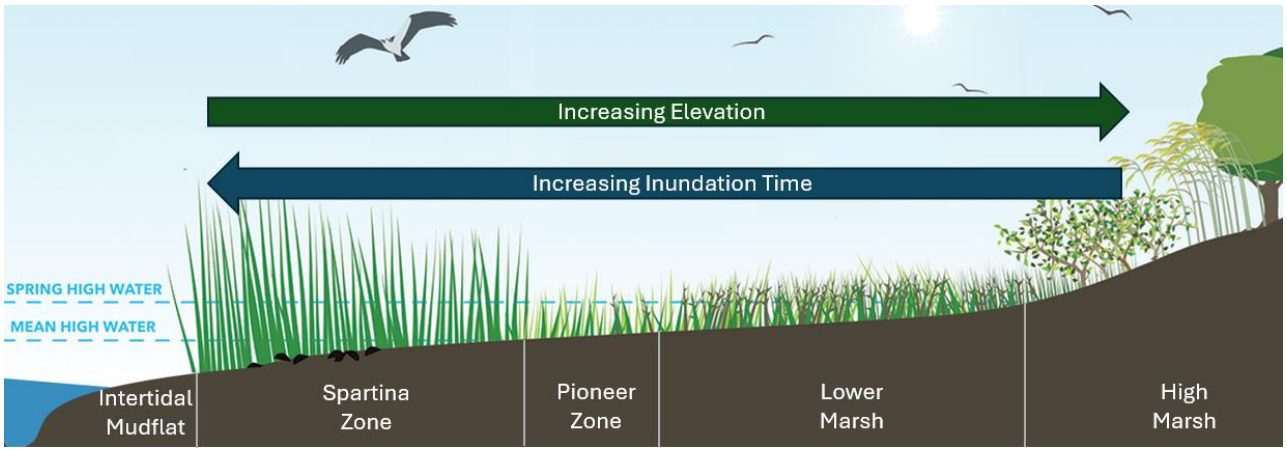

Figure 1.4 - Salt marsh zonation showing individual zones, elevation and inundation time gradients, along with high tide water lines.

Salt marshes are valuable habitats, known for their high productivity and the unique biodiversity which they sustain, for example as feeding grounds for migratory birds (Greenberg *et al.,* 2014). They also provide numerous ecosystem services, such as natural coastal defences (Mcowen *et al.,* 2017), cycling of nutrients (Sousa *et al.,* 2010), sinks of organic carbon (Alongi, 2020; Sousa *et al.,* 2010; Teixeira *et al.,* 2014) (Teixeira *et al.,* 2014) (Teixeira *et al.,* 2014), as well as being important staging grounds for the growth of young fish (Deegan *et al.,* 2005). These marshes can also have some commercial value through recreation and tourism (Gedan *et al.,* 2009), however this can be potentially damaging to the ecosystem if not properly managed. Perhaps the most valuable of all these resources are the coastal protection and stabilisation, and their role in carbon storage. Salt marshes act as a soft coastal defence by reducing the impact of storm events and reducing coastal flooding through wave dampening (Rao *et al.,* 2015; Shepard *et al.,* 2011). By dissipating the wave energy through interaction with their dense vegetation they can reduce it by as much as 85% compared to a barren mudflat (Möller, 2006). Salt marshes are also crucial habitats in climate change mitigation through sequestering atmospheric carbon (CO_2) and storing it as organic carbon (above and below surface biomass and soil organic matter). A combination of slow biomass degradation due to the anoxic conditions caused by regular inundation, along with their high productivity results in carbon storage values up to five

times higher than regular terrestrial forests (Chmura *et al.,* 2003). This has led to their recognition as "blue carbon" environments, and thus as ecosystems they are important for alleviating climate change. Despite often forming in protected or secluded coastlines, globally these habitats are under threat from anthropogenic sources such as tourism, fishing, pollution, increased marine traffic, waste deposition, the building of facilities such as harbours and construction of new embankments (Hansen and Reiss, 2015), and a general "squeeze" caused by land reclamation for agriculture and urbanisation (Gedan *et al.,* 2009). Combined with the effects of global warming and rising seas, many salt marshes are gradually receding (Crooks *et al.,* 2011; Duarte *et al.,* 2008).

1.6 Microplastics in Salt Marshes

In additional to the abovementioned threats to salt marshes, their potential to accumulate microplastics is a growing concern. The physical processes involved in salt marsh formation and continual accretion are inherently biased towards microplastic trapping (Helcoski *et al.,* 2020). The same hydrodynamic properties that lead to sediment deposition have also been tied to an increased trapping of microplastics (Vianello *et al.,* 2013), therefore it is hypothesized that salt marshes are effective concentrators and long-term sinks for microplastics.

Salt marshes can also accumulate macroplastics, which through processes such as biodegradation, thermal degradation and oxidation, will degrade over time into smaller and smaller plastic pieces, eventually leading to the formation of micro and nanoplastics (Weinstein *et al.,* 2016). Whilst the complete degradation of polymers can take up to hundreds of years (Chamas *et al.,* 2020), microplastic formation occurs on a much more rapid time scale. In coastal waters these degradation processes are thought to be the major source of microplastics entering ecosystems (Gray *et al.,* 2018). Much like with microplastic accumulation through sedimentation, salt marshes are also ideal habitats for microplastic formation through degradation due to a combination of factors. Subject to the mechanical forces of wave movement and turbulence whilst immersed, coupled with exposure to oxygen in the air, UV radiation and increased temperatures, salt marshes are ideal habitats which promote the breakdown of plastics (Weinstein *et al.,* 2020). Detritivorous organisms

are commonplace in all salt marshes, and some such as amphipods have been shown to digest and fragment carrier bags into microplastics (Hodgson *et al.,* 2018). Consequently, the breakdown of macroplastics is of just as much concern as the direct input of microplastics from the water systems.

Yet, while microplastics have been a growing research topic in marine ecosystems over the past decade (Law and Thompson, 2014), salt marshes are still a relatively new area of study. However, even from the few studies published there is a lot of evidence to show that microplastics are present and affecting salt marshes, and as such, as an ecosystem they warrant further research to fully understand the immediate and future effects of microplastics.

1.6.1 Occurrence of Microplastics within Salt Marshes

The main research questions in this area surround concentration and distribution; are microplastics observed in salt marshes, how much, and where? Many studies have been carried out in China (J. Li *et al.,* 2020; Wu *et al.,* 2020; W. Yao *et al.,* 2019), whilst other studies in the UK (Stead *et al.,* 2020) and Portugal (Cozzolino *et al.,* 2020) also report microplastics within salt marshes. Perhaps the most important result of these studies is the presence of microplastics, which occur in every sediment sample taken. The exact sampling methods differ between studies, but there is overwhelming evidence with microplastics found in over 100 separate sediment samples. However, despite highlighting their presence in different marshes around the world, it is hard to accurately compare the relative amounts of microplastics. Therefore, further studies are still required to build up knowledge on the abundance of microplastics within these environments and demonstrate whether salt marshes are key ecosystems for microplastics accumulation.

1.6.2 Spatial Distribution of Microplastics with Salt Marshes

Whilst spatial variation was not a key focus in many of these studies, they provided a range of samples from mudflats to inner marsh habitats. When comparing these areas, J. Li et al. (2020) and (Wu *et al.,* 2020) found a larger amount of microplastics in the vegetated habitats when compared to the neighbouring unvegetated region. This vegetative trapping is supported by a study on the sea surface microlayer (SML) (Stead *et al.,* 2020). Buoyant microplastics float on the water's surface, and so measurements taken from the SML of salt

marsh creeks found a decrease in the microplastic abundance between the incoming flood tide and outgoing ebb tide. This suggests that the salt marsh vegetation slowed the water enough for microplastics to be trapped, complementing the previous results. The extent of this vegetation effect however is highly relative, as in their study (Cozzolino *et al.,* 2020) found no discernible difference between microplastic abundance in the salt marsh sediments and nearby unvegetated sediments. Whilst this result could have been due to differences in sample handling and microplastic validation, it suggests that the effects of vegetation are likely to vary with plant communities and density between different marshes. It also highlights that plants are not the only factors that impact microplastic accumulation. Relative elevation, local climate and hydrodynamic factors can also play a part in the sedimentation process. Therefore, as quoted by Stead et al. (2020) "additional studies on a variety of salt marshes, through a range of methods and in different locations, are needed in order to confirm that there is a consistent trapping effect for microplastics by salt marshes".

1.6.3 Temporal Distribution of Microplastics in Salt Marshes

Salt marshes vertically accrete over time, and so the depth profile of the sediment can represent the history of the marshes. The distribution of microplastics throughout the sediment was found to be negatively correlated with sediment depth, the majority of microplastics being found within the top 2 cm of sediment (J. Li *et al.,* 2020; Wu *et al.,* 2020). Whilst the annual rate of sedimentation varies between marshes, this result matches the hypothesized trend that microplastic concentration within the sediment layers would mirror their increasing annual global usage. Using ^{210}Pb dating on their sediment cores (J. Li *et al.,* 2020) were able to determine the ages of the different portions of their samples, and with a few exceptions due to extreme weather events, found that microplastics mirrored China's increasing consumption of plastics over time. This top-heavy distribution of microplastics has been found in all studies that look at temporal variation Click or tap here to enter text.(Matsuguma *et al.,* 2017; Wu *et al.,* 2020) and suggest that microplastics remain effectively trapped in their sediment layer. Other components can still influence temporal variation however, and further studies are needed to explore the effects of different sedimentation processes and anthropogenic input.

1.6.4 Degradation of Microplastics in Salt Marshes

The harsh environmental conditions present in salt marshes are thought to be favourable for plastic degradation, however the rate and extent of this is unclear. A series of studies conducted by (Gray *et al.,* 2018) and (Weinstein *et al.,* 2020) tested the breakdown of macroplastics under these conditions, inspecting the main polymer types in litter as well as some biodegradable plastic alternatives. The studies were conclusive in that evidence of microplastic formation occurred in as little as 4-8 weeks. Alongside the formation of microplastics the samples did show another concerning aspect, namely habitation and consumption by salt marsh organisms. Biofilms and salt marsh periwinkles were found on all samples, along with oysters and barnacles which settled on the plastic's surface. Whilst these cannot be attributed to any increase in plastic loss through biodegradation, organisms are exposed to and interacting with these plastic sources very quickly after their deposition.

Understanding the processes of plastic breakdown is a key step in determining the potential environmental pathways they may access. It should also be noted that these studies looked at the plastic degradation under the optimal conditions found in the intertidal zones. Exposed to both wave action and high UV this area was predicted to have high breakdown potential. However, this might not be the case for the more vegetated high marsh areas which receive less inundation (as well as more plastic contamination from terrestrial sources). As such this method of microplastic generation may well be more specific to the pioneer and lower marsh zones, leading to potentially higher numbers of microplastics there.

From the existing knowledge of salt marshes there is a clear indication of the presence of a wide range of microplastics. There is some evidence of several spatial and temporal trends, and the current studies suggest that vegetation plays a role in trapping microplastics. Macroplastic degradation has been shown to be a major microplastic source for salt marshes, and organisms are found to be interacting and consuming them. Whilst this information highlights the residence of microplastics within salt marshes, it barely scratches the surface of their full distribution and impacts.

1.7 The future of Microplastic Research

In March 2022, the UN Environment assembly passed a resolution to develop an instrument on plastic pollution. This covered various points; including, the recognition of microplastics within plastic pollution, the impacts to marine environments, and the need to improve understanding of plastics both from distribution, impacts and experimental perspectives. Once this treaty on plastic pollution is fully realised, it will incorporate the monitoring of microplastics on a global scale. However, to reach this an understanding of the current microplastic abundances are around the world is needed. As such, studies which provide local and environmental knowledge of microplastics are critical towards the completion of this instrument, particularly in areas or habitats that have previously been understudied. This thesis studies microplastics in salt marshes, not only providing new environmental data for the UK and Europe but also adding to the growing collection of information for this ecosystem worldwide.

1.8 Research Questions

In order to expand our understanding of microplastics in salt marshes, fundamental studies showing the presence and distribution of microplastics in these habitats are needed. This thesis sets out to provide baseline information regarding the sampling and handling of salt marsh samples and then studying the distribution of microplastics and potential factors which affect this.

The main research questions are:

- Can we develop a methodology suitable for the extraction, counting and analysis of microplastics from salt marsh sediments?
- Are microplastics present in salt marsh sediments from two different sites?
- What is the spatial distribution of microplastics across salt marsh sediments? Are there any physical of vegetation factors which influence this distribution?
- What is the temporal distribution of microplastics within salt marsh sediments? Are there distribution patterns, and do any physical or environmental factors explain this distribution?

1.9 Thesis Outline

In this thesis I focus on studying the presence and abundance of microplastics in salt marshes, using different sites to ask questions around spatial and temporal distribution, and the contribution of various factors to these results (Figure 1.5). However, it is impossible to ask such questions without first ensuring a valid methodology for the sampling, extraction, counting and analysis of microplastics.

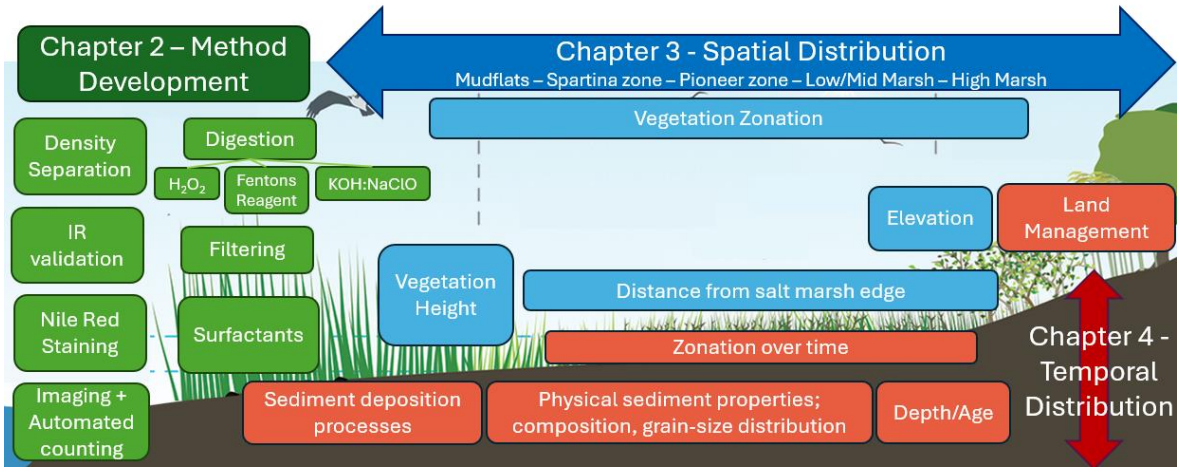

Figure 1.5 - Main research topics of Chapters 2,3 and 4, showing various topics/steps focussed on in each chapter.

In **Chapter 2** I therefore develop a method for microplastic extraction, focussed on optimising the protocol for salt marsh samples. Using pre-existing lab protocols and methods from other microplastic studies (Maes *et al.,* 2017; Prata *et al.,* 2019), I created a framework for the methodology consisting of; density separation, chemical digestion, particle counting via Nile Red staining and analysis with IR spectroscopy. Each step is then tested, trialling different solutions and other variations to the method, to optimise each protocol regarding handling salt marsh samples. The best practices for each step are then combined into a final methodology, which is then written up as the standard operating procedure (SOP).

In **Chapter 3** I then apply this methodology to the first of my field samples to study spatial distribution. Using surface sediment cores from Blakeney Point salt marsh, I test for microplastic abundance in over 100 samples across the salt marsh and study the spatial distribution of microplastics across the marsh. The results are then compared with

explanatory variables such as vegetation height, elevation, distance from the marsh edge, and vegetation zonation, to observe if the microplastic abundance is affected by these factors and whether or not they explain microplastic distribution across the marsh.

In **Chapter 4**, I use deep sediment cores taken from a salt marsh from the Wadden Sea to study microplastics on a temporal scale. Using pre-existing radionuclide data for dating, I can compare microplastic abundance with time and study the variation in microplastic distribution in the past 60 years. I then look at the changes in marsh management over time to determine whether this impacts the microplastic distribution, as well as physical factors such as grain-size and sediment composition. Finally, I consider the sediment depositional processes for each sample and compare microplastic abundance to discern whether microplastics behave and deposit in a similar manner to sediments.

In **Chapter 5** the findings of chapter 3 and 4 will then be discussed, to draw wider conclusions and implications regarding microplastics in salt marshes. Comparing this to existing literature, I will highlight the significance of my results, whilst suggesting new research areas that should be the focus of future studies.

Chapter 2 - Developing a Method for Microplastic Extraction and Analysis from Salt Marsh Sediments

Abstract

With the widespread interest in microplastics continuing to increase, various methods are being developed to study them in different environmental compartments (e.g. biota, sediment, or water). However, there is little uniformity across the various studies, particularly when it comes to the extraction and analysis of microplastics. When approaching the diverse nature of sediments along the mudflat to salt marsh gradient, a new methodology was required. This work focussed on developing a method that would be suitable for extracting microplastics from a variety of salt marsh sediments, including sand, mud, and samples with dense vegetation. Using sediment-microplastic isolation (SMI) units and zinc chloride, a density separation step was successfully demonstrated with an average recovery rate of 91 +/- 2% for various plastic forms in spiking studies. Digestion was found to be a crucial component of the methodology, using a mixture of KOH:NaClO to remove over 90% of the organic matter in samples. Nile Red staining was found to be an effective method for staining particles after removing contaminants and thus could be used to implement the rapid automated counting of microplastics. Finally, these tests were validated with micro-FTIR, demonstrating the ability to effectively differentiate between organic matter and plastics, as well as being able to match and identify different polymer types by comparison with an online spectral library.

2.1 Introduction

2.1.1 Methods in Microplastic Studies

Whilst the full extent of microplastics is almost impossible to measure, their frequent detection in sediments, water, and air samples from all around the globe mean that they are likely a worldwide contaminant (Park and Park, 2021). As plastic usage continues, there are growing concerns as to the potential impact of microplastics, and thus research interests are now focussing on how microplastics can be measured in the environment (Barboza and Gimenez, 2015). However, there remains a significant issue when comparing datasets with microplastic information from various studies, due to the lack of standardised or harmonised methods in their sampling, extraction, and analysis (Prata *et al.,* 2019).

One of the biggest reasons for the lack of established protocols, is the sheer variety of techniques, often necessitated by the different environments being sampled. The medium of the sample can often dictate the method. For example, water samples are commonly collected using nets of various mesh size (e.g. surface water manta nets (Tamminga *et al.,* 2018), plankton nets (Yona *et al.,* 2019), and deeper water bongo nets (Di Mauro *et al.,* 2017) pumps (Harrold *et al.,* 2022), and even *in situ* sieving (Dubaish and Liebezeit 2013). Meanwhile, biota and sediment samples often need more complex methods, requiring extraction and digestion stages to release and isolate microplastics prior to their identification. Furthermore, restrictions such as the costs as well as the appropriate infrastructure, can often dictate which methods can be used in different studies (Prata *et al.,* 2019). Therefore, despite the difficulties arising from the lack of comparable data and the need for validation, a wide variety of methods are still employed today in environmental microplastic studies.

2.1.2 Methods within Sediment Studies

When considering sediments as a matrix for microplastic monitoring, there is still a huge variety in the different methods being used. Whilst some attempts have been made to harmonise protocols by organisations such as OSPAR and NOAA (Marine Debris Program, 2015; Marine litter in the Northeast Atlantic Region : assessment and priorities for response., 2009), there is still the absence of a standardised method for both marine (Imhof *et al.,* 2012) and terrestrial sediments (Dioses-Salinas *et al.,* 2020). Protocols are then left

up to the researcher to determine, often using a pre-existing protocol or adapting one to the specific needs of their samples. Nonetheless, certain analytical steps are generally conducted such as contamination reduction procedures, particle extraction using density separation, chemical digestion for the removal of biogenic compounds, and the quantification of particles using microscopy and spectroscopic techniques (Hanvey *et al.,* 2017).

2.1.2.1 Sampling

Collecting the samples is the first step in any microplastic study, however the sampling approach is often dictated by the nature of the sampling site. Most sediment studies use a bulk based sampling strategy, taking a defined volume or area of sediment (Hidalgo-Ruz *et al.,* 2012) back for laboratory analysis. However, some studies also choose to use *in situ* sampling, manually collecting plastics with forceps or processing sediments by sieving them directly in the field (Hanvey *et al.,* 2017). For terrestrial sediments, this volumetric approach is normally completed by collecting sediment cores. Whilst several coring techniques exist (e.g. barrel cores, augers), they allow for the simple extraction of given volumes and depths of sediment. Marine sediments present a greater challenge, and therefore more specialist sampling equipment is required, such as a box corer or sediment grab (Galgani *et al.,* 2022; Harrison *et al.,* 2012). Inconsistencies between these methods however lie in the reported units of the sediment, with some studies reporting number of particles per surface area (m^2) (Ivar Do Sul and Costa, 2014), sediment volume (m^3, or mL and L) (Turra *et al.,* 2014) or even by weight (g, kg) (Maes *et al.,* 2017; Ng and Obbard, 2006). If the bulk density and volume of a sediment sample is known, per weight can be calculated and vice vera. However, even weight can be contentious, as studies show a 45%:55% breakdown in reporting per dry weight and per wet weight respectively (Prata *et al.,* 2019). This can make data difficult to compare, despite studies sharing identical methodologies, which supports the need for harmonisation or at least specific reporting of bulk density so the values may be inter-converted. Furthermore, sediment depth is an important consideration when sampling. Microplastics have generally been shown to be most abundant in the surface sediments, with over 50% of all microplastics in the top 5 cm of the sediment, and 95% of MPs found in the top 15 cm (Carson *et al.,* 2011). Reporting the depth of sediment samples

is therefore important in microplastics studies, especially when sampling with deep sediment cores.

2.1.2.2 Extraction

Once a sediment sample has been collected, the microplastics must be removed to be counted and analysed. Whilst the exact order of steps can vary, all studies have an extraction step to separate their microplastics from the matrix of sediment and other materials. The various techniques are summarised in Table 2.1.

Table 2.1 Summary of different techniques used in extracting microplastics from sediments, showing pros and cons of each technique

Technique	Pros	Cons
Physical Separation	Fast, Simple, Cheap	Ineffective on smaller plastics, High likelihood of false positives
Density Separation	Good recovery rates (80%+), Wide range of solutions to choose from, Simple apparatus	Solutions expensive, Extraction time varies on solution (up to 24 hours)
Oil Separation	High recovery rates (90%+),	Plastics hard to recover from within sediment, Plastics stick to filters
Pressurised Solvent Extraction	Very effective for small particles	Results dependent on plastics solubility, Potential altering of plastic properties
Electrostatic Separation	Method unaffected by plastic type, Good for processing large sample amounts	Technique is highly specialised, Instrument is very expensive

Physical Separation

The simplest technique for extraction is physical separation. Sediment samples are passed through steel sieves of various sizes, and then the collected material is sifted through and microplastics are separated out visually. Whilst this is a fast and simple technique, visually picking out microplastics means this is only effective for larger microplastic pieces (>1 mm) and is often biased towards brighter coloured particles, therefore missing a large portion of the potential microplastics within a sample. This issue is even more pronounced for fibres, where their small diameter (~20 μm) means that even large fibres can be lost during sieving. Therefore, sieving is often a prelude step to extraction which removes some of the larger

organic material and meso/macroplastics and is then followed up with another extraction process.

Density Separation

Density separation is the most widely used technique when it comes to extracting microplastics from sediments, with one review (Bellasi *et al.,* 2021) finding 73% of studies reporting this process. These techniques follow four general steps: immersion of sample in a high-density solution (> 1.2 g/cm^3), mixing of sample and solution for a set period of time, a set settling time, and collection of the floating materials/supernatant solution. Plastics have a range of densities (0.8 – 2.2 g/cm^3) (Table 2.2), however inorganic sediment particles have on average higher densities (2.65 g/cm^3) (Bergmann *et al.,* n.d.). Thus, upon being mixed in high density solutions, plastic being lighter will float to the surface whilst sediments will settle to the bottom. Various solutions have been proposed, with Figure 2.1 summarizing the most commonly used solutions in literature.

Table 2.2 Densities of different microplastics and microfibres (synthetic and natural), showing behaviour in water (Preston-Whyte *et al.,* 2021)

Microplastic and Microfibres	**Density (g/cm^3)**	**Behaviour**
Polypropylene (PP)	0.9	Float
Low density polyethylene (LDPE)	0.95	
High density polyethylene (HDPE)	0.95	
Freshwater	1	
Polystyrene (PS)	1.05	
Seawater (salinity 35g/kg, 25^{o}C, pressure = 1000)	1.06	
Nylon 66, nylon 6	1.14	Sink
Polyamide (PA)	1.16	
Acrylic	1.19	
Polycarbonate (PC)	1.2	
Wool	1.3	
Polyvinyl chloride (PVC)	1.3-1.45	
Polyester	1.39	
Cellophane	1.42	
Regenerated cellulose	1.44	
Natural cellulose	1.5	

Viscose rayon	1.52
Cotton	1.55

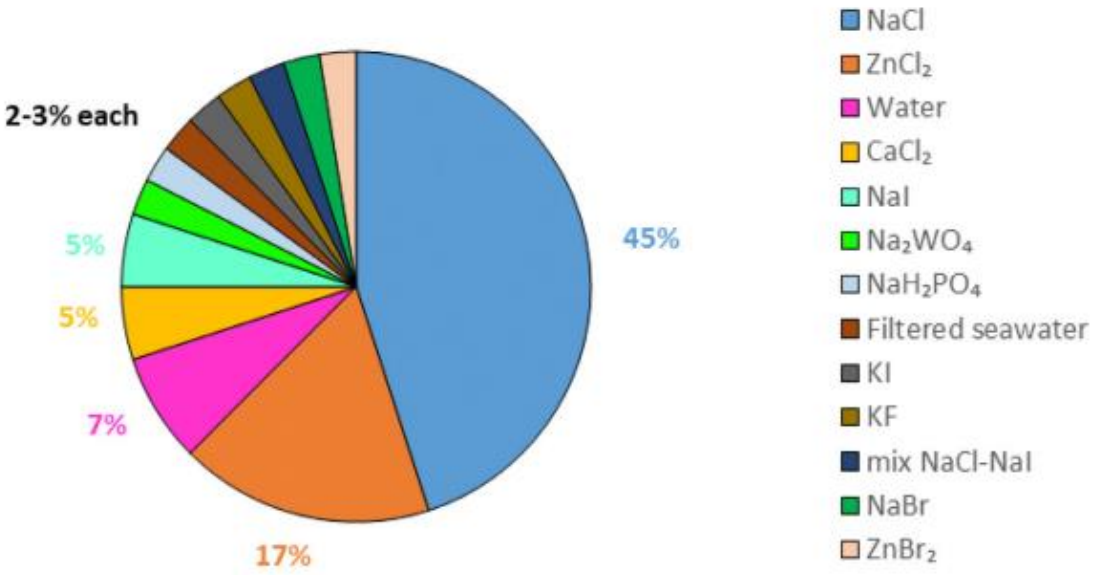

Figure 2.1 - Chemical solutions used for density separation steps in considered microplastic extraction studies; showing list of all solutions used and % occurrence (Bellasi *et al.,* 2021).

Whilst many of the salt solutions are very similar, they all have their individual benefits, and potential flaws. NaCl (1.2 g/cm^3) is the most used solution in these extraction steps. This is due to its high availability, low cost and over all ecofriendly nature. However, due to its overall lower density this solution has been shown to have poor recovery rates in heavier polymers (e.g. PVC, PET, HDPE) (Quinn *et al.,* 2017). Variations upon NaCl have been proposed, such as adding sucrose to the solution to increase the density and so improve recovery of high-density polymers (Bellasi *et al.,* 2021). Whilst the trials showed solid recoveries, this method has yet to be used on particles <500 μm. Furthermore, the sucrose mixture increased the viscosity of the solution as well, which may result in reduced filtering speed and efficiency in later steps.

NaI (1.6 g/cm^3) and $ZnBr_2$ (1.7 g/cm^3) both showed high recovery rates (99%) in heavier polymers, however NaI reacts with the commonly used cellulose based filters, whilst $ZnBr_2$

is expensive and hazardous to the environment (Quinn *et al.,* 2017). $ZnCl_2$ is another widely used solution, with a range of densities (1.4 – 1.7 g/cm^3). This solution again demonstrates high recovery rates (95.8%) (Coppock *et al.,* 2017) and can be recycled and used again, however it is corrosive and has environmental concerns. Many of the solutions are used in conjunction with each other, either mixing or as sequential separation steps (Rivoira *et al.,* 2020).

Mixing of the sample and solution can be carried out in a few different ways. Some studies use a mechanical shaker (Ng and Obbard, 2006) with the sample in a beaker. Sediment Microplastic Units (SMIs) are widely used in extraction protocols and are mixed with magnetic stirring and manual shaking. A few studies also employ the use of a centrifuge to both mix and separate the microplastics in one go (Woodall *et al.,* 2014). Mixing time is often subject to the method, however when allowing the solution to settle enough time must be given to allow microplastics to float up. There is no agreed timeframe for this however, and so studies report times from 5 minutes to over 12 hours (Klein *et al.,* 2015). In general, it is assumed that the sample is left until the solution has at least cleared of sediment by visual inspection.

The final stage of extraction is to separate and collect the floating microplastics. For SMI units, this involves closing off the central valve and pouring off the supernatant solution, an effective and simple method that requires only proper rinsing of the apparatus to ensure all microplastics are collected. Other studies pipette the supernatant, which can be time consuming and can easily miss smaller microplastics. Finally, one study (Nuelle *et al.,* 2014) used an overflow method, adding more salt solution so that the beaker filled up, and microplastics floating in the overflow were caught in a second vessel. Whilst effectively collecting all the microplastics, this requires further apparatus and increases the amount of chemicals required in the method. Once collected, the supernatant solutions will be used in further steps to count and analyse the microplastics. Despite the various approaches to density separation, high recovery rates have been demonstrated in many different apparatus configurations and solutions, and therefore is the recommended protocol when it comes to extracting plastics from sediments.

Oil Separation

As an alternative to density separation, oil-based methods have been proposed due to the natural hydrophobic properties of plastics. First proposed by (Crichton *et al.,* 2017), the oil extraction protocol (OEP) has been used with several different types of oil, including Canola, olive, (Karlsson *et al.,* 2017) and pine oil (Imhof *et al.,* 2012). In principle, when in contact with an oil the hydrophobic plastics will suspend into the oil whilst sediment will not. The oil layer can then be extracted and filtered normally. This process is considered as an alternative to density separation and plastics suspend in oil regardless of density (due to the hydrophobic properties of both plastics and oil). However, whilst these studies report good, spiked recovery rates, they also found that particles remain easily trapped within the sediment (Crew *et al.,* 2020) as well as being challenging to remove from the filters (Nuelle *et al.,* 2014). Furthermore, oily residues are often left on the extracted plastics which may interfere with later spectroscopy analysis (Bellasi *et al.,* 2021). When tested on environmental samples recovery rates were found to decrease below that of density separation studies (Crew *et al.,* 2020).

Pressurised Solvent Extraction (PSE)

Like oil-based methods, pressurised solvent extraction works by using a hydrophobic solvent in which plastics suspend. Samples are immersed in the solvent solution, then exposed to high temperatures (180 - 190 ^{o}C) and pressures (100+ bar). Solvents are then removed from the resulting residue via evaporation under nitrogen gas, and the remaining microplastics dried and analysed. (Fuller and Gautam, 2016) used dichloromethane to successfully recover spiked polymers included PET, PS, PP and PE. Further tests by (Stile *et al.,* 2021) found that PSE results were comparable to density separation, and often higher when considering small particle size fractions. However, the results are very variable depending on the solvent used and the different solubilities of different polymers (Saliu *et al.,* 2021). This method also alters the surface of the microplastics, changing size and morphology. Therefore, SPE is effective in identifying different types of microplastics but cannot provide further information.

Electrostatic Separation

This more specialised method was used to separate plastic from a sediment matrix based on electrostatic properties (Felsing *et al.,* 2018). The sample is exposed to a high-voltage field, where particles become electrostatically charged. By rotating the sample on a drum, particles are then separated and are collected based on their discharge speed; non-conductive materials (plastics) being much slower than conductive materials (sediment and sand). This method is unaffected by plastic density and once set up is very straight forward with few procedural steps. Whilst (Felsing *et al.,* 2018) reported a separation efficiency of nearly 100%, the equipment needed is highly specialised and expensive and therefore should only be considered if processing high amounts of samples.

2.1.2.3 Removing Organic Matter

Nearly all environmental samples contain biological matter, and sediments can include varying levels of different organic matter such as fine roots. Since they have naturally low densities and similar properties to plastic, organic materials are often also extracted during separation. As small particles, they are easily mistaken for plastics and thus can often lead to the overestimation of plastics in environmental studies. Therefore, protocols often need a step to remove organic material from their samples (Miller *et al.,* 2017). This is mostly carried out in the form of a chemical digestion step, before or after extraction, which chemically removes the organic material without damaging the plastics. However, there are various types of digestion chemicals available, and the exact needs of a protocol may depend on the environment and sediment from which the sample was collected.

Acid Treatments

Strong acid solutions can be used to effectively digest organic matter, yet this is also at the increased risk of damage to the plastics. Karami *et al.,* (2017) found that a solution of 37% hydrochloric acid (HCl) effectively removed over 95% of organic debris from the sample, but at the cost of also degrading any PET present. Nitric acid (HNO_3) is also used in several studies, where it was found to be particularly effective in dissolving biological samples (Naidoo *et al.,* 2017). However, this treatment was found to alter particle properties with some microplastics losing their hard surface for a rubbery exterior, as well as causing the disfiguration or loss of nylon, PS, LDPE, PET and HPDE. Claessens *et al.,* (2013) also used this

acid solution, and found a general yellow staining in all their polymers. Furthermore, this acid treatment is run at 60 ^{o}C for optimal digestion efficiency, but such temperatures have also been shown to damage through melting some microplastics depending on the polymer type (Munno *et al.,* 2018). Finally, a study using perchloric acid ($HClO_4$) found it to degrade the polymers PA, PS and PVC, whilst also having an impact on the Raman spectra of these polymers even when no physical damage was detected (Enders *et al.,* 2017). Whilst acid digestion may be effective in removing organic matter, it runs the risk of removing microplastics as well, thus potentially leading to an underestimation of results.

Alkali Treatments

Like acid-based solutions, alkali digestions have also been used to remove organic matter in several different studies. Potassium hydroxide (KOH) has been used in the digestion of biota (Dehaut *et al.,* 2016) and organic matrices (Munno *et al.,* 2018) leading to the successful identification of polymers. Whilst no direct damage to the polymers was observed, the KOH treatment still resulted in the discolouration of many polymers, including nylon, PE and PVC. Sodium hydroxide (NaOH) has also been used as a digestion chemical; however, it caused similar coloration issues in PVC and PET (Dehaut *et al.,* 2016). Alkaline solutions also tend to struggle digesting hard organic material such as shells and bone fragments, and often leave fatty deposits in the sample (Kühn *et al.,* 2017). Whilst not outright degrading plastics like the acid protocol, nonetheless alkaline mixes still have prevalent side effects that can disrupt the counting of plastics. Polymers with ester and carbonate linkages, PC and PET for instance, are susceptible to saponification. In concentrated alkaline solutions these functional groups will undergo alkaline hydrolysis, thus damaging the plastics (Schrank *et al.,* 2022). A combined acid and alkaline digestion protocol was proposed by (Roch and Brinker, 2017), which was used for the rapid digestion of organic material leading to a microplastic recovery rate of >95%. However, this still resulted in changes to weight, size and colour of various polymer types.

Oxidising Treatments

Oxidising agents are some of the most widely used digestion treatments, having been used in several microplastic studies (Karami *et al.,* 2017; Nuelle *et al.,* 2014; Qiu *et al.,* 2016). These report digestive levels comparable to, if not more effective than, acid digestion, with

negligible impact on the plastics. Hydrogen peroxide (H_2O_2) is the most common treatment, with most studies using between 30 - 35% concentration. This treatment can also be run under varied conditions to improve the digestion efficiency such as increasing temperature (Avio *et al.,* 2015; Cole *et al.,* 2014). However, Gulizia *et al.,* (2022) showed that at temperatures of 60 ^{o}C and above polymer properties begin to change, which results in changes to their spectroscopic fingerprints. Other side effects of the treatment have been noted, such as the discoloration of PET (Karami *et al.,* 2017) and the degradation of nylon. A variation of this hydrogen peroxide treatment is the addition of an iron (II) catalyst, with the resulting solution known as a Fentons Reagent (Hemond, 2014). This has been used in several wastewater studies, with sequential Fentons digestion steps proving effective in removing organic matter (Dyachenko *et al.,* 2017), with minimal impact observed on PP, PET, PS and PVC (Maw *et al.,* 2022). However, a temperature control process is often required with this reagent, to ensure the reaction temperature never exceeds 50 ^{o}C. Despite this being NOAA's recommended digestion protocol for both water and sediment samples (Marine Debris Program, 2015), it has still not been widely used in further sediment studies, perhaps due to being more complex than the standard oxidation treatments.

Enzyme Treatments

Whilst most treatments are simple chemical solutions of some kind, enzymes have also been used as a potential digestion method. Due to the very specific targeted nature of enzyme degradation, this process can be used with minimal impact to plastics within the sample (Courtene-Jones *et al.,* 2017). A variety of different enzymes are used, often depending on the make-up of the sample matrix. Examples include Proteinase-K (Cole *et al.,* 2014), Collagenase, Tripsin and Papain (Courtene-Jones *et al.,* 2017), Protease, Cellulase, and Chitinase (Löder *et al.,* 2017). These were all used to successfully digest organic material, with efficiency of 97%, 72% and 98% respectively. However, when inspecting sediment samples, the wide variety of organic compounds that can be present mean that a more complex enzyme protocol may be required. Mbachu *et al.,* (2021) proposed several sequential digestions, consisting of Cellulase, Hemicellulase, Lipase and Protease, to target the most common organic materials in soils. This multistep digestion was effective, having a 94% digestion efficiency, and other sediment-based studies have found combining multiple enzyme steps with other digestion solutions (H_2O_2) to be most

practical when approaching sediment samples (Crichton *et al.,* 2017). However, despite the efficiency of the treatments and overall minimal impact towards plastic, there are still some major drawbacks to enzymatic protocols. These treatments are very complex and expensive when compared to other chemical digestions (Catarino *et al.,* 2017), their multi-step processes can add a significant period of time to the overall methodology, as well as increasing the risk of contamination through each step (Prata *et al.,* 2019).

2.1.2.4 Identification and Characterisation

Microplastics are naturally hard to analyse due to their small (often microscopic) size. A variety of identification and analysis methods do exist, but much like with sample processing methodologies, these can often depend on the facilities and equipment available during the study.

The simplest method is visual identification, and for large microplastics (2-5 mm) even the naked eye is sufficient (Shim *et al.,* 2017). For samples in the μm range, different types of microscopies exist including light (Eriksen *et al.,* 2014), fluorescent (Maes *et al.,* 2017) and even Scanning Electron Microscopy (SEM) (Lobelle and Cunliffe, 2011). These techniques can be used to identify microplastics with relative confidence, however they lack any form of chemical confirmation. As such it is not uncommon for samples to be misidentified, with some studies finding the misclassification of 70% of particles after performing chemical analysis (Hidalgo-Ruz *et al.,* 2012). Several methods have been employed to improve the rates of visual identification, including the use of the hot needle test (Shim *et al.,* 2017) and various staining protocols. Of these, Nile Red was found to be the most effective staining solution (Maes *et al.,* 2017), clearly highlighting plastics whilst having no impact on later FTIR based spectroscopic analysis. This technique is particularly good for sediment samples, as most biological material does not stain or stains poorly, and plastics are stained regardless of their state of degradation (Shim *et al.,* 2016). There are still drawbacks however, as some polymers (PET, PVC) along with fibres can have very weak staining (Erni-Cassola *et al.,* 2017; Tamminga, 2017). The benefit of visual techniques lies in their low cost, high speed, and ease to perform. They can be effective for physical characterisation, and being simple procedures, they are often used as the starting step in a series of analytical techniques (Hidalgo-Ruz *et al.,* 2012).

Chemical confirmation is essential when analysing microplastics, as they are easily confused with inorganic matter and biological debris. For this a lot of studies use some form of spectroscopic method. The most common analytical techniques are Infrared (IR), Raman, and Thermoanalytical techniques (such as pyrolysis-Gas Chromatography Mass Spectrometry (GC-MS) (Dümichen *et al.,* 2017), Differential Scanning Calorimetry (DSC) (Elert *et al.,* 2017) and Thermogravimetric Analysis (TGA) (Mansa and Zou, 2021)) all of which provide information about the chemical structure of the plastic sample (Shim *et al.,* 2017). Thermoanalytical techniques are highly accurate, able to identify samples even within a complex sediment mix, as well as handling a high number of samples. However, they can be quite slow (often several minutes per particle) and are destructive techniques which offer no physical information regarding the size, shape and numbers of the samples (Dümichen *et al.,* 2015). The industry standard instead focusses on the other two techniques, specifically micro-FTIR spectrometry (Song *et al.,* 2014) and Raman Spectrometry (Araujo *et al.,* 2018). These use a combination of light absorption and scattering to provide a spectrum that is unique to the chemical structure of the sample. This spectrum can then be compared with a database and when matched will identify the polymer composition of the microplastic sample. As widely used instruments in a variety of different fields, many research labs have access to one or both techniques. Additionally, given their popularity, resources supporting microplastic analysis with these techniques are widely available, such as protocols or spectral libraries specifically for plastics (De Frond *et al.,* 2021). There is also an increase in open access libraries that do not require the purchase of a licence to use. As non - destructive techniques (Raman may risk damaging smaller microplastics is using powerful laser settings) they allow for repeated analysis of samples leading to very high accuracy (Prata *et al.,* 2019). Combined with their low detection limits (10 - 20 μm for FTIR, 5 μm for Raman), this allows for the analysis of the majority of microplastics that may be found in any given study. However, they are not without their faults either. Spectroscopic instruments have high costs associated with purchase and upkeep, and so may not be affordable if the equipment is not already available. As complicated instruments, significant training and lab time is required to be proficient with the technique. Additionally, these techniques are quite time consuming, and so measuring particles in large data sets may take hours or even days.

Further variations within these techniques exist, including Attenuated Total Reflection (ATR) FTIR, Focal Plane Array (FPA) FTIR, Raman-imaging, and automated Raman-mapping. ATR-FTIR ensures direct contact between the sample and measurement accessory of the spectrometer, and so provides more accurate information than transmission or reflectance FTIR, especially in irregular, opaque or samples with a lot of background material (Courtene-Jones *et al.,* 2017). FPA uses a series of sensors to take spectral recordings of an entire filter (or selected area), resulting in the automated mapping and identification of particles (Primpke *et al.,* 2017). Whilst this technique shows great potential for particle analysis directly on filters, sample analysis can often be lengthy (>8 hours per filter for larger systems) and software (e.g. Simple) are needed for the processing of the large number of FTIR spectra generated. Raman-mapping can also do something similar, using automated identification to pick out particles on a filter, and then test each particle individually. Whilst this is valuable for accurately identifying multiple particles in one session, this process also requires expensive specialist filters (Araujo *et al.,* 2018) and very clean backgrounds, so may not be suitable for certain environmental samples. With such a range of models and settings, FTIR and Raman techniques can be optimised for specific study needs. Both instruments provide accurate chemical identification of plastics, and as complimentary techniques, can be used in conjunction with one another for the greatest accuracy. There is a general assumption in the field that such spectroscopic identification is definitive, but, given the relatively poor spectral quality often achieved when measuring small microplastics, there is still considerable ambiguity and uncertainty, and arbitrary "hit quality index" values are often set (e.g. >0.7 or 0.8) for quality of match with a library spectrum. This is still a crude measure, however, since it is a purely statistical approach and does not take account of the chemical and informational importance of certain features within a spectrum. This is an area where the use of AI is likely to significantly improve outcomes in the future.

Whilst all these instruments have been used to identify microplastics within water and sediment samples, the recommended analytical procedure is to use a combination of techniques. Most commonly this involves physical characterisation using visual microscopy, before performing chemical confirmation on a sub-set of samples using either FTIR or Raman Spectroscopy (Prata *et al.,* 2019). For techniques using the Nile Red staining method,

a complimentary spectroscopic technique is also needed to account for the mis-staining of other particles.

2.1.2.5 Quality Assurance and Quality Control

Perhaps the greatest issue with the lack of standardised methods for microplastic analysis, is the absence of an agreed protocol for dealing with sample contamination. With plastics so widely used, samples could be contaminated in all environments, even from just the air (Dris *et al.,* 2017). To minimise contamination, some procedures have generally been accepted and are observed in most studies. These include: using glass or metal equipment over plastic (where possible), wearing cotton lab coats and minimising the use of synthetic textiles during sampling, rinsing all equipment before and after use, and ensuring samples are properly covered and handled in controlled environments (Prata *et al.,* 2019). This last step is particularly important, as a laminar flow cabinet has been shown to reduce contamination by 50% (Wesch *et al.,* 2017), whilst an estimated 90% of contamination comes from sample exposure during extraction, digestion, and analysis steps (Torre *et al.,* 2016).

Overestimation or underestimation of quantified microplastics is also a significant risk, which is mostly addressed through some form of laboratory control sample. However, many studies do not carry out such controls, or do not report them, with (Hanvey *et al.,* 2017) finding only 16% of 43 analysed studies reported some sort of validation. The use of spiked controls and lab blanks are a critical part of testing a protocol, as they show both the reliability of the method and the validity of the results.

2.1.3 Current Methodologies within Salt marsh Studies

As coastal ecosystems, salt marshes may be considered, at least from a methods perspective, as both terrestrial and marine habitats. This means that there is a wide variety of protocols to choose from, however even previously established conventions for these different sediment types are often still insufficient due to the challenges that salt marsh sediments present. Concerns for salt marsh samples include the ability to: handle various sample types, effectively remove vegetation so that is does not hinder counting, and readily identify microplastics from heavily organic samples. In the few microplastic studies that have been carried out in salt marshes, the protocols show clear methodological differences

resulting from different sites, equipment, and research objectives. Eight such studies were selected to summarise the previously used methodologies for this unique ecosystem (Table 2.3).

Table 2.3 Summary of methodologies used in salt marsh microplastic studies showing; sample medium (d.w. - dry weight, w.w. – wet weight), microplastic extraction technique and solution, chemical digestion solution, and microplastic confirmation technique

Sample Medium	Microplastic Extraction	Chemical Digestion	Microplastic Analysis	Reference
Water	None	30% H_2O_2	Microscope + micro FTIR /ATR	(Stead *et al.,* 2020)
Sediment (d.w.)	Density Separation (NaCl)	30% H_2O_2	Microscope + micro FTIR	(Wu *et al.,* 2020)
Sediment (d.w.)	Manual removal, Density Separation (NaCl)	None	Microscope + micro FTIR /ATR	(Lautaro *et al.,* 2023)
Sediment (d.w.)	Sieving, Density Separation (NaCl)	Fentons Reagent	Microscope + ATR-FTIR	(Almeida *et al.,* 2023)
Sediment (d.w)	Sieving, Density Separation (ZnCl2)	Fentons Reagent	Microscope	(Lloret *et al.,* 2021)
Sediment (d.w.) + Water	Density Separation (NaCl)	None	Microscope + micro FTIR /ATR	(Pinheiro *et al.,* 2022)
Sediment (d.w.)	Density Separation (CaCl2)	30% H_2O_2	Microscope + micro FTIR	(J. Li *et al.,* 2020)
Sediment (w.w.) + Water	Sieving	30% H_2O_2	Visual Identification + micro FTIR	(W. Yao *et al.,* 2019)

2.1.3.1 Sampling

Due to the regular inundation of salt marshes, both the tidal water and the marsh sediment can be good targets for microplastic sampling. Yet only 3 of the 8 salt marsh studies used water as a sampling medium. Stead et al. (2020) used the sea surface microlayer (SML) as the focus for their study, using water samples to demonstrate how microplastic

concentrations in this layer changed with the incoming and receding tides. However, in the other studies, a few water samples were collected (either in glass jars or filtered through syringes) to compare microplastic concentrations against (Pinheiro *et al.,* 2022; W. Yao *et al.,* 2019). The primary focus in over 90% of salt marsh studies has been on sediment samples. This is mostly due to the research aims of each study, with objectives focussed on themes like vegetation trapping ability, distribution, and retention of microplastics in the sediment. Within these sediment samples, however, there is still a split between dry and wet sediment reporting. Whilst most of the studies dried their sediment samples to a constant weight, Yao et al. (2019) reported their results as per wet weight of sediment. This may have been due to the large number of samples collected, resulting in the need for a faster methodology and analysis.

2.1.3.2 Microplastic Extraction

The extraction protocols for salt marsh sediment samples are the most consistent section of the methods, with studies employing a mix of sieving and density separation. Most begin with pre-sieving their samples (25 mm – 5 mm mesh sizes), while Lautaro et al. (2023) is the only study to apply manual removal of microplastics from samples. This is due to the focus of the study being on macro and larger (>1 mm) microplastics. After these preliminary steps, a density separation is then carried out in all protocols. Sodium chloride (NaCl) was the most common solution used (density 1.2 g/cm^3), found in half of the studies. Zinc chloride ($ZnCl_2$) and calcium chloride ($CaCl_2$) (densities of 1.5 – 1.7 g/cm^3) were used in a single study each (J. Li *et al.,* 2020; Lloret *et al.,* 2021). All these solutions have been demonstrated as effective in floating up microplastics (Prata *et al.,* 2019), and therefore the choice of chemical is likely up to the preferences of the researchers. This unanimity across different studies however shows that density separation is a highly valued and effective technique for extraction of microplastics from salt marsh sediments.

2.1.3.3 Digestion

Whilst the exact nature of the sediment will vary depending on its location around the world, and even its local distribution within the marsh itself, salt marsh samples tend to be heavily vegetated with lots of organic matter within the soil (Mcleod *et al.,* 2011). Therefore, it is not surprising that a digestion step was carried out in 75% of the salt marsh studies. All studies were consistent in the choice of digestion chemical, employing the use of the

oxidising agent hydrogen peroxide (H_2O_2). 30% H_2O_2 was used to immerse the extracted samples under varying conditions (50-70 ^oC, 2-24 hours). In some studies, an iron catalyst was added to make the solutions a Fentons Reagent, thus speeding up the process of digestion (Almeida *et al.,* 2023; Lloret *et al.,* 2021). H_2O_2 has been shown to be effective in removing organic matter, whilst having the least amount of impact on the plastics within the sample (Prata *et al.,* 2019). This may have been an important consideration for many of these studies, which go on to record visual characteristics of the plastics. For the studies which did not carry out digestion steps (Lautaro *et al.,* 2023; Pinheiro *et al.,* 2022), their focus was primarily on larger microplastic pieces and so could better differentiate their plastics from organic debris due to their increased size and recognisable physical appearance.

2.1.3.4 Microplastic Analysis

Whilst the nature of the sample has less impact on the analysis of extracted microplastics, salt marsh studies nonetheless have very similar protocols when it comes to microplastic identification. This is primarily achieved through visual identification, with all eight studies doing so with the aid of a stereomicroscope (4x – 120x magnification). Studies identified various physical characteristics, including morphology, size, transparency and colour. Whilst many studies imaged their particles, only Almeida et al. (2023) employed the use of automated characterisation of their samples. Automated particle counting allows for the rapid estimation of particles within a sample and allows for an efficient evaluation of particles below the visual limit of detection. This is particularly useful in environmental samples where smaller particle fractions dominate, and numerous samples are tested. However, this is also highly susceptible to contamination and so requires filters to be as clean as possible. Visual identification is used as it is cheap and effective for many samples. However, it also has a lot of bias and can lead to the misidentification of samples, especially in smaller particles as expected to observe in environmental samples. To validate their identification, many studies analysed a sub-set of their samples with either ATR-FTIR (>500 mm) or micro-ATR FTIR spectrometry (<500 mm), with only Pinheiro et al. (2022) and Lloret et al. (2021) forgoing any form of chemical analysis. FTIR is a common technique employed in microplastic confirmation, due to its wide availability and extensive databases. It is therefore not surprising to see it used so widely in salt marsh studies.

2.1.3.5 QA/QC

Perhaps the sole unanimity of protocols lies within the control measures employed. Despite the differences in sites, sampling and extracting techniques, digestion and analysis, core control procedures are shared in all the studies. These include avoiding plastic equipment where possible, carrying out work in contained environments and sealing samples/equipment when otherwise exposed, wearing cotton lab coats and nitrile gloves, rinsing all equipment and filtering solutions, and carrying out procedural blanks alongside the samples. These have been highlighted as the major quality control steps (Hanvey *et al.,* 2017) for any microplastic study, and salt marshes should be no exception. It is therefore encouraging that salt marsh studies are taking sufficient precautions, whilst at the same time highlighting that additional, salt marsh specific controls are not considered necessary.

2.2 Aims and Objectives

With no universal protocol for extracting microplastics from sediment samples, this methodology would have to be designed and adapted from the current processes available, and precedent within the research group. Based on the trends observed in previous salt marsh studies, the equipment available and the research goals for the project, there existed a framework from which testing could begin, focussing on the microplastic extraction, digestion and analysis steps.

The main aim of this chapter was to develop a successful extraction methodology for microplastics within salt marsh samples. This method will include steps which have previously been tested and can therefore be used directly, as well as some steps which required trialling and adaptation. This begins with i) testing the density separation, is the use of SMI units still effective when considering heavily vegetated samples? Following this ii) propose a digestion step: is digestion necessary for salt marsh samples, and which treatment processes prove most effective? Finally, iii) test the use of Nile Red staining method, specifically if it can still be used in heavily vegetated samples, and whether the various treatments still enable the successful automated counting of microplastics.

Following the results of each stage, the best practices will be taken forth and combined into a lab SOP, which will be the methodology used going forward when handling the eventual samples from our salt marsh study.

2.3 Methodology

2.3.1 Materials

Table 2.4 List of Chemicals showing Formula, Manufacturers/Suppliers and Purity

Chemicals	**Molecular formula**	**Manufacturer/Supplier**	**Purity (%)**
Potassium hydroxide	KOH	VWR/VWR	-
Sodium hypochlorite	NaClO	VWR/VWR	14% active chlorine
Hydrogen Peroxide	H_2O_2	VWR/VWR	30%
Ethanol	C_2H_6O	Acros organics/ThermoFisher scientific	95% purity
Nile Red	$C_{20}H_{18}N_2O_2$	Acros organics/ThermoFisher scientific	99% purity
Zinc chloride	$ZnCl_2$	VWR/VWR	-

Table 2.5 List of Instruments/Machinery showing: Model, Manufacturer and any further components

Instrument	**Model**	**Manufacturer**	**Add-ons**
Shaker	Rotamax 120	Heidolph	-
Orbital Shaker Incubator	ES-80	Grant Instrument	-
Incubating Mini Shaker	Incubating orbital mini shaker	VWR	-

FTIR Spectrometer	Hyperion 2000	Bruker	ATR crystal, Fluorescence measurements (Hg lamp)
Imaging Rig	N/A	Custom Made	Rig Model + Canon EOS 6000

2.3.2 Pre-existing Steps

Whilst the aim of this chapter was to test and develop a method, for certain basic steps, existing lab protocols were followed. These were used all throughout the project, and unless otherwise specified, the processes remained the same.

2.3.2.1 Procedural Controls and Contamination Prevention

Throughout the procedure 100% cotton lab coats were always worn, along with cotton clothing underneath. Nitrile gloves were worn, and all work was carried out inside laminar flow cabinets. All solutions and extraction equipment were similarly stored in laminar flow cabinets when in use.

All glassware and apparatus used in this protocol was washed three times with MilliQ water before every use. Where possible plastic equipment was minimised, and when impossible to remove, plastics were washed three times with MilliQ and regularly checked for any wear and damage. MilliQ water was used since it has been previously filtered and can be kept in 5L glass containers, so avoids contamination from taps.

All solutions were filtered (Cellulose Nitrate 2 µm) before use and stored in glass flasks with ground glass stoppers between procedural steps. Where possible, glassware for each sample was reused throughout the protocol to ensure no cross contamination of samples, and anything missed in early rinsing step was caught later.

The following additional steps were observed when possible, for contamination minimisation in the laboratory:

- Clean lab floor as well as bench surfaces (mop and/or paper towels). Wait at least one hour after completion for any residual dust to settle.
- Frequently clean the place where analysis is performed using a slightly damp lint-free cleanroom cloth or equivalent.

- Minimize the number of people allowed in the laboratory.
- Use of recovery tests (i.e. positive controls) with spiked sediment samples with known quantities of reference materials to investigate efficiency of applied extraction techniques.

2.3.2.2 Extraction using SMI Units

An SMI (Sediment Microplastic Isolation) unit consists of two sections of PVC pipe (sealed bottom, open top) connected with a ball valve in the centre. Both sections of pipe are connected to the valve via two exterior rings which screw tight creating a seal between pipe and valve. The SMI is then capped with either parafilm or aluminium foil (Figure 2.2). Whilst this design was first described by Coppock et al. (2017), the SMI units used in this research are smaller (diameter 2.8 cm, volume 300 mL). This made the units easier to construct and prepare, as well as allowing for multiple (up to 12) units to be ran in parallel.

Sediment samples are placed in a pre-cleaned 250 mL beaker, immersed in a small volume (20-30 mL) of zinc chloride solution (pre-filtered, 1.54 g/cm^3) and allowed to mix on an orbital shaker (100 rpm) for at least an hour. The sample is then poured into a set up SMI unit (pre-cleaned and rinsed with MilliQ water) and filled up with further zinc chloride solution till the level is 5-6 cm above the central valve (Figure 2.2). The valve is then rotated to released trapped air, before being kept in the open position. Sealing the top (parafilm in the method development, later swapped to rinsed aluminium foil in Chapters 3 and 4), the SMI is then inverted several times to ensure the sediment is thoroughly mixed throughout the solution. The SMI unit is then returned to its upright position and left to allow the sediment to settle and other material to float to the surface. Once the solution has lost its brown colour and all the sediment is settled on the bottom (Figure 2.2), the central valve is turned, separating the solutions in the top and bottom halves. The top solution (containing any floating material, vegetation, and plastics) is then poured into a separate beaker for later filtration. The top half of the SMI is then rinsed with zinc chloride solution, collecting any stuck material, which is added to the collection beaker. The SMI is then refilled with zinc chloride solution to the same level as before, the valve is opened and the process of

mixing, separating, and collecting is repeated. A total of three extractions are carried out to ensure all potential material is removed from the sediment.

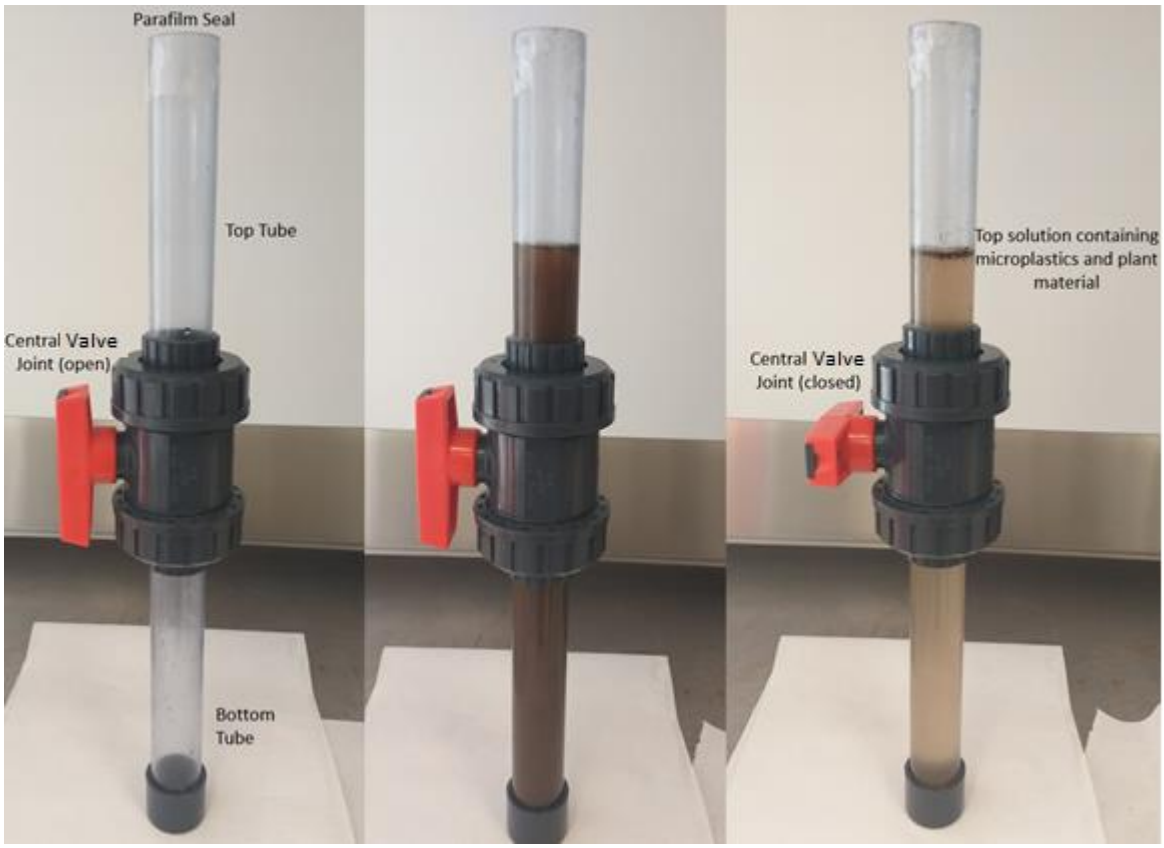

Figure 2.2 - Sediment Microplastic Isolation (SMI) units: empty showing components (left), after mixing (mid), and after separation (right).

2.3.2.3 Filtration

A glass conical flask, filter core and funnel piece are all pre-cleaned and triple rinsed with MilliQ water. The glassware is then assembled along with a membrane filter, held in place using a metal clamp. The filtration apparatus is then connected to a vacuum pump via a glass manifold (Figure 2.3) and the top sealed off using either a pre-rinsed petri dish, or clean aluminium foil. The extracted solution is poured into the apparatus, filtering off the solution and leaving only the collected material. The collection beaker is rinsed several times (using pre-filtered zinc chloride solution) and the rinsate filtered. The glass funnel is then rinsed (with zinc chloride solution) and filtered until all solution is gone and the collected material is dry. Unless otherwise stated, 47 mm cellulose nitrate filters (2 mm pore size) are used to collect any material from the filtration.

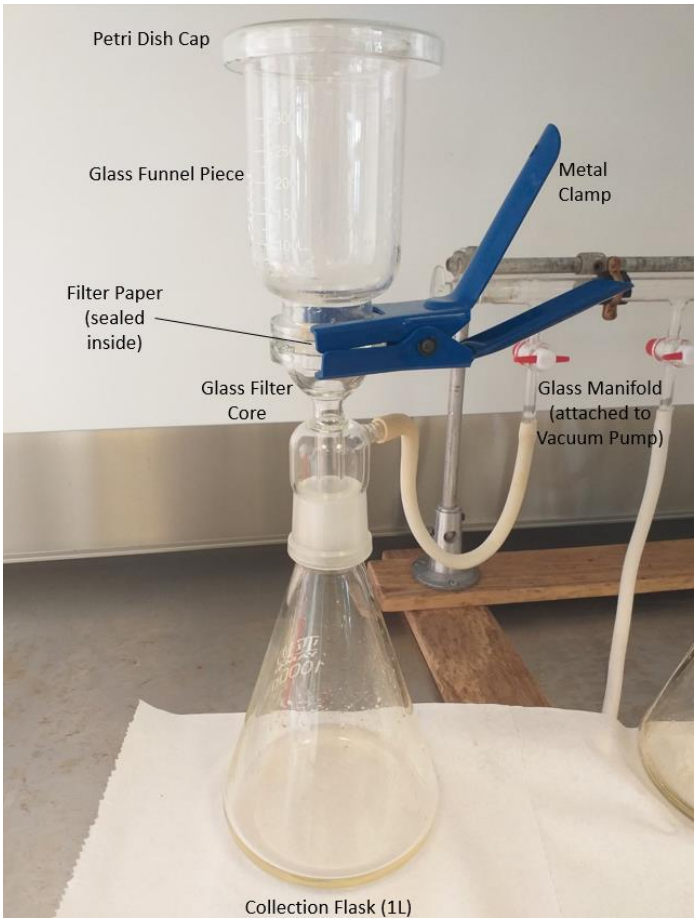

Figure 2.3 - The Filtration Apparatus, showing all individual components and final set-up as would be used in a laminar flow cabinet.

2.3.2.4 Recycling Zinc Chloride

Since zinc chloride is environmentally hazardous and can be costly, rather than immediately disposing any used solutions, they are instead recycled for future use. Zinc chloride from the SMI units is collected by pouring into a pre-cleaned flask. The SMI unit is tipped gently so as to not disrupt the sediment, and poured until the solution becomes dark brown. This remaining solution containing the sediment (approximately 20mls) is then flushed down the sink with lots of water. The collected zinc chloride solution is then filtered using the above filtering process. Finally, filtered zinc chloride is collected in a large flask and then subsampled for a density check. 100ml of solution is weighed in a volumetric flask and the density calculated. If the density is lower than 1.54g/cm^3, the refiltered zinc chloride is blended with a higher density solution (1.8g/cm^3) until the desired density is reached. This process of recycling is repeated several times, until the solution takes on a strong yellow colour. At this point the zinc chloride is stored for disposal and a fresh batch is made.

2.3.2.5 Nile Red

Nile Red solution is made by dissolving Nile Red powder in n-propanol at the following ratio:

Nile Red (mg): n-propanol (mL)

1:1

Volumes used vary depending on the amount of Nile Red required, however it is commonly made up in 20 mL batches. The solution is made up in a glass vial, and then thoroughly mixed to ensure all Nile Red powder has dissolved. Finally, this solution is filtered through a 0.2 µm syringe filter and transferred into a clean glass vial. The vial is then covered with aluminium foil until use.

For a staining solution, Nile Red solution is diluted into an Ethanol/Water mix (50:50) in the following ratio:

Nile Red solution (µL): Ethanol/Water mix (ml)

10:1

Using a micro-pipette, the Nile Red solution is added to the necessary volume of Ethanol/Water. The solutions are then mixed and wrapped with aluminium foil if not immediately used. The final solution can be left for up to two weeks during which staining efficiency should not be affected. After two weeks a new batch of staining solution was made up.

2.3.2.6 Staining

Pre-cleaned and rinsed glassware was set up in the same way as the filtration method (Figure 2.3). One apparatus per sample is made up, with an additional set-up for filtering solutions.

Samples are pre-cleaned and rinsed in the filtration apparatus. Once dry, the vacuum is turned off and a small volume (approximately 5 mL) of staining solution is added to the filter head until the sample is fully immersed. The solution is then left for 30 minutes to ensure effective staining of the sample. The solution can then be filtered off, and the filter removed for imaging or storage.

2.3.2.7 Imaging

Microplastics on the filters were counted using a fluorescence imaging technique (Maes *et al.,* 2017). Pre-stained filters were placed onto a motorised camera rig (Figure 2.4), containing a UV torch (Crime-Lite 420-470 nm Blue) and a modified Canon EOS 6000 (with a MP-e 65 mm macro lens and Hoya 55 mm Orange filter). By running a script in Mach 3 CNC, the camera and rig followed a programmed course taking a total of 24 photos exactly aligned edge to edge in a 6x4 array (Shutter Speed: 1/20, Aperture: f5.6, Iso:800). The images are then stitched together using the program ImageJ to create a single image of the fluorescing filter. Microplastics are then counted on the software ImageJ, using an automated particle identification tool which measures the relative brightness of the particles in comparison to the background filter. In this final array, an individual pixel is approximately 1.5 µm, so by selecting a minimum particle size (measured by area) of 9 (3x3) pixels (to avoid "bad pixel" bright spots on the camera imaging sensor due to electronic faults), the minimum detection limit of microplastics was 15 – 20 µm. Microplastics were then categorized into size groups; <50 µm, 50-99 µm, 100-199 µm, 200-399 µm, 400-999 µm, 1000-5000 µm. Imaged filters are then sealed in clean petri dished and stored for future use.

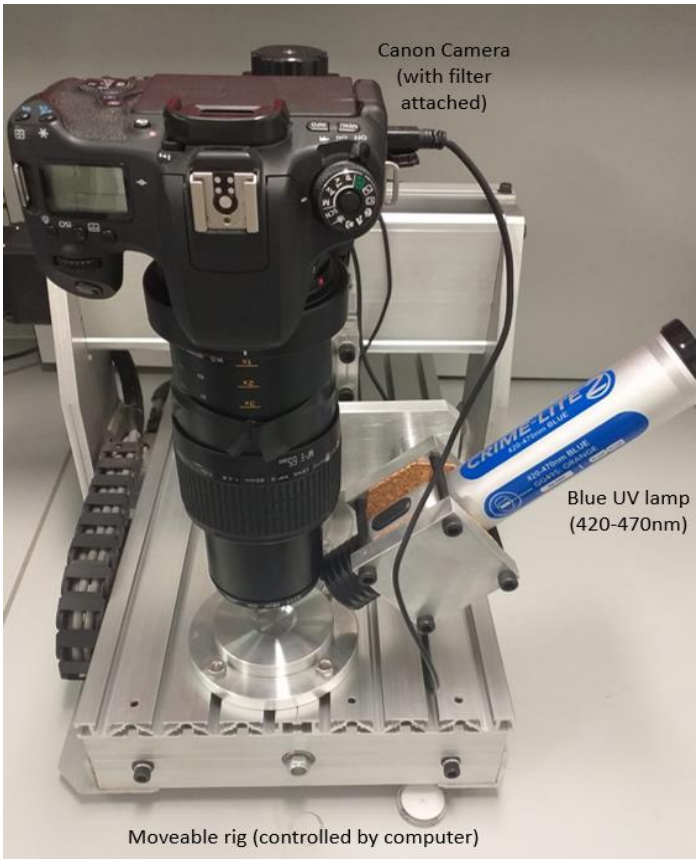

Figure 2.4 - Imaging Rig Components, showing the movable rig attached to the camera, and stationary UV lamp.

2.3.2.8 IR Analysis

Chemical conformation of microplastics was carried out using FTIR spectrometry. The instrument used was a Bruker Hyperion 2000 IR spectrometer, with built-in microscope and added fluorescence lamp (Figure 2.5). Samples can be analysed in reflectance, transmission or (micro) attenuated total reflectance (ATR) modes. Previously stained filters were placed in the landing tray under the microscope, whilst the instrument is set up using the OPUS software. Samples are found using the microscope (using a combination of 5x and 20x magnification lenses), with the fluorescence lamp being used to highlight the pre-stained microplastics, and imaged. Once particles have been found, the microscope is swapped to the ATR crystal, and a background sample run. Finally, details of the analysis are input on the OPUS software (exact measurement location, number of measurements, number of scans per measurement, saved data) before the measurement is left to run (2-5 minutes depending on the number of measurements and scans). Once the spectra have been run,

they can be analysed and compared with the built-in spectral library. Spectra are saved and stored for any further analysis. Once the spectra have been obtained, the landing resets and the filter is repositioned under the microscope. The next particle can then be analysed, going through the above steps once again.

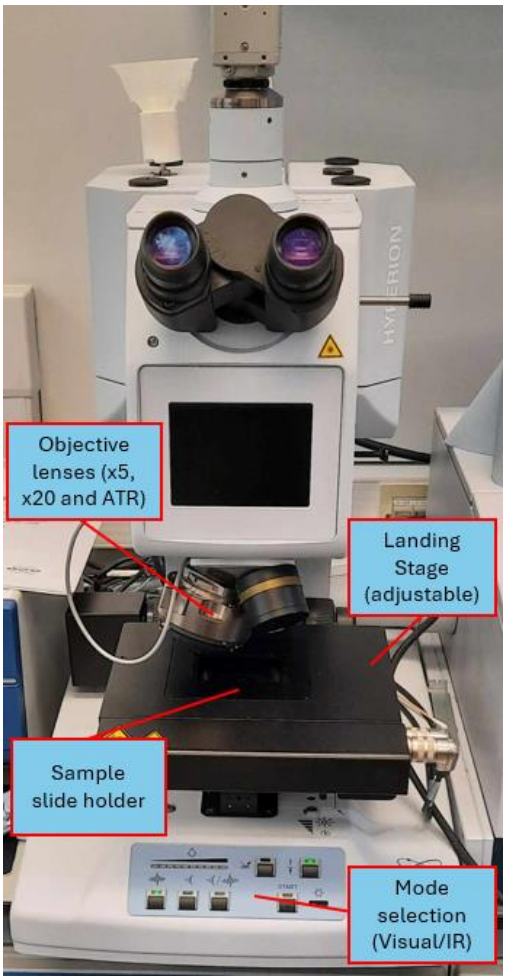

Figure 2.5 - Hyperion 2000 ATR spectrometer with components labelled.

2.3.2.9 Salt marsh samples

All salt marsh samples used in Chapter 2 were collected during an initial fieldtrip to observe potential marshes for future sampling. Samples were collected on 14/05/2021 from the salt marsh at Blakeney Harbour. The sampling site was north of the harbour carpark, situated approximately 30 m west of the North Norfolk Coastal Path (52.964237, 1.017565). Samples consisted of metal cores (approximately 40 cm long, 5cm diameter) knocked into the ground with a mallet, then extracted with salt marsh sediment and vegetation contained within (Figure 2.6). Samples were then wrapped in aluminium foil, transported in cardboard boxes, then stored in the lab freezer until used. When using sediment, large slices of the

core were cut off and left to defrost in a laminar flow cabinet. These were then dried in a vacuum oven (50^{o}C) to a constant weight, and then the appropriate amount of sediment for the experiment was weighed out. Any spare sediment was once again wrapped in aluminium foil and placed in the freezer.

Figure 2.6 - Initial salt marsh samples, showing; salt marsh location (left), core knocked into the ground (mid), and extracted core containing salt marsh sediment (right).

2.3.2.10 Storage

Unless otherwise stated, sediment and filter samples were stored in pre-cleaned petri dishes in a freezer at -20 ^{o}C to halt any ongoing sediment processes and preserve the microplastics in the sample. Chemicals were stored in their respective lab areas, or if solutions were made up, were kept in a separate, clean, laminar flow cabinet.

2.4 Impacts of Covid

The majority of the work done for this chapter was carried out during the restrictions of the covid outbreak. Whilst lab and fieldwork and was permitted, it was heavily regulated. Some restrictions included: limited lab space – use of a singular laminar flow cabinet for all work, limited lab time – had to schedule time with other lab users as no more than 2 individuals could be in the lab at the same time, limited equipment access – each lab users had their own designated lab equipment, however for things like glassware this was often quite minimal as the labs supply was split between several users. These restrictions had a significant impact on the experiments planned as part of the method development. Due to the space and time restrictions, most experiments were limited to only a single replicate. Staining and chemical analysis of microplastics was also not possible for several experiments due to demand for the instruments. Finally, in many cases experiments had to

be run on a sample-to-sample basis due to flow cabinet space preventing further apparatus set-ups. This means that some replicates were ran using different batches of sediment or solutions from the first samples in the experiment.

As such, the steps tested in this Chapter were used predominantly to demonstrate the proof of concept of each stage. Due to limited sample sizes, changes to the methodology were only considered if they showed significant variation within the experiment. Furthermore, results were compared with similar microplastic studies in order to judge the effectiveness of any steps tested. If any further restrictions occurred as part of the covid safety measures, they are noted in the individual experiment methodologies.

2.5 Method Development – Testing Experimental Steps

2.5.1 Microplastic Extraction

Whilst density separation protocols have been used in several salt marsh studies (Pinheiro *et al.,* 2022; Wu *et al.,* 2020; W. Yao *et al.,* 2019), the chemicals and equipment used in each study was different. Despite having been demonstrated as an effective extraction method for sediments (Coppock *et al.,* 2017), SMIs have yet to be used on salt marsh samples. How much sample can be loaded, does vegetation potentially obscure particles, or trap microplastics in the sediment and prevent them from floating up in the solution? These experiments aimed to test the efficiency of SMI's for extracting microplastics from salt marsh samples.

2.5.1.1 SMI Recovery Rates

The aim of this test was to determine the effectiveness of SMI units when handling salt marsh samples. This was achieved by testing the recovery rates for different types and sizes of plastic spiked in salt marsh sediment.

This trial compared similar plastics to those we might expect to find in salt marsh samples. Fibres and fragments are the most abundant microplastics in salt marsh studies (Almeida *et al.,* 2023; Pinheiro *et al.,* 2022), and so nylon fibres (1-10 mm length, 1 mm width) and chipped polystyrene fragments (<2 mm) were selected as the test samples. These would represent two very different shapes of microplastics (fibres would be more easily stuck in

vegetation) (McIlwraith *et al.,* 2024), whilst also being distinctly fluorescent (when stained with Nile Red) and so could be easily counted.

Smaller plastics were not tested as these would be much harder to manually make and count out, whilst the selected plastics would also not be confused with any plastic contamination that may already have been in the salt marsh samples.

Method

Three different lengths of fibre were tested: 1-2 mm, 2-4 mm, and 10 mm lengths (all <1 mm diameter), along with two sets of fragments. Each test was replicated three times. These microplastic were made up in the laboratory and stored in glass vials till used.

Salt marsh sediment samples were taken from pre-sampled cores (2.3.2.8), using only the top 2 cm of each core to ensure there was a significant amount of biomass present in the sample. The sediment was measured into a beaker and immersed in zinc chloride solution (1.54 g/cm^3), where microplastics were then manually counted out and added to the sample. The exact number of microplastics was different in each sample, with a random number between 25-50 selected. Using two people the microplastics were added independently, then the samples switched so that there was no knowledge of the microplastic content in each sample, and bias toward counting removed. Finally, the samples were transferred to the SMI units, and the SMI Extraction procedure followed.

Following extraction and filtration steps, the recovered plastics were stained with Nile Red and then imaged under blue UV light, and the particles manually counted.

Results and Discussion

The results show that the SMI units had high recovery rates, ranging from 75.6 – 100%, with a combined average across all samples of 91 ± 3.5% recovery (Table 2.6).

Table 2.6 Average recovery rates of different fibre and fragment samples (n=3) extracted from salt marsh sediment, using SMI units

Sample (n=3)	**Average Recovery Rate (%)**
Fibres 1-2 mm	93 ± 3.7
Fibres 2-4 mm	89 ± 11.6
Fibres 1 cm	93 ± 5.0
Fragments Set A	86 ± 7.6

Fragments Set B	95 ± 3.0
Total Average Fibres	92 ± 2.0
Total Average Fragments	90 ± 4.7

With little variation between the various fibres and fragment tests (standard deviation 2 – 4.74%), this shows that the SMI units are producing consistent results across different sample types. When comparing these values to studies in the literature, we observe similar results. Whilst exact figures vary from study to study, density separations normally report extraction efficiencies in the range of 90-99% (Quinn *et al.,* 2017). SMI unit studies report values of recovery > 80% (Nel *et al.,* 2019), and the combination of SMI units along with zinc chloride has been shown to produce average recovery rates of up to 95.8% (Coppock *et al.,* 2017). These samples show similar recovery averages, and so whilst a little lower, are still well within an acceptable range of performance. The variance in recovery rate might be due to the difference in sample type, with the published studies being carried out on marine sediments. Compared with our samples, they lack the vegetation that may otherwise disrupt the floating and counting of microplastics. Furthermore, it was noted that the greatest loss of plastics came not from failure to extract, but rather particles adhering to the glassware during filtration even after rinsing. These particles were observed during the experiment, however, were not manually added to the final counts since this would not be possible for sub 1 mm particles, and therefore their loss should be represented. To minimise this loss in future, glass filter funnels with more gently sloped gradients were used and rinsed 3 times to wash down as much microplastic as possible. This issue was also further explored in section 2.5.4. Regardless, even with the loss of this portion, recovery rates were still deemed acceptable (>90%) and thus the SMI method was proven effective for microplastic extraction specifically in salt marsh samples.

2.5.1.2 SMI Unit Separation Duration

Many variables differ between SMI studies, such as size, salt solution and the duration during which solutions are left to settle. The latter can often be very varied, depending on the time it takes for plastics to float up, and for the sediment to settle. This varies greatly from coarse sediments such as sand to fine silts and muds. Reported settling times therefore vary from 5 minutes (Corcoran *et al.,* 2015) to over 12 hours (Klein *et al.,* 2015).

Salt marsh sediments can have considerable difference in composition even across a small area (Bradley and Morris, n.d.). The sediment is predominantly made up of sand, clay, and silt particles, however the exact fractions change depending on location within the marsh, resulting in varied sediment densities and settling velocities across different samples. With a potential broad range of settling rates, we tested the optimal duration of separation to ensure maximum extraction efficiency was obtained, whilst minimising the amount of sediment collected alongside the microplastics.

Method

Samples were made up using the pre-collected salt marsh sediments and then spiked with a mix of fibres and polystyrene beads (using the randomised blind counting from section 2.5.1.1 to avoid any bias). Samples were then transferred to the SMI units and the standard extraction protocol was followed. The selected durations for separation were 3, 4, 6, 8, 16, and 24 hours, with a replicate for each time period (Set A and Set B). After each time period, the set up was visually observed for zinc chloride solution clarity before extracting microplastics. Visual clarity was recorded as; murky – solution is still very muddy, predominantly brown colour, cloudy – solution is a paler yellow but still has distinct particles floating in it, and clear – solution is off white and clearly transparent. Following this the samples were extracted, filtered, stained, and imaged as per the standard methods.

Due to space limitations the replicate set (B) was ran one week after the first (A). The same experimental conditions were used for both runs; however new batches of chemical solutions may have been used for the replicate run.

Results and Discussion

Recovery rates of each Set were plotted against duration to see if the length of time left to settle had any impact on microplastic extraction (Figure 2.7). The recorded observations of zinc chloride clarity for each duration test were recorded to determine how quickly the sediment settles after mixing (Table 2.7).

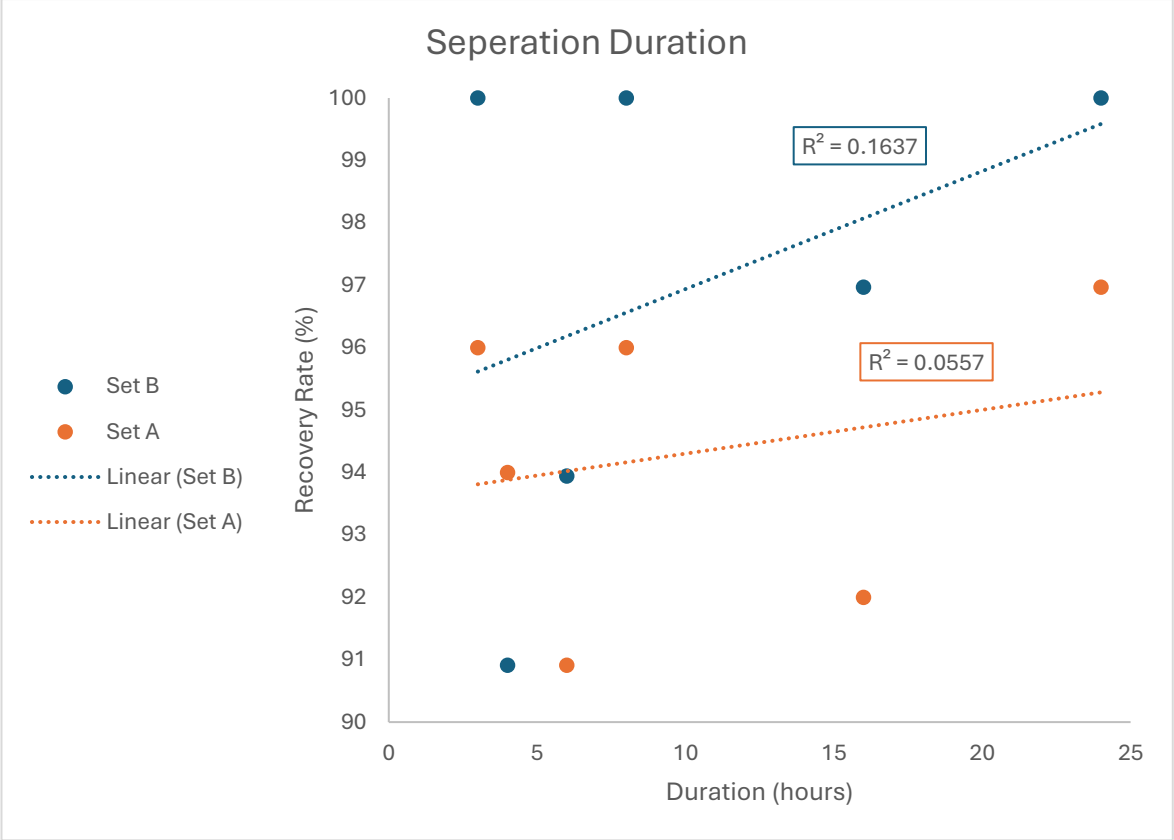

Figure 2.7 - Recovery rates of spiked fibre/fragment mix from salt marsh sediment using SMI units with different separation durations.

Table 2.7 Visual clarity of zinc chloride solution after different separation durations

Separation Duration (hours)	**Visual Clarity**	
	Set A	**Set B**
3	Murky	Murky
4	Murky	Murky
6	Murky	Murky
8	Cloudy	Cloudy
16	Cloudy	Cloudy
24	Clear	Clear

Simple linear regression was used to test if duration significantly affected the recovery rate of microplastics. For Set A, the regression was found to be statistically non-significant (R^2 = 0.0557, $p > 0.05$). For Set B, the regression was again found to be statistically non-significant (R^2 = 0.1637, $p > 0.05$). Therefore, it was concluded that increasing duration did not significantly increase the recovery rate of microplastics.

From the visual clarity observations, it was found that whilst the majority of the sediment settled in the first 6-8 hours, the solution still remained cloudy and it wasn't until 24 hours after mixing that the solution returned to its original colour and all the sediment had settled into the bottom half of the SMI.

All the durations showed high recovery rates of over 90%, with 100% recovery being found in samples at 3 hours, 8 hours and 24 hours (Figure 2.7). These recovery rates are comparable with the previous test (Section 2.5.1.2, 12-hour separation), as well as the Coppock et al. (2017)Coppock et al. (2017)Coppock et al. (2017) SMI study, suggesting that increasing the separation time should have no impact on extraction efficiency. Based on these, it can be concluded that microplastics likely float up during the first 3 hours of separation. However, despite this the selected duration for the final protocol was 24 hours. This was not based on the recovery rates, but rather the rate at which sediment settled during the extraction. In the 3–8-hour samples, sediments were observed to still be settling in the solution, evidenced by the brown colour of the zinc chloride. This suspended sediment was therefore extracted along with the microplastics in the supernatant solution, and so ended up on the filter alongside them. Whilst this layer of sediment was found to not interfere with the spiked control samples, this is because they were intentionally larger and brightly fluorescing particles. When considering potential particles in environmental samples may be much smaller and more easily obscured, it was deemed necessary to minimize the amount of sediment which ends up on the filter. After 24 hours the solutions were visually observed to have returned to their regular transparent state, with all sediment having deposited on the bottom. Thus, while duration did not affect the extraction efficiency of microplastics, it did reduce the sediment contamination on the final filters and so was adapted into the protocol accordingly. This will likely have a significant effect on the counting of small microplastics in real samples, as well as making the recycling of the zinc chloride solution (section 2.3.2.4) much easier.

2.5.2 Digestion Tests

The current salt marsh literature showed a clear need for a digestion step as part of the protocol, with most studies utilising this step. Following the examples of previous salt marsh studies, the most widely used digestion treatments were selected, hydrogen peroxide (J. Li *et al.,* 2020) and Fentons reagent (Almeida *et al.,* 2023). In addition to this, an alkaline

mixture (30% KOH:NaClO v:v) (Bakir *et al.,* 2023; Enders *et al.,* 2017) was tested as recent studies demonstrated its effectiveness in digesting biota and sediment (Enders *et al.,* 2017), and this could provide a good pilot for its effectiveness in salt marsh samples. Acidic treatments were not trialled due to concern over damage to the microplastics within the sample (Claessens *et al.,* 2013), whilst enzymatic treatments were deemed too costly and time consuming to be considered. The aim of this experiment was to determine which digestion treatment would be most effective for the salt marsh methodology.

Previous studies also vary in when the digestion is carried out (Pfeiffer and Fischer, 2020). Even within the salt marsh literature, studies are split into two categories, digestion preceding the extraction (Lloret *et al.,* 2021; Wu *et al.,* 2020), or following it (Almeida *et al.,* 2023; J. Li *et al.,* 2020). In pre-extraction, the entire sediment sample undergoes digestion, or post-extraction when only the material collected from density separation is digested. Early digestion allows for an easier extraction of microplastics, as the sediment is looser and less likely to trap material. Doing digestion after extraction however results in significantly lower sample volume, thus is much easier to handle and is more economic.

When considering these salt marsh samples, the decision was made to introduce the digestion step after extraction, without running any tests. This is because many of the samples contained high amounts of biomass, and so considerable quantities of digestion solution would need to be made up. Furthermore, the largest material is often the hardest to digest and so would still be left in a pre-extraction digestion. However, by extracting and then sieving the samples, tougher materials were removed (thick stems, twigs etc) before digestion occurs.

2.5.2.1 Digestion - Solution Volume

Within the literature, volumes of digestion solutions varied considerably, from 10 mL up to 100 mL (in pre-treatment digestion) depending on the sample volume but was often just not reported. As a post-extraction treatment, the goal was to reduce the amount of solution needed to minimise the risk and cost. We hypothesize that a greater volume of solution will digest more material, so a balance must be found ensuring digestion is completed whilst reducing the solution used. Therefore, volumes of 10, 20 and 30 mL were selected to test on small sediment samples (10 g).

Initial tests were performed after the filtration step, adding the digestion solutions directly into the filtration apparatus. This removed the need to transfer filters between glassware and meant the digestion solution could then be immediately filtered off once complete.

Methods

The three solutions tested in the protocol were; 30% KOH: 0.5M NaClO v:v, 30% H_2O_2 and Fentons Reagent (H_2O_2 with an iron (II) sulphate catalyst).

Solutions were made up in advance using the following recipes.

H_2O_2: bought directly from Thermo Fischer Scientific at 30% in water (H_2O_2 already in solution).

Fentons Reagent: 0.1M iron (II) sulphate solution was made up by weighing out 2.78 g of $FeSO_4.7H_2O$ and dissolving it in a small amount of MilliQ water. The solution was then made up to 100 mL using further MilliQ water. 20 mL of the iron (II) sulphate solution was then mixed with 20 mL of 30% H_2O_2 and gently heated for the 30 minutes. Finally, the pH was measured using a pH probe and adjusted with concentrated sulphuric acid until the pH was 3.0. Fentons reagent solutions were made up separately from the other digestion solutions in a fume cabinet due to the potential of the reagent frothing up and spilling if the solution overheated.

KOH NaClO: 30% KOH solution was made up by weighing out 30 g of solid KOH granules and then agitating them in MilliQ water until dissolved. The solution was then made up to 100 mL with MilliQ water. 1M NaClO was bought and then diluted with MilliQ water (50 mL of each solution). The 100 mL solutions of KOH and NaClO were then gentled mixed using a magnetic stirrer plate.

Salt marsh sediment samples were prepared following the extraction and filtration protocols as outlined above. For each digestion solution, volumes of 10, 20 and 30 mL were added to the filtered samples. The solutions were then left for 72 hours and then filtered off. This was repeated so each solution and volume had two replicates. The results of digestion were estimated visually. Each filter was split into 8ths, and individual sections checked for obscuring vegetation (if at least half the section had vegetation present it was considered covered), with the total amount of covered sections then totalled (Figure 2.8).

The effect of digestion was therefore characterised by how much vegetation remained; considerable vegetation present (++) i.e. the filter is at least 50% obscured by vegetation (5+ sections covered), vegetation present (+) i.e. 25 to 50% vegetation coverage on the filter (2-4 sections covered), and vegetation reduced (-) i.e. less than 25% vegetation coverage on the filter (<2 sections covered).

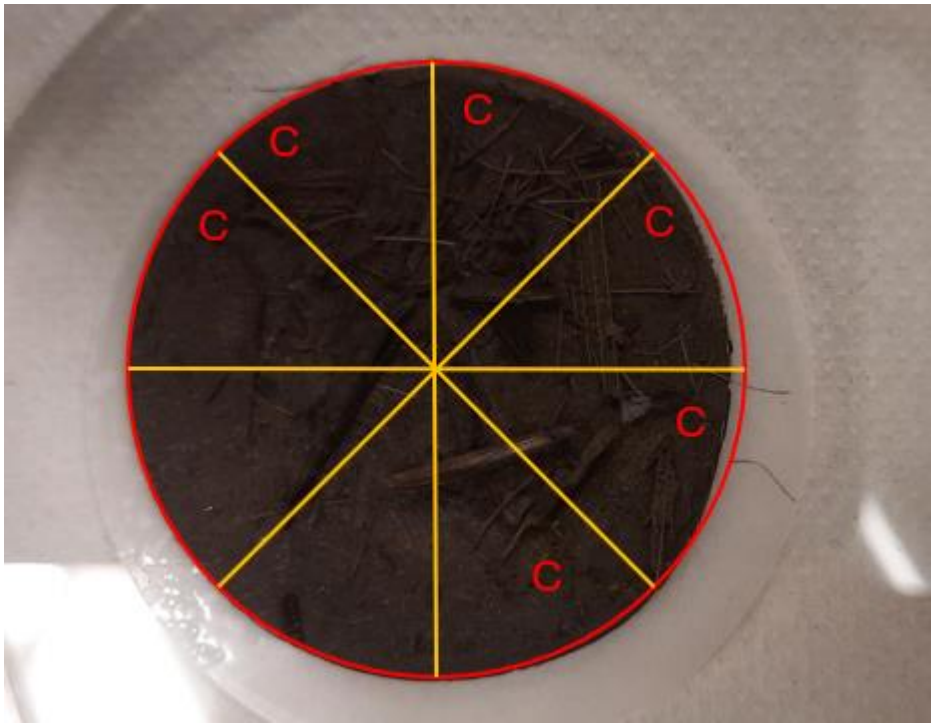

Figure 2.8 - Filter after digestion test showing how the surface was visually broken into sections and checked for obscurity. C represent sections that were observed to be at least 50% covered by vegetation.

As a further test, the 30 mL samples for each solution were then stained with Nile Red and imaged following the imaging protocol. The presence of vegetation and its potential staining was then observed and recorded.

For each digestion and solution, a replicate (Set B) was run on the following week following the exact same procedure. This was due to limitations from covid restrictions (section 2.4), but also the need to make up Fentons reagent in smaller batches (maximum 100 ml) due to working in a confined fume cupboard. Due to the lab work time restrictions, along with poor initial results from all digestion solutions, a 3rd replicate of this test was not run.

Results and Discussion

The results (Table 2.8) show that all the digestion solutions and all volumes had at least 25% of the filter still covered by vegetation after completion. All digestion solutions were equally effective in digesting some of the plant material, but all of them still had visible organic matter left on the filter. For the solutions, both 10 and 20 mL seemed least effective. 30 mL saw slight improvements for each solution however, digesting up to 50% in some samples, and therefore this was the volume used going forward in further tests.

Table 2.8 Vegetation coverage of filters after immersion in digestion solutions for 72 hours; considerable vegetation present (++, >50%), vegetation present (+, 25-50%), vegetation reduced (-, <25%)

Digestion Solution	**Filter Coverage (%)**	
	Set A	**Set B**
Fentons Reagent (10ml)	++ (>50)	++ (>50)
Fentons Reagent (20ml)	++ (>50)	++ (>50)
Fentons Reagent (30ml)	+ (25 – 50)	+ (25 – 50)
30% H_2O_2 (10ml)	++ (>50)	++ (>50)
30% H_2O_2 (20ml)	++ (>50)	++ (>50)
30% H_2O_2 (30ml)	++ (>50)	+ (25 – 50)
30% KOH: 0.5M NaClO (10ml)	++ (>50)	++ (>50)
30% KOH: 0.5M NaClO (20ml)	++ (>50)	+ (25 – 50)
30% KOH: 0.5M NaClO (30ml)	+ (25 – 50)	+ (25 – 50)

The stained 30 mL filters (Figure 2.9) show the presence of vegetation in each of the treatments, which is then being stained and fluorescing. This fluorescence is brighter than the background so stands out and could be mis-identified as plastics by the automatic particle counting.

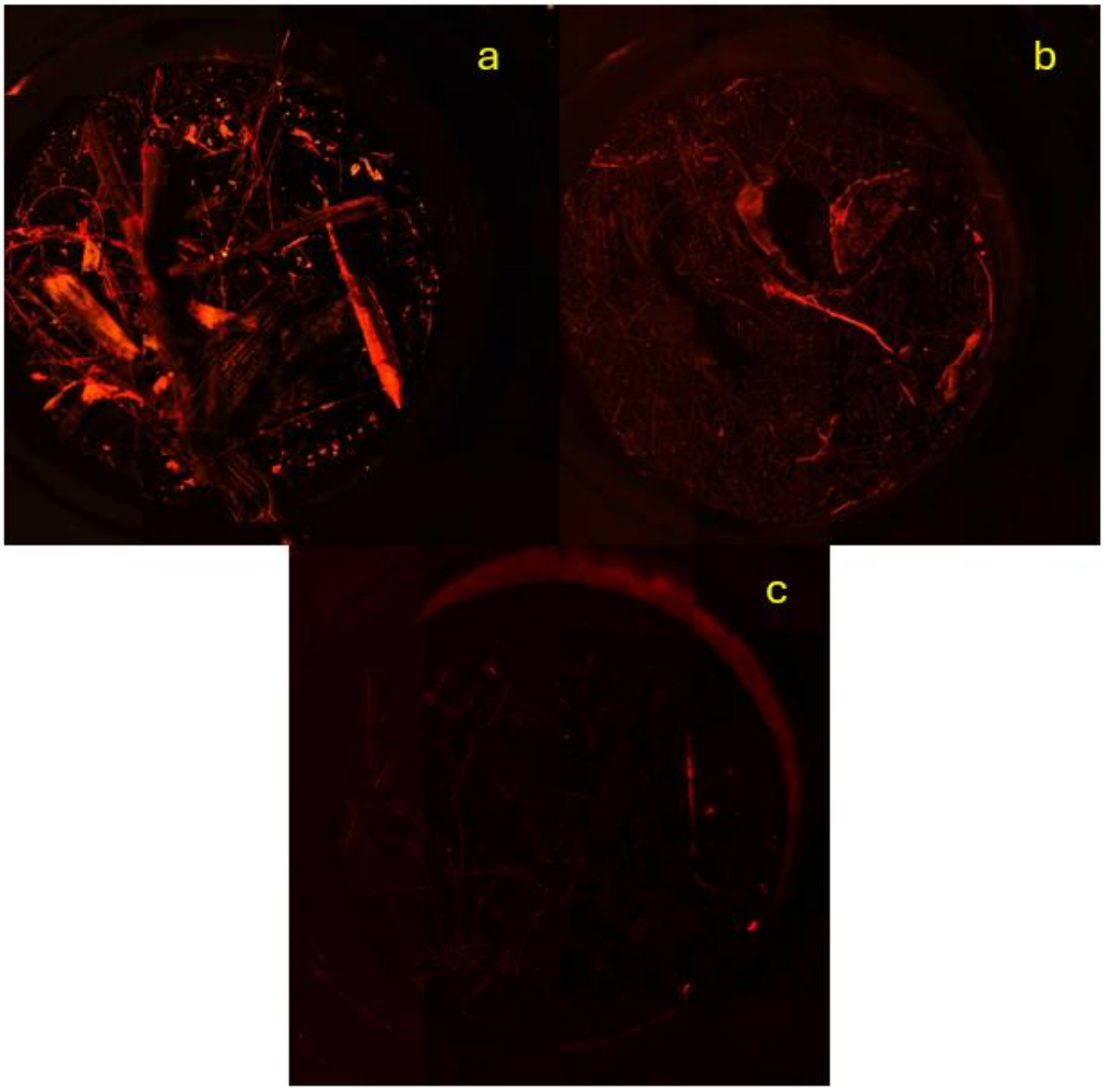

Figure 2.9 – Nile Red stained filters after 72 hours in digestion solutions (30ml); H_2O_2 (a), KOH NaClO (b) and Fentons Reagent (c).

When comparing the different volumes, it was observed that all volumes of each treatment were insufficient, with the best results still showing up to 50% vegetation present on the filter. This is evidenced in the fluorescent images, with vegetation pieces being large enough to detect by eye. However, compared to the 10 mL test of each treatment, this is a considerable improvement, with over 50% of the filter covered in these tests. Increasing the volume of digestion solution caused a visibly notable decrease in the amount of the vegetation. When comparing the different treatments, no solution proved significantly better than the others, with no protocol reducing vegetation to less than 25% coverage. With these treatments having demonstrated efficiencies from 75 - 100% digestion of

organic material (Tan *et al.,* 2022), used in this way the digestion solutions were found to be insufficient, and so further testing was required.

2.5.2.2 Digestion – Agitation

Whilst often not reported, agitation has been used during the digestion step as a way of improving the treatment's efficiency. (Lloret *et al.,* 2021) employed the use of a magnetic stirrer during digestion, whilst (Tan *et al.,* 2022) carried out all treatments in a shaker-incubator. By immersing the filters in digestion solution, and then agitating the samples, the hypothesis was that the increased contact between organic material surface and the solution should result in more effective digestions.

Method

Samples and solutions were prepared, extracted, and filtered as per standard procedure. However, rather than adding the digestion solutions directly into the filtration apparatus, 30 mL of each digestion solution was measured into pre-cleaned beakers. The filters were then transferred into the solutions, and the beakers sealed with aluminium foil. The beakers were then placed on a shaker (Rotamax 120, Heidolph) at 100 rpm and left for 72 hours.

After digestion, the solutions were collected, with material scraped off the filters if necessary. The solutions were then refiltered once again, and the level of vegetation coverage observed. Finally, a filter of each treatment was then stained and imaged to determine how much the contamination might affect the counting.

Results and Discussion

The results show that the digestion solutions influenced the vegetation coverage (Table 2.9). Compared with the results of the previous digestion test (Table 2.8), agitation led to a substantial improvement in the total vegetated matter digested with no filter having more than 50% coverage by vegetation, and at least half of all agitated filters having less than 25% coverage. This was most noticeable in the KOH:NaClO treatment, where both filters showed the greatest reduction in vegetation and thus the cleanest filters.

Table 2.9 Vegetation observed after immersion in digestion solution (30ml) for 72 hours with and without agitation; considerable vegetation present (++, >50%) vegetation present (+, 25-50%), vegetation reduced (-, <25%)

Digestion Solution (30mL)	**Filter Coverage (%)**			
	With Agitation		**Without Agitation**	
	Set A	**Set B**	**Set A**	**Set B**
Fentons Reagent	- (<25)	+ (25 – 50)	+ (25 – 50)	+ (25 – 50)
30% H_2O_2	+ (25 – 50)	+ (25 – 50)	++ (>50)	+ (25 – 50)
30% KOH: 0.5M NaClO	- (<25)	- (<25)	+ (25 – 50)	+ (25 – 50)

Each of the stained images (Figure 2.10) show the presence of vegetation, however the coverage is overall quite low. The H_2O_2 treatment shows the greatest amount of vegetation stained, however it is still present and observable in the other two solutions as well.

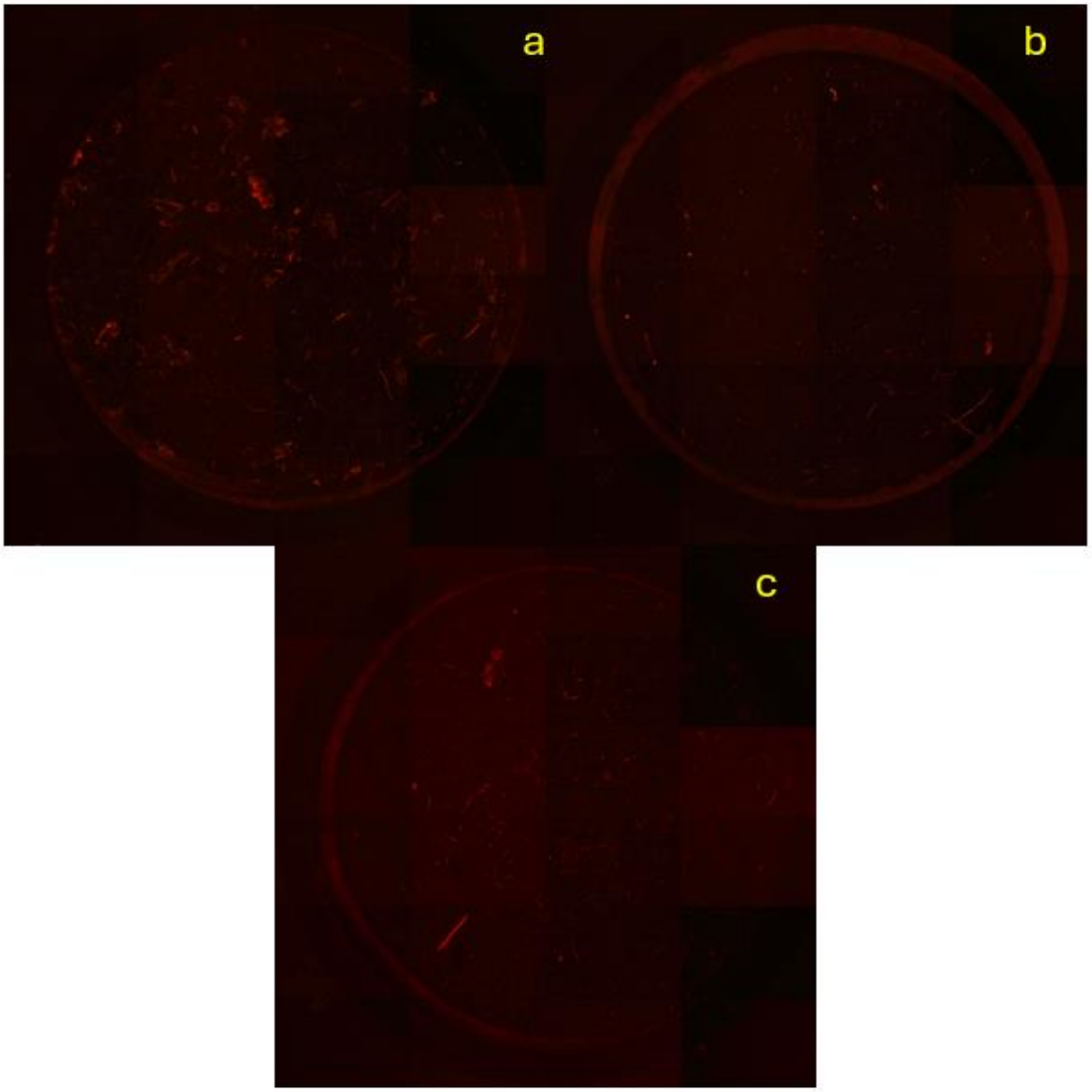

Figure 2.10 – Nile Red stained filters after 72 hours in digestion solution (30 ml) under agitation; H_2O_2 (a), KOH NaClO (b) and Fentons Reagent (c).

Each solution saw an increase in the digestion efficiency, suggesting that the agitation has had a positive effect. When comparing the treatments however, there is no clear difference between the solutions. Whilst the Fentons Reagent and Alkaline treatments show the best results, the measurements are based on visual observations and so cannot accurately quantify this improvement. Furthermore, when considering the staining results, it is apparent that there is still enough vegetation coverage to show up in the imaging and counting process. Such particles could be manually removed from the counting; however, this is only possible for large, obvious pieces. Smaller particles would be impossible to

distinguish, and therefore to eliminate the possibility of potentially miscounting microplastics, greater digestion efficiency was deemed necessary. Therefore, whilst agitation improved digestion efficiency, vegetation coverage was still too high and so further steps need to be trialled to make the protocol suitable for the automated particle counting process.

Additionally, during this test it was noted that the cellulose nitrate filters were not dissolved in the H_20_2 and Fentons Reagent treatments. To re-filter the sample, they had to be manually scraped off with a spatula and then rinsed to get any remaining material. This was a complicated process and lead to loss of the sample, and thus potentially loss of microplastics.

2.5.3 Digestion Tests – Part 2

With the introduction of agitation showing improved efficiency, further factors were considered that might improve the amount of vegetation digested. Increasing the concentration of the various solutions was decided against, since these concentrations are widely used across the multiple sediment studies (Prata *et al.,* 2019). Changing the concentration of each solution reduces the comparability of our results to other studies, as well as potentially increasing the likelihood of damage to the microplastic samples. As damage to microplastics would be hard to test and measure, the concentrations were kept the same to eliminate any possibility of increasing risk.

Therefore, increasing the duration of digestion was tested instead, running the digestion under heated conditions, and sieving out portions of the organic biomass.

2.5.3.1 Digestion - Treatment Duration

Like the other factors, there is a wide range of different times over which digestion protocols are used, varying from 30 minutes (Lloret *et al.,* 2021), to 24 hours (J. Li *et al.,* 2020) and even up to a week (Cole *et al.,* 2014). Previous samples in the other tests were left to digest for 72 hours. This was selected as it coincided with the wait time between experimental extraction runs. However, this might not be enough time for digestion to run to completion, and therefore to test whether digestion is still ongoing, a longer experiment for one week

duration was designed, which was further extended to two weeks for one set of samples. It was hypothesized that increasing duration time should increase the digestion efficiency.

Method

Samples and solutions were prepared as before in the agitation test, with samples immersed in the digestion solutions and left in beakers on a Rotamax shaker. Samples were left for a week, after which the solutions were observed for organic matter. For Set A (1 week), the samples were refiltered, coverage recorded and then stained and imaged using the standard protocols. For Set B (2 weeks), they were immersed in the filtered off digestion treatments and then put back on the Rotamax for another week. After this week the recording was repeated, and the staining and imaging carried out.

Results and Discussion

After the one-week extended digestion, all samples showed less than 25% coverage of the filters (Table 2.10). H_2O_2 treatment was the only solution showing more than 25% coverage after the first week, but after the second week it too had a reduced coverage. Other Filters from Set B with <25% coverage had no noticeable difference between weeks 1 and 2, nor was there an observable difference between each of the solutions filters categorised this way. The stained filters still showed that vegetation was present enough to be stained and be visible during the counting (Figure 2.11).

Table 2.10 Vegetation coverage on filters after immersion in digestion solutions (30mls) for extended periods (1 week and 2 weeks); considerable vegetation present (++, >50%), vegetation present (+, 25-50%), vegetation reduced (-, <25%)

Digestion Solution (30ml)	**Filter Coverage (%)**		
	Set A (1 Week)	**Set B (1 Week)**	**Set B (2 Weeks)**
Fentons Reagent	- (<25)	- (<25)	- (<25)
30% H_2O_2	- (<25)	+ (25 – 50)	- (<25)
30% KOH: 0.5M NaClO	- (<25)	- (<25)	- (<25)

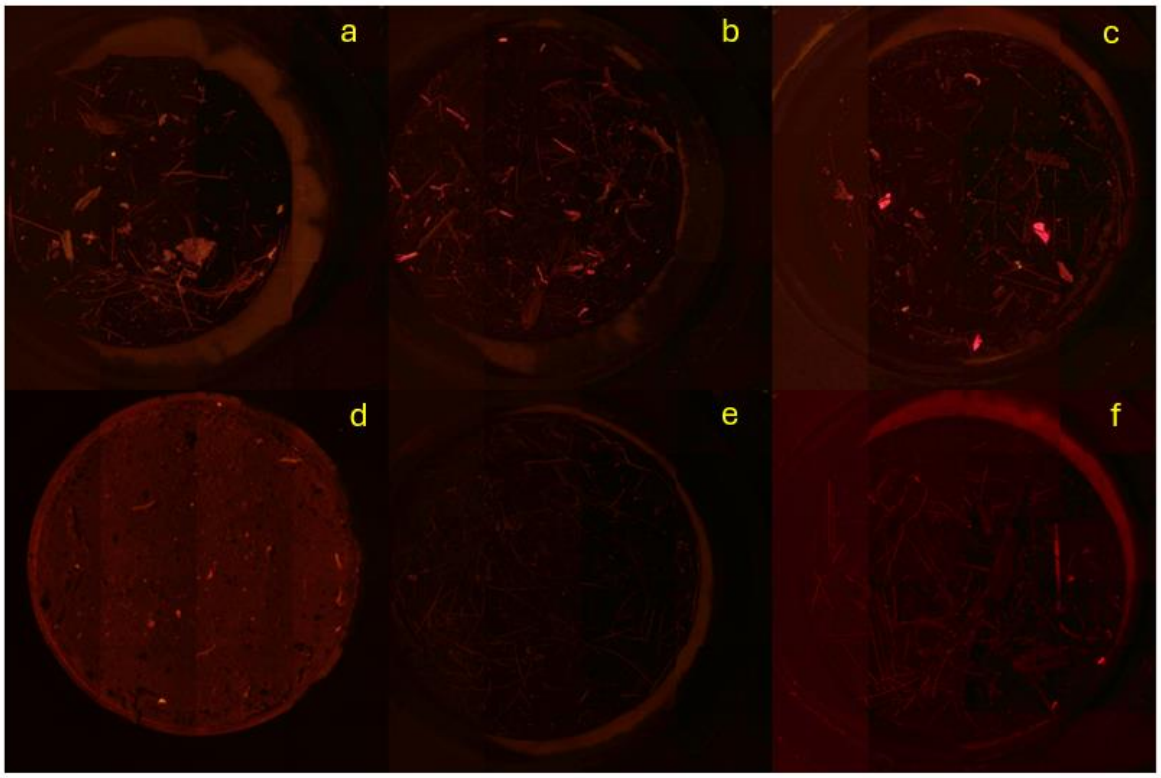

Figure 2.11 – Nile Red stained filters after 1-week (top) and 2-weeks (bottom) in digestion solutions(30ml) under agitation; H_2O_2 (a,d), KOH NaClO (b,e) and Fentons Reagent (c,f).

As H_2O_2 was the only treatment to show a reduction in efficiency between the 1- and 2-week samples, this suggests that both KOH:NaClO and Fentons Reagent run their digestions to completion in under one week. However, despite all digestions being run to completion, each of the filter's still showed vegetation was present during the imaging. It was concluded that increasing the duration of digestion is still insufficient for improving the digestion efficiency of the treatments. Even with the increased duration the filters in the Fentons Reagent and H_2O_2 treatments remained partially dissolved, thus contaminating the sample further. Finally, with extraction taking 3-4 days, having an increased duration digestion step would extend the methodology further, meaning that from sediment extraction to final imaging, 2 weeks would be required. This length of methodology is too long when considering processing hundreds of samples. Therefore, based on insufficient improvements to digestion and the increased length of the protocol, extending the duration of the digestion beyond 72 hours was not a justifiable improvement and was removed from the methodology.

Despite not improving the methodology, the duration test did allow us to observe the effects of running the digestions to completion. None of the digestions were effective enough, even when run for twice as long as the expected completion time. Being unable to increase the concentration of the digestion solutions, a test was proposed to decrease vegetation extracted, whilst increasing the treatments temperature to digest more material.

2.5.3.2 Digestion - Sieving Step and Increased Temperature

Sieving

From the results of the treatment duration test, we were able to observe what the potentially completed digestion looks like. Whilst this demonstrated the best digestion efficiency so far, it still failed to remove all the organic material, particularly struggling with tough plant parts such as pieces of twig and seed capsules. Furthermore, whilst coverage of the filter was being measured, this did not represent the percentage of vegetation successfully removed. The effect of digestion was often visually noticeable in terms of reducing the organic material; however such was the initial quantity in the sample pre-digestion, it would remain prevalent even after the treatments. To reduce this initial volume of plant material, a sieving step was proposed.

Sieving steps are widely implemented across sediment studies (Hanvey *et al.,* 2017), often before extraction to remove organic materials from the sediment. Initially a sieving step was not considered for this protocol, as it introduced additional equipment, rinses and transfers of the sample which could result in increased contamination. However, in samples taken from the Lower/Mid and High marsh zones, vegetative matter was particularly prevalent, and was extracted in such volumes that it could not be totally digested. Introducing a post-floatation sieving step should reduce the amount of vegetation on the filters, thus allowing for a more effective digestion.

To overcome the issues of having to transfer the samples, a sieving apparatus was designed that would slot into the pre-existing filtration set-up. This resulted in a small brass sieve that could be clipped into the top of the filtration apparatus (Figure 2.12). This could therefore

catch and remove the larger vegetation in the extracted supernatant solution, without needing to introduce any new glassware or transfer steps.

Figure 2.12 - Custom made brass sieve, shown set-up and sitting in filtration apparatus.

Temperature

Like all the other variables, the temperature at which digestion was run was often specific to each study, ranging from room temperature (Cole *et al.,* 2014) up to over 80 ^{o}C (Enders *et al.,* 2017). However, in H_2O_2 treatments, temperature has been demonstrated to be a determinant factor (Prata *et al.,* 2019) and therefore important to consider when testing the different solutions.

There are additional concerns surrounding temperature in microplastic protocols, with (Munno *et al.,* 2018) finding temperatures of over 60 ^{o}C damaging and destroying microplastics. Therefore, for this experiment 50 ^{o}C was selected and tested to determine if temperature had any impact on the digestion efficiency of the treatments.

Method

Salt marsh sediment samples were set up as per the extraction protocol. During filtration, brass sieves (3 mm mesh size) were clipped into the top of the filtration apparatus, and the

extracted solutions poured through them. The filtered material was then rinsed with zinc chloride several times to ensure that no microplastics remained stuck in the vegetation. The sieve was then removed, and the vegetation manually checked for larger microplastics pieces. Those recovered were then placed back into the filtration apparatus. The remaining solution was then filtered as per the normal filtration protocol.

Once the filters had been collected, they were transferred to beakers containing 30 mL of one of the three digestion solutions, with each treatment being tested twice. Beakers were then sealed with aluminium foil and placed in a shaker incubator (ES – 80 or incubating orbital mini shaker, 50 ^{o}C, 100 rpm). They were then left for 72 hours and allowed to digest.

After digestion, the samples were visually observed, before being refiltered. The filters were then assessed for vegetation coverage, and finally stained and imaged. The results of digestion observation, and staining, were then combined to determine whether the protocol had been effective, and which of the digestion treatments were most effective.

Results and Discussion

Following the sieving step and digestion step, each of the filters showed minimal vegetation coverage (Table 2.11), with each filter having <25% of the filter visually obscured. For the majority of samples however (--) digestion was so efficient that vegetation matter was hard to detect visually and therefore an accurate % coverage could not be given. However, for the H_20_2 treatment the filter had not dissolved, and the sample had to be scraped off. In the Fentons Reagent treatment the filter had only partially dissolved and could not be removed from the solution.

Table 2.11 Vegetation coverage of filters after immersion in digestion solutions (30ml) for 72 hours, comparing sieving, temperature and agitation with agitation only; considerable vegetation present (++, >50%), vegetation present (+, 25-50%), vegetation reduced (-, <25%), vegetation unobserved (--, <5%)

Digestion Solution (30ml)	**Filter Coverage (%)**			
	Sieving, Temperature and Agitation		**Agitation Only**	
	Set A	**Set B**	**Set A**	**Set B**
Fentons Reagent	-- (<5)	-- (<5)	+ (25 – 50)	+ (25 – 50)
30% H2O2	-- (<5)	- (<25)	++ (>50)	+ (25 – 50)

30% KOH: 0.5M NaClO	-- (<5)	-- (<5)	+ (25 – 50)	+ (25 – 50)

From the stained images (Figure 2.13) it can be observed there has been a significant reduction in the vegetative material, with no large discernible pieces observed. What little vegetation is present is fluorescing much weaker and thus is lost in the background. In particular, the KOH NaClO filter is very clean, only showing the bright dots of microplastics on an otherwise dark sediment background. The Fentons Reagent filter shows a lot of particles present, the result of the incomplete digestion of the filter which then stained and fluoresced.

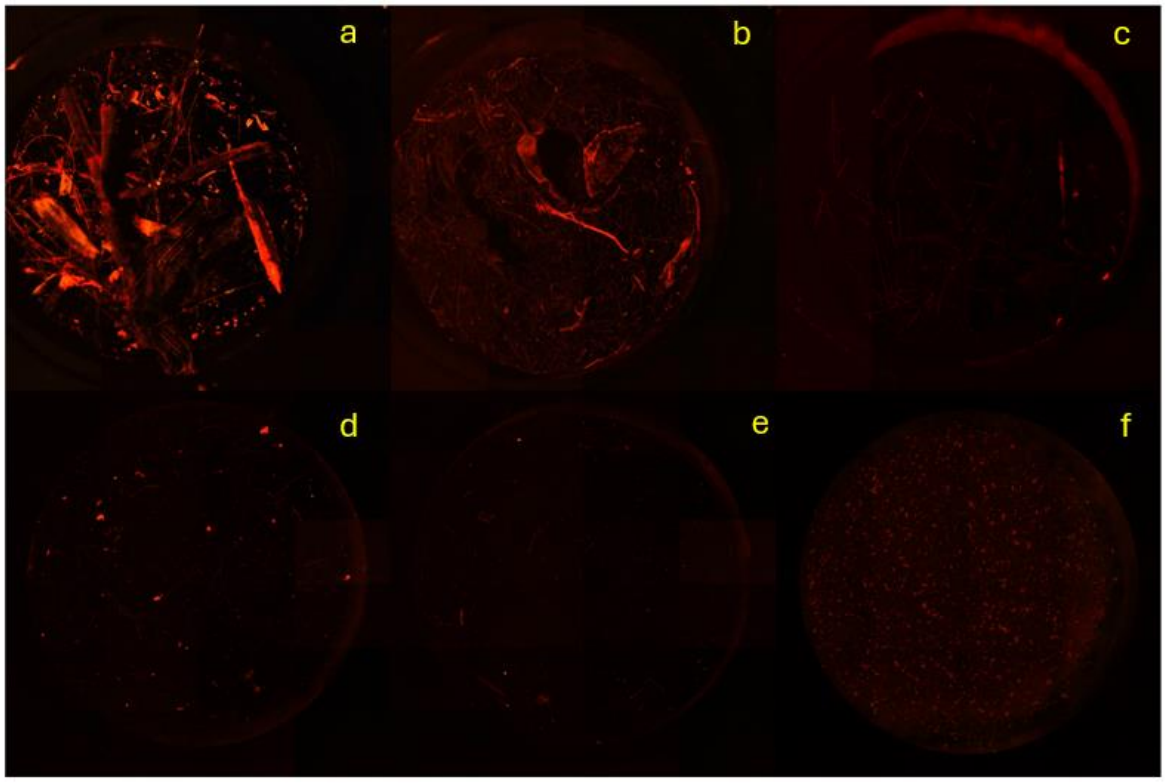

Figure 2.13 – Nile Red stained filters after sieving and immersion in digestion solutions (30ml) (bottom) compared to initial digestion treatment (top); H_2O_2 (a,d), KOH NaClO (b,e) and Fentons Reagent (c,f).

In many samples, digestion was so efficient that vegetation was hard to detect visually, mirrored by the lack of vegetation staining in the fluorescent images. Compared with just agitation, the samples went from approximately 25% coverage, to less than 5%. With an

estimated digestion efficiency between 80-99%, these results are now comparable to other sediment studies (Enders *et al.,* 2017). With the removal of large hard plant pieces, a reduced volume of vegetation, and increased energy from the higher temperature, each treatment can nearly fully dissolve the organic matter extracted from the samples. When studying the stained images, evidence of organic matter is still present, represented by faint patches on the background. However, when compared with previous filters the level of background is severely reduced, and individual plastic pieces can be clearly identified. With vegetation now only present in low quantities that weakly fluoresce, it can be easily distinguished from the brighter plastic particles, thus allowing us to effectively use automated software when counting the particles. With this test finally producing the desired digestion efficiency, both the sieving step and increased temperature were concluded to be effective and adopted into the final methodology.

2.5.3.3 Digestion Treatment Comparison

Throughout every test, each of the digestion treatments have been very similar to one another, with only H_2O_2 showing slightly reduced efficiency in the agitation and duration tests (Sections 2.5.2.2 and 2.5.3.1) when compared to the other two. Examining the final sieving and temperature test, each treatment was able to achieve a digestion efficiency of 90% or greater, with Fentons and the alkaline treatment achieving almost complete visual digestion. Rather than base the choice on digestion rates, other factors were considered when finalising the digestion treatments. When reviewing the impacts of each treatment on the microplastics, the oxidising treatments were found to have the least overall impact (Prata *et al.,* 2019). Hydrogen peroxide was found to discolour PET (Karami *et al.,* 2017), whilst Fentons Reagent, as a derivation of H_2O_2, had minimal observed effects on various tested polymer types (Maw *et al.,* 2022). The alkaline treatment has wider implications, with discolouration found in nylon, PE, PVC and PET (Dehaut *et al.,* 2016). However, since neither report the major degradation of plastics both are suitable treatments for salt marsh samples. Furthermore, since this protocol uses a staining process, colour is not a parameter that is measured, and so potential discolouration should have no effect on the data.

The deciding factor ended up being the ability of each treatment to dissolve the filters used during extraction and filtration. Cellulose nitrate filters were used to not contaminate the samples with further plastic. However, as observed in the agitation, duration and even

temperature experiments, only the KOH:NaClO treatment was able to completely digest the filter. In the H_2O_2 and Fentons reagent treatments, the filter was either partially digested or not damaged at all. The latter results in material having to be scraped off the filter after digestion, a procedure that is inefficient and leads to loss of the sample. Meanwhile partial digestion is even worse, as the cellulose nitrate filter fragments stain. These then show up in the imaging process, and either covers the sample, or fragments fluoresce enough to be mistaken for plastics. As the handling of the sample and counting of microplastics are crucial steps in the procedure, the H_2O_2 and Fentons Reagent treatments were found to be too disruptive to the extraction process and might influence the amount of microplastics recorded. KOH:NaClO was therefore selected as the digestion treatment for this methodology and used in all experiments going forward.

2.5.4 Further Analysis

One issue highlighted in the early salt marsh experiments, was the tendency for fibres to remain stuck to the glassware during filtration. Whilst all glassware was rinsed thoroughly to catch any such particles, fibres were still observed to be stuck on the lowest parts of the glassware after filtration. To overcome this, a test of trying different chemical surfactants was proposed. Surfactants lower the surface tension of surfaces, thus allowing particles to be moved easier. Increased mobility in microplastics have been observed in previous surfactant studies (Jiang *et al.,* 2022; Shen *et al.,* 2021).

In this test the surfactants Span20, Tween20, Triton X-100, a water/ethanol mixture (50/50), and a control sample of MilliQ Water were used. Surfactants were selected based on availability.

Method

Spiked samples were made up using 30 nylon fibres of different lengths (1-2 mm, 2-4 mm, 10 mm, 10 of each length) floating in a beaker of zinc chloride solution. The solution was then filtered, and then the glassware rinsed with the surfactant. After rinsing the glassware three times the number of fibres remaining either in the sample beaker or in the filtration glassware were counted. For each surfactant two replicates were run.

Results and Discussion

From the results it was observed that fibres remained present on the glassware in all the surfactants (Figure 2.14). The most successful surfactants were the Ethanol/Water mix and Tween20, with an average of 5 and 4.5 fibres retained respectively. However, the MilliQ Water control sample also showed an average of 4.5 fibres retained. Overall based on the fibres adhered none of the surfactants showed better results than the MilliQ water, as was used in the current protocol.

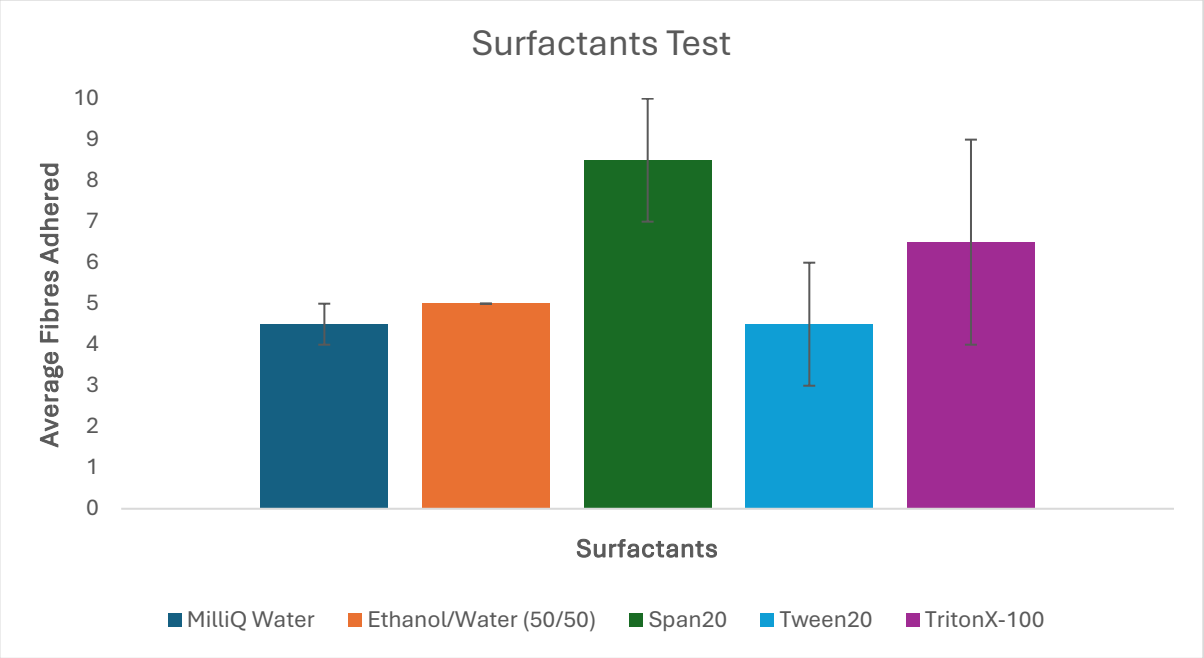

Figure 2.14 - Average number of fibres adhered to glassware post-extraction after different surfactant treatments.

Whilst the initial expectation was to notice an improvement based on previous studies, there are some important differences within this experiment. Primarily, the type of plastic used, with (Jiang *et al.,* 2021; Shen *et al.,* 2021) only studying effects on PE and PP, whilst here the spiked controls were PS and nylon. Furthermore, there was no overlap between the surfactants used between these studies and ours, and (Jiang *et al.,* 2022) has demonstrated that different surfactants can have different effects on MP adhesion. The biggest difference however was the medium of samples, with both (Shen *et al.,* 2021) and (Jiang *et al.,* 2022) studying the effect of MPs within columns. Compared to this controlled

lab sample, our protocol involves mixing microplastics with sediment and then immersing them in zinc chloride, resulting in potential changes to the microplastics surface. If any zinc chloride residue remains on the surface, then its hydrophilic nature may discourage suspension within the surfactant.

Whilst Tween20 showed the same results as the control rinse, a follow up staining procedure with Nile Red showed that filters rinsed with Tween20 had an overwhelming amount of background staining, making any form of automated particle counting impossible. Based on this and the poor results of the other surfactants, any surfactant rinse was removed from the final procedure and the MilliQ rinses were retained. To prevent particles sticking to the glassware, rinses were done in small volumes whilst the vacuum pump was still running, thus ensuring there was no build-up of water in the filtration head, and so eliminating any chance for microplastics to be carried up by the water level and left on the glassware. Furthermore, after the testing of the digestion step the issue of particles remaining stuck to the glassware was no longer observed. This suggests that the previously observed adhesion was likely either due to the larger nature of the spiked samples, or the presence of a film/residue that digestion would later remove.

2.5.5 Microplastic Analysis

2.5.5.1 Nile Red Staining Solution

Nile red dye can be made up in a variety of different solvents, including acetone (Maes *et al.,* 2017), ethanol and dichloromethane (Shim *et al.,* 2016). Initially, in this protocol Nile Red was diluted using MilliQ water, as this had been pre-filtered to reduce contamination, and introduced fewer chemicals into the overall method. However, as Nile Red is a naturally hydrophobic molecule (Sitepu *et al.,* 2012), it was found to precipitate out of solution shortly after being used, thus rendering it unusable for any future procedures. Whilst Nile Red is soluble in several hydrophobic solvents, many of these have been shown to degrade the filter membranes used during analysis (Shim *et al.,* 2016). Additionally, there is concern around the dye being too effectively solvated in solution. With such affinity for hydrophobic solutions the dye may not be encouraged to adsorb on a plastic surface and thus not effectively stain the sample (i.e. the equilibrium favours solvated dye over adsorbed dye).

To balance these two issues, a series of mixtures of MilliQ Water and ethanol was proposed. It was hypothesized that finding the right solvation/polarity balance, solutions should still effectively dissolve and retain the Nile Red solution, whilst not impeding it from staining hydrophobic particles upon the filter.

Method

A range of ratios between the two solutions was tested: (water/ethanol) 100/0, 75/25, 50/50, 25/75, 0/100. Nile Red solutions were prepared at 1 mg mL^{-1} for each mixture, with each ratio having three replicates. The solutions of Nile Red were then used to stain some control filters with a mix of different plastics (nylon fibres, PS beads, PVC fragments), whilst the bulk solutions were kept in glass vials. These vials were then left for a period of two weeks to be observed.

Results and Discussion

The results of the Nile Red in various mixtures can be seen in Table 2.12. In each replicate, the Nile Red in pure water solution showed evidence of precipitation in under 24 hours. The 75% water 25% ethanol was not much better, having a shelf time of only 1-2 days. However, the 50/50, 25/75 and 0/100 mixes all showed the same longevity, with no signs of precipitation after two weeks. When comparing the Nile Red staining, no visual differences were detectable between all the solutions.

Table 2.12 Effect of different solvent mixtures on suspension duration of Nile Red

Water/Ethanol Mix	**Days remaining fully Dissolved**		
	Batch 1	**Batch 2**	**Batch 3**
100/0	0	0	0
75/25	1	1	2
50/50	14	14	14
25/75	14	14	14
0/100	14	14	14

Increasing the ratio from 0 to 50% ethanol enhanced the reagent lifetime by at least 14 days in each replicate. However, since this ratio retained Nile Red in solution for the full duration

of the test, no further effects are observed when increasing the ethanol ratio to 100%. Whilst these solutions may be able to retain Nile Red for even longer periods of time, fresh solutions were made up every two weeks so retention for longer times was not a necessary requirement. When considering the impact of hydrophobic solutions reducing staining, increasing ethanol ratio had no observable effect on the staining capability of the solution. Regardless, as the 50/50 MilliQ Water and ethanol mixture retained the Nile Red fully dissolved without risking impacts toward staining, it was adopted for the methodology and used in any further procedures requiring Nile Red solution.

2.5.5.2 Chemical Validation

Spectroscopic techniques are widely used in microplastic studies, as they provide highly detailed spectra of particles, allowing for in depth analysis and accurate sample identification (Hanvey *et al.,* 2017). Fourier Transform InfraRed Spectroscopy (FTIR) was the main method of analysis used in this project, due its availability, capabilities and demonstrated use in previous salt marsh studies (Almeida *et al.,* 2023; Lloret *et al.,* 2021). Using a Bruker Hyperion 2000 a range of samples could be measured, using the benchtop ATR for larger fragments and fibres, and micro-ATR FTIR for anything too small to be transferred off the filter. Whilst the technique has been used in a variety of microplastic studies, each with their own model and instrument, it was still deemed practical to the efficiency of the instrument and its built-in sample library. Therefore, a selection of known plastics and materials were collected and measured in both the benchtop and micro-ATR.

Method

For the benchtop ATR, samples approximately 5 mm in length were cut from each test material. A total of three different ATR spectra were collected for each sample, and the spectra compared with a built-in library to give a match and hit quality for each material. The hit qualities were then averaged and recorded.

For the micro-FTIR, the previous samples were then finely cut into small pieces (<1 mm), which were then filtered onto a cellulose nitrate filter along with a fine layer of sediment (to be representative of environmental samples). If the particles were obscured, the filters were stained with Nile Red so that they could be found using fluorescence on the microscope images. Using the micro-ATR, spectra were obtained for at least two fragments

of each material. The spectra were then compared with the library, and the Hit Qualities recorded and averaged.

Results and Discussion

From the ATR spectra, the built-in library was able to match each of the materials to spectra of the same material in its data base (Figure 2.15). The hit quality varied, with the lowest match being Plant Material (184 and 232 Hit Quality for benchtop and micro-ATR respectively) and the highest being High Density Polyethylene (987 and 896 respectively). For the benchtop ATR, all plastics except for Nylon had a hit quality of 600 or higher. Plastics in the micro-ATR were generally lower than the benchtop results, the only exception being Cotton.

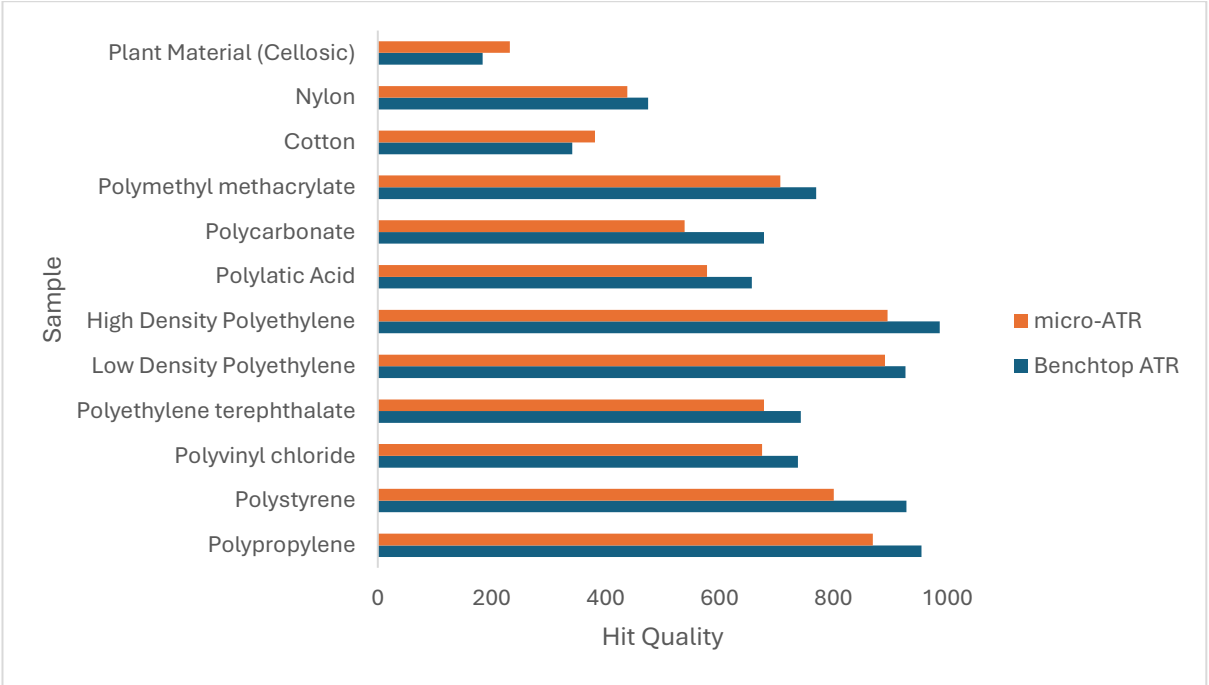

Figure 2.15 - Hit Qualities of various polymers and materials as determined by FTIR - ATR spectrometry, showing both micro and benchtop ATR results.

Within microplastic validation, various degrees of matching certainty are accepted. For general microplastic indentation via FTIR, the (MSFD Technical Subgroup on Marine Litter, 2013) (MSFD Technical Subgroup on Marine Litter, 2013) (MSFD Technical Subgroup on Marine Litter, 2013) suggests that a match of 70% (0.7) is a strong indicator of successful

identification. For ATR-FTIR the spectra are generally more accurate due to direct contact with the sample removing background noise (Courtene-Jones *et al.,* 2017) allowing for more complex matching systems. Hit Quality Index characterises spectra using an algorithm based on all the various peaks (measuring peak height, wavelength and peak area), which it then uses to match with spectra of known polymers (Renner *et al.,* 2017), and has accepted hit qualities ranging from 0.6 – 0.8. For our tests, the target was at least a 60% match (or 600 hit quality) to ascertain the particle as plastic or not. The benchtop ATR was able to achieve this standard, with only the nylon fibres, cotton samples and plant material falling below this threshold. In the case of plant material, whilst the built-in search function was able to match it as cellulosic, the varied nature of organic matter resulted in very poor hit quality. Additionally, this sample was taken from the sieving step and therefore had undergone several chemical treatments likely altering its properties and thus its spectra. However, as a non-plastic this poor match was advantageous, as it is clearly distinct from the other material identified by the spectra. Any potential plant material that is tested should therefore result in similarly poor spectra and will not be falsely identified as plastic. With the micro-ATR samples, a reduction in the hit qualities was observed, meaning several plastics were just around or below this threshold. This drop is likely due to the change in the sample, with the micro-ATR samples being measured on filters with a background of sediment. Whilst the ATR makes direct contact with the tested particle, it can still pick up some interference from the surrounding sediment, especially when particles are very small. This is seen as noise on the spectra and so can make it harder for software to make a successful match to spectral libraries. Another reason for the lower hit qualities may be due to how they are calculated to include peak area. This means that where the signal strength is lower in samples, peaks have reduced height and area, reducing their overall hit score due to noise and uncertainty in the baselines. However, these spectra can still be positively identified by matching the wavelength of the peaks and comparing it to those of the known spectra. Finally, the library identification is limited by the spectra it has available to compare. These samples were not measured with microplastics in mind and therefore may not be a sufficient repository of spectra for identifying worn microplastics. The overall low qualities of micro-ATR spectra generated for known polymers was concerning, especially when considering the impure state of real samples. Therefore, it was determined that hit quality alone could not be used to positively identify microplastics, and that a visual

comparison of the peak wavelengths should also be included when deciding whether a sample is plastic or not. As the main purpose of chemical validation was to identify any non-plastic material, this method was deemed acceptable in being able to distinguish the poor spectra of organic material, with cleaner plastic spectra being studied further for more polymeric information.

For the final methodology, spectra were taken from the library and uploaded to OpenSpecy. Spectra were then compared against their much larger online library. To alleviate the issue of background noise, the acceptable match was raised, only accepting particles with a 75% or higher polymer match as plastics. Particles with a 60 - 74% match were looked at, and then if the wavelengths of the peaks were found to line up with those of the known polymer, they were then also accepted.

2.6 Final Methodology

Following these tests, a final methodology was established incorporating the results of the extraction, digestion and staining tests (Figure 2.16).

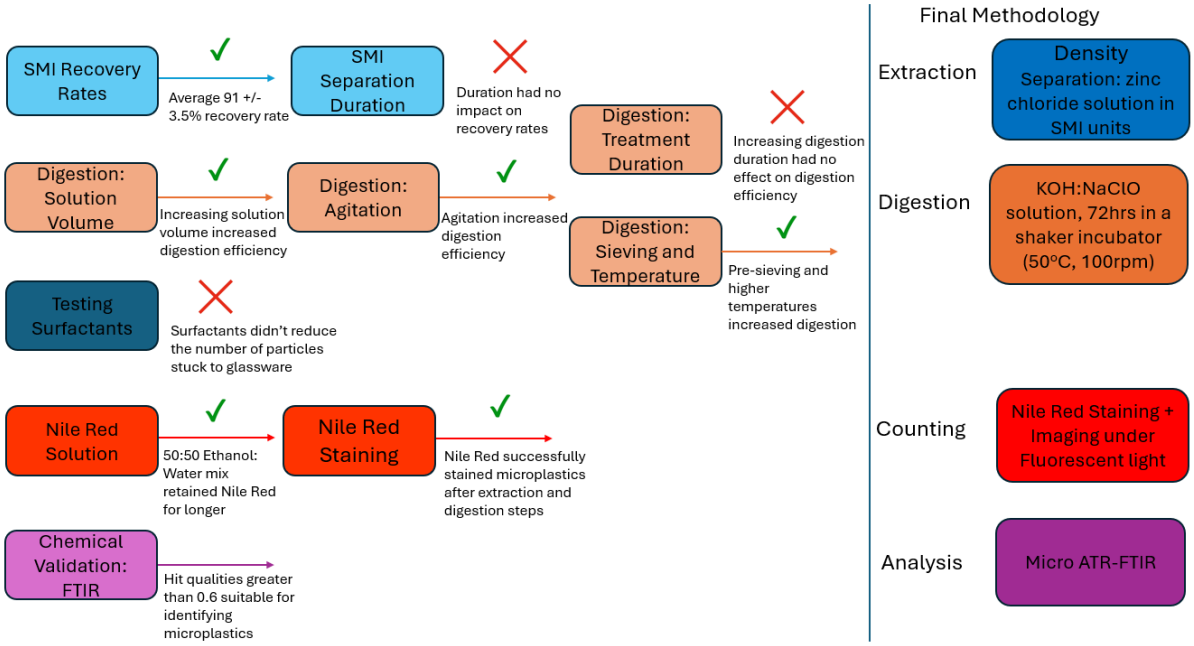

Figure 2.16 - Flowchart summary of the different steps trialled as part of the method development, showing which were incorporated into the final methodology.

The final methodology was written into a Standard Operating Procedure (SOP; Appendix 5) and used as the primary method in Chapters 3 and 4.

2.7 Future Work

Whilst this methodology was successfully used to extract and analysis microplastics from over 100 sediment samples, using it so extensively has also demonstrated areas in which the methodology could be improved.

Firstly, whilst extraction rates from the SMI units were high, due to the intricate nature of the valve system, despite rinsing it was still possible that microplastics could get trapped in the SMI unit. Furthermore, with the SMI units being made from PVC pipe, it is possible for contamination to occur. Recently, a newer extraction protocol been suggested that would be suitable to test for this methodology. Overflow density separation still utilises the high-density zinc chloride, allowing sediments and microplastics to settle after shaking in a glass vessel. After settling, the vessel is then put into a larger glass container, to which more zinc chloride is then slowly added to the original vessel. This results in the solution overflowing, in which this supernatant solution contains the microplastics and is collected separately in the larger glass container (Crutchett and Bornt, 2024). This methodology uses simple glassware and pre-existing solutions that were already part of our methodology. However, it should be tested for suitability before being adapted into this procedure, especially when considering that microplastics are not the only thing present in the supernatant solution of salt marsh samples.

Another issue highlighted from this study, was the presence of calcium-based shell fragments from various salt marsh organisms. These small pieces can be on the same scale as microplastics, and due to their tough structure, often are not dissolved as part of the digestion step. Furthermore, these can also sometimes be stained by the Nile Red, thus creating false positives and potentially altering the microplastic values recorded. However, this can potentially be addressed with the addition of a weak acetic acid digestion step (as recommended by Cefas Microplastic Laboratory). Whilst a further step may increase the risk of contamination, if effective it will significantly reduce the risk of false positives in the automated counting stage. Therefore, this should be experimentally tested to see if it still

works in the salt marsh sediments and can be combined with our pre-existing digestion stages.

2.8 Conclusion

This chapter detailed the step-by-step process by which a methodology is trialled, developed, and then implemented for salt marsh samples. For extraction of microplastics, density separation using zinc chloride was the selected approach. The use of sediment microplastic isolation (SMI) units were trialled and found that they were still very effective even when dealing with heavily vegetated samples, achieving an average recovery rate of 91 +/- 2% for various plastic forms. This result is very similar to recovery rates from other studies, and therefore sufficient to proceed with the use of SMI units throughout the rest of the methodology. The final extraction step was: 10-20 g immersed in roughly 250 mL of zinc chloride (1.54 g/cm^3) and mixed in a SMI unit. The unit is left for 24 hours so the sediment settles, then supernatant is collected, and the step repeated three times. Digestion of organic material was quickly established to be a necessary step, and so the treatments of H_2O_2, Fentons Reagent and KOH:NaClO were tested. Initial digestion results were poor, however through experiments on volume, duration, and treating under agitation and temperature, digestion efficiencies of up to 90% were obtained for each treatment. No treatment was found to be significantly more effective at digesting plant material than the others, however the KOH:NaClO treatment allowed for the complete digestion of cellulose nitrate filters used in the process, resulting in a more streamlined transition from filtration, to digestion and then filtration again. The final digestion step was: A beaker containing 50 mL of 30% KOH: 0.5M NaClO, sealed in a shaker incubator (100 rpm, 50 ^oC) for 72 hours. Finally, Nile Red was tested to allow for the implication of an automated particle counting software. After each digestion experiment images of Nile Red stained filters were captured and observed for contamination. The various treatments were observed to have no effect on the staining capabilities of the Nile Red, and particles were still clearly identified as being much brighter than the background. After the development of a successful digestion step, the presence of vegetation was low enough that it did not obscure the filter during staining, and therefore automated counting was possible. This was then validated using micro-ATR FTIR, which when tested was found to be able to distinguish between various plastics and

organic matter. Plant material was shown to be spectrally distinctive with very low hit qualities (<300), whilst plastics could be loosely matched on the spectrometer, before being compared to more diverse online libraries allowing for the successful identification of plastics. The use of automated counting could therefore be applied to salt marsh samples, with false positives being successfully identified through chemical confirmation.

With the successful testing of each of these steps, they were then combined with the pre-existing protocols for filtration and staining, resulting in a final Standard Operating Procedure for salt marsh samples (Appendix 5). This SOP was then used as the basis for all samples going forward, successfully extracting and analysing microplastics from over 150 different salt marsh samples.

Chapter 3 - Presence and Spatial Distribution of Microplastics in a Norfolk Salt Marsh

Abstract

Microplastics are gaining increasing interest for their potential impacts on organisms and ecosystems. Coastal habitats are widely known for their high microplastic prevalence, however within them, salt marshes remain relatively understudied. This work focussed on studying the presence and spatial distribution of microplastics across a salt marsh, and then trying to explain the distribution using physical factors present within the environment. The study found microplastics present in all areas across the salt marsh, averaging 21,000 ± 2,300 particles/m^2 (3,400 ± 390 particles/kg d.w.) per sample in the size range ~20 to 5000 μm. A pattern in distribution was observed, with the Lower/Mid Marsh zone having over twice as many microplastics as any other vegetation zone. However, when considering the factors vegetation height, elevation and distance from the edge of the salt marsh, only vegetation height showed a significant correlation with microplastic abundance. Despite this, none were found to have any discernible trends when compared with the distribution of microplastics. These results highlight the widespread presence of microplastics in salt marshes, and that their trapping distribution may be a result of the combined effects of several different factors.

3.1 Introduction

Found at the transition point between land and sea, salt marshes are coastal ecosystems identified by their regular tidal inundation, and distinctive saline resistant vegetation (Bakker, 2014; Baugh *et al.,* 1990). These are important habitats, known for their high productivity and the unique biodiversity which they sustain. They also provide numerous ecosystem services, such as natural coastal defence (Mcowen *et al.,* 2017), cycling of nutrients, being important staging grounds for the growth of young fish (Deegan *et al.,* 2005) and feeding sites for migratory birds (Greenberg *et al.,* 2014). Furthermore, salt marshes are now garnering attention as potential sinks of blue carbon (Teixeira *et al.,* 2014), making them important ecosystems when considering climate mitigation strategies. However, these habitats are also under multiple threats such as the building of facilities such as harbours, and a general reduction caused by land reclamation for agriculture and urbanisation, as well as waste deposition and emerging contaminants (Gedan *et al.,* 2009).

One such potential emerging contaminant is microplastics, which have been steadily gathering interest in both terrestrial (Rillig and Lehmann, 2020) and marine ecosystems (Pauna *et al.,* 2019). Now recognised as a global issue, microplastics are broadly defined as pieces of plastic litter which are in the size range of below 5 mm down to the micrometre scale (Thompson *et al.,* 2004). Microplastics have been found in nearly every ecosystem around the world, from arctic ice to alpine snow and even the trenches of the oceans (Bergmann *et al.,* n.d.; Lusher *et al.,* 2015b; Peng *et al.,* 2018). This global reach leads to a major concern; their widespread availability to organisms at numerous different trophic levels where they can easily be mistaken for food. Various studies have found microplastics that were ingested by different organisms, including birds (Mallory, 2008), fish and crustaceans (Bakir *et al.,* 2020), turtles (Mascarenhas *et al.,* 2004), and even in plankton (Lin, 2016). Habitats themselves are also at risk, as microplastics are hypothesized to have direct impacts on both plants and sediments as well (Rillig, 2019). Furthermore, microplastics not only have direct impacts, but can also act as vectors for additional hazardous contaminants, such as metals, pathogens, and chemical contaminants such as persistent organic pollutants (POPs) and polychlorinated biphenyls (PCBs) (Caruso, 2019). This has led towards increased environmental concerns, particularly around coastal

habitats. While we recognise that these ecosystems are potentially vulnerable, few studies have properly investigated the full impacts that plastics can have.

In coastal environments, plastic litter is the most predominant form of waste, varying from 60-80% of all marine litter, potentially even as high as 90% in some regions (Derraik 2002). Coastal wetlands have previously been shown to be effective in trapping marine litter (Martin *et al.,* 2019), due in part to their hydrodynamic properties. Salt marshes form when hydrodynamic and ecological properties overlap; the resulting low energy environment formed when tidal water and vegetation me*et al*lows for the deposition of sediment to occur. The process of sediment deposition however is very complex and is controlled by various factors such as tidal regimes, creek hydrology, sediment load, and the disruption to water flow caused by plants and the marsh surface (Leonard, 1997; Allen and Duffy 1998; Yang, 1999; French *et al.,* 2000). Empirical modelling studies were able to relate depositional patterns to different environmental variables, observing marsh elevation, distance from tidal channels/ seaward marsh edge, and vegetation coverage all had significant effects on the sedimentation rates (Fagherazzi *et al.,* 2012). The influence of vegetation has been widely studied, with Silva et al. (2009), Cahoon et al. (1996), Brown (1998) and Pont et al. (2002) all considering the marsh plants when studying vertical accretion. Whilst the extent of impact is hard to measure, they were able to show that differences in vegetation coverage resulted in different sedimentation rates. Other factors have also shown to be important, with Silva et al. (2009) highlighting the importance of both the concentration of sediment load, and the sediment type. Looking specifically at the spatial distribution of sedimentation, Temmerman et al. (2003) found that increasing surface elevation and distance from the marsh edge had a strong negative effect on the sedimentation rates. Particles within the creek must be carried up over the marsh, and therefore the further they travel the more likely they are to be deposited/trapped. Increasing distance results in particles having to travel further without being deposited, whilst increasing elevation reduced the amount of water (and thus transported sediment load) reaching these sites. Both factors can also be used as a proxy to discuss the effect of flooding frequency by the tide. Area's furthest from the creek, and areas with high elevation, receive significantly less tidal inundation than those at the creek edge. Thus, by studying the impact of these factors we will be able to suggest the influence that inundation

time has as well. As such, these physical factors have an important role in sediment deposition, especially when considering the local distribution within the marsh.

These same properties that lead to sediment deposition have also been tied to an increased trapping of microplastics (Vianello *et al.,* 2013). Vegetation has been highlighted to have an important role, with flume studies demonstrating the impact of vegetation in riverine, tidal and wetland ecosystems (de los Santos *et al.,* 2021; Gallitelli *et al.,* 2023; McIlwraith *et al.,* 2024) which along with the physical trapping effects of vegetation, makes salt marshes inherently biased towards microplastic trapping (Helcoski *et al.,* 2020). Furthermore, their dynamic conditions provide the right environment for larger plastic litter to degrade into microplastics very quickly (Weinstein *et al.,* 2016). Combining all these sources makes salt marshes potential microplastic propagators and collectors.

Despite this, as habitats they are understudied when it comes to understanding their role within microplastic circulation. Whilst many studies show salt marshes sites of plastic waste contamination (Gilligan 1992, Uhrin 2011, Viehman 2011), their focus is on larger macroplastic litter. This can be an important indicating factor, with (Weinstein *et al.,* 2020) showing that the combined factors of; mechanical forces, UV radiation, and exposure to oxygen and increased temperatures, can lead to the degradation from macro to microplastics in as little as 4-8 weeks. Microplastics were found extensively in studies carried out on Chinese salt marshes (J. Li *et al.,* 2020; Wu *et al.,* 2020; W. Yao *et al.,* 2019), as well as confirmed in marshes in Portugal (Cozzolino *et al.,* 2020) and the UK (Stead *et al.,* 2020). The quantities varied depending on location, however, were significant when compared with terrestrial habitats.

The current literature around microplastics in salt marshes is sparse and limited to independent studies focussing on a variety of different questions. The lack of globally accepted protocols makes comparison between data sets difficult with multiple methods being applied across studies. Whilst there is strong evidence for the presence and accumulation of microplastics, the extent of their distribution within a salt marsh is still unknown. Previous studies have focussed on heavily polluted marshes, and so there is little understanding as to what the natural accumulation of plastics in non-urbanised salt marshes might be. Whilst they may be collected by the same trapping processes as sediment, their fate and distribution across a marsh could be attributed to other processes.

3.2 Aims and Hypotheses

This study aims to fill in that knowledge gap by broadening the understanding of microplastic distribution in salt marshes. The main objectives are: i) to investigate the occurrence and abundance of microplastics in a remote UK marsh, ii) to investigate the role of vegetation cover and height on plastic distribution, iii) to investigate the effect of other parameters, namely elevation and distance from the salt marsh edge (creek side) on microplastic distribution and iv) investigate the potential relationship between the abundance of microplastics and vegetation zonation.

We hypothesize that; i) microplastics should occur in salt marshes and will have some variation within their distribution. We assume that microplastics will behave similarly to sediments, and therefore microplastic accumulation will be influenced by the same marsh factors. Therefore, we hypothesize that ii) vegetation height, elevation, and distance from the edge of the salt marsh (creek side) will show significant correlation with our microplastic abundance and iii) may be used to explain the microplastic distribution. Finally, we hypothesize iv) that as a parameter which incorporates all these factors, vegetation zonation will also have some significant impact on the microplastic distribution.

3.3 Methodology

3.3.1 Study Site

Blakeney Point (52° 58' 37.92" N, 0° 58' 40.08" E) is situated at the western end of Blakeney Spit, a 12 km stretch of the North Norfolk coastline, UK (Figure 3.1). Salt marshes dominate the southern face of the spit, covering approximately 15 km^2 of land. The marshes are submerged by water from a single main Channel – Blakeney Channel – which joins the North Sea and is fed by the small River Glaven (drainage 137.1 km^2) from the east. As part of the National Trust Blakeney Nature Reserve, this area has restricted access and so receives minimal footfall. There is little industrial impact in the area, with the only major water use being Blakeney Harbour, mooring small fishing boats and yachts along the channel.

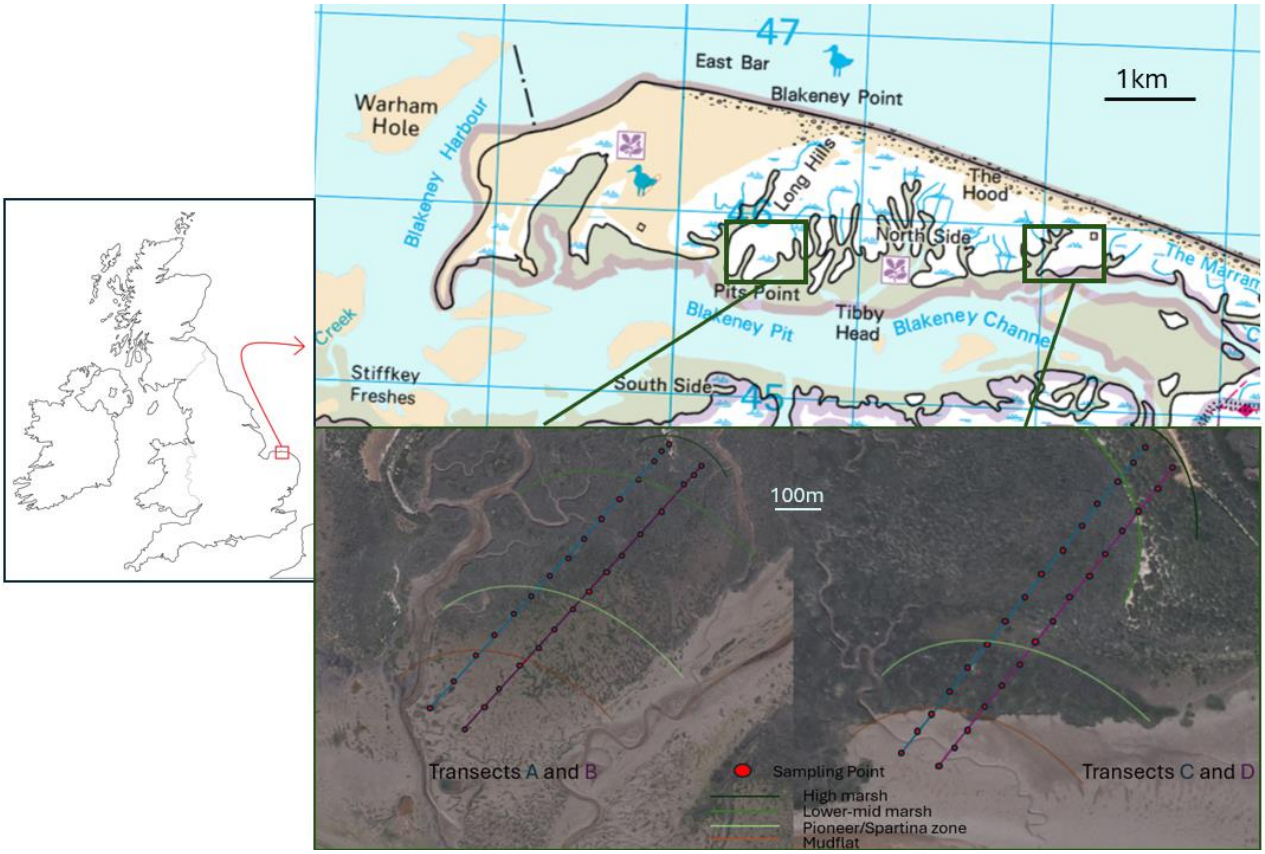

Figure 3.1 - Map of Blakeney Point, showing geographical location within the UK, as well as transect sites and sampling points with vegetation zones denoted.

At Blakeney Point, over 300 different plant species have been recorded (Pearson John, 2007). These species make up communities that change as you travel across the marsh, compiling into different zones of vegetation. Zones were determined by visual observation of vegetation species during fieldwork, done by Dr Stefanie Nolte with assistance from a local botanist Richard Porter. The zones observed were Pioneer zone (dominant species; *Salicornia sp*.) Spartina zone (dominant species; *Spartina anglica*) Lower/Mid marsh zone (dominant species; *Halimione portulacoides*) and the High marsh zone (dominant species; *Suaeda vera*) (Table 3.1).

Table 3.1 - Dominant vegetation species for each zone across all transects showing % coverage of each species. Dominant species were only considered if coverage was > 5%.

Zone	Transect	Dominant Vegetation Coverage (%)
Pioneer	A	*Salicornia sp* (10)*, Spartina anglica* (5)
Pioneer	B	*Salicornia sp* (10), *Spartina anglica (5), Suada linearis* (5)
Pioneer	C	*Salicornia sp* (10), *Aster tripolium* (10), *Suada linearis* (5)
Pioneer	D	*Salicornia sp* (10*) Suada linearis* (10), *Aster tripolium* (5)

Spartina	C	*Spartina anglica* (60), *Suada linearis* (10)*, Aster tripoluim* (10)
Spartina	D	*Spartina anglica* (80) *Salicornia sp* (10)
Lower/mid marsh	A	*Halimione portulacoides* (70), *Suada vera* (10)
Lower/mid marsh	B	*Halimione portulacoides* (60), *Spartina anglica* (30), *Salicornia sp* (5)
Lower/mid marsh	C	*Halimione portulacoides* (50), *Aster tripolium* (20), *Spartina anglica* (20), *Salicornia sp* (5)
Lower/mid marsh	D	*Aster tripolium* (40), *Halimione portulacoides* (30), *Salicornia sp* (15), *Suada linearis* (5)
High marsh	A	*Suada vera* (90), *Halimione portacoides* (5)
High marsh	B	*Suada vera* (75),
High marsh	C	*Suada vera* (50), *Armeria maritima* (10), *Elytrigia atherica* (10)
High marsh	D	*Suada vera* (80)*, Elytrigia atherica* (10)

3.3.2 Sample Collection

A total of 100 cores were collected from the Blakeney Point salt marsh in May 2021.

The corers were cut from Aluminium, 20 cm long with 5.4 cm diameter, and collected roughly the top 10-15 cm of the sediment. These were collected along four transects in two different areas (Figure 3.1), with a later 10 cores collected in August 2022 for further studies. Transects were drawn using a 200m measuring tape, starting from beyond the high marsh a line was walked down to the exposed mudflat. The separate zones were marked out, then samples were taken approximately every 10-15m metres ensuring that each zone had at least 3 samples. If a zone was particularly large, then a 2nd set of 3 samples were taken in order to provide an even distribution across the zone. Where the sampling locations along the transect landed in creeks or the centre of dense vegetation, the core was moved the minimal distance required to avoid the obstruction whilst still remaining on the transect line.

Samples were taken by pushing the corer into the ground, or if the sediment was hard, using a wooden mallet to knock the corer into the ground. To extract the corer, a trowel was used to loosen the sediment surrounding the corer so that it could be pulled out without difficulty. Sediment compaction was not measured for these samples since the focus of the study was the presence of microplastics, and depths beyond the surface layers were not as important. The cores were then wrapped in aluminium foil placed in a cardboard box, before being transported and stored in a freezer at -20 ^{o}C until ready for analysis. Every three cores a blank control core (empty corer placed on the marsh) was taken, as well as an

additional sediment core (taken in the same way)to be used for alternative sediment analysis.

Metadata were also collected alongside each core including: a photograph of the core and its surrounding vegetation, vegetation species, vegetation height and presence of macroplastic in the sampling location (determined visually, with any macroplastics within a few metres of the sample noted and photographed). Elevation data was determined later, extracted from a topography map of the site using GIS and previously collected GPS locations.

3.3.3 Planned Sampling

Alongside sediment samples, a further selection of water samples was planned for collection, including surface samples from the creek adjacent to the salt marsh, and water samples both upstream in the River Glaven, and downstream where the creek meets the North Sea. However, due to the extra time and safety requirements needed for these steps, as well as permissions needed from the County Council and Wildlife Trust, it was not possible to complete these samples as part of this study.

3.3.4 Microplastic Extraction

Microplastics were extracted from samples using the following protocols. This methodology was based on an existing Cefas SOP, with steps tested and modified to accommodate for the differences in vegetation and sediment types found in salt marshes. An overview of the method is given here, with the full SOP being given in Appendix 5.

3.3.5 Procedural Controls and Contamination Prevention

Throughout the procedure 100% cotton lab coats were always worn, along with cotton clothing underneath. Nitrile gloves were worn, and all work was carried out inside laminar flow cabinets. All solutions and extraction equipment were similarly stored in laminar flow cabinets when in use.

All glassware and apparatus used in this protocol was washed three times with MilliQ water before every use. Where possible plastic equipment was minimised, and when impossible to remove, plastics were washed three times with MilliQ and regularly checked for any wear and damage. All solutions were filtered (cellulose nitrate filter, 2 µm pore size) before use

and stored in glass flasks between procedural steps. Where possible, glassware for each sample was reused throughout the protocol to ensure no cross contamination of samples, and to minimise sample loss.

3.3.6 Materials

The list of chemicals and solutions used in the methodology for extracting microplastics from salt marsh samples are listed in Table 3.2.

Table 3.2 - List of Chemicals used in extracting microplastics, showing; manufacturers, suppliers and purity of solution

Chemicals	Molecular formula	Manufacturer/Supplier	Purity (%)
Potassium hydroxide	KOH	VWR/VWR	-
Sodium hypochlorite	NaClO	VWR/VWR	14% active chlorine
Ethanol	C_2H_6O	Acros organics/ThermoFisher scientific	95% purity
Nile Red	$C_{20}H_{18}N_2O_2$	Acros organics/ThermoFisher scientific	99% purity
Zinc chloride	$ZnCl_2$	VWR/VWR	-

3.3.7 Preparation of Samples

Cores were extracted from the corer using a large wooden rolling pin (diameter 5cm) to push the sediment from the bottom of the corer. Once a few centimetres of sediment had been pushed out the top of the core, a wooden ruler was used to measure the top 1cm, starting from where sediment began (Figure 3.2). Then, using a sharpened steel paint scraper the top 1 cm (and any surface vegetation) was sliced off and placed into a pre-weighed glass petri dish. These samples were then dried in a vacuum oven (<50 ^{o}C) for 48 hours, or if still wet, dried in 4-hour periods until a constant weight was obtained. The wet

and dry weights for the samples were then recorded. Finally, samples were immersed in a small volume of zinc chloride solution (density 1.54 g/cm^3) and left on an orbital shaker (Heidolph Rotamax 120, 100 rpm) for 12 hours (Figure 3.3) in clean beakers covered with rinsed foil. This helped break up the dry mud – with a spatula being used to gentle break apart any large clumps that remained.

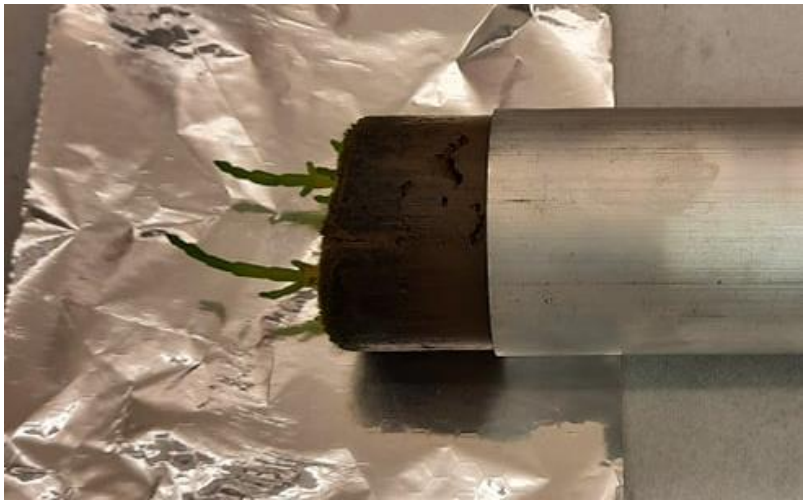

Figure 3.2 - Mud being extracted from the corer, showing top few centimetres of sediment (and surface vegetation) having been pushed up and out of the corer

Figure 3.3 - Samples immersed in approximately 20 ml zinc chloride solution, left to be agitated on the orbital shaker for 12 hours.

3.3.8 Microplastic Extraction from Sediment

Microplastics were extracted from the sediment using a customized density separation protocol, designed specifically to deal with the densely vegetated salt marsh samples. Such

samples can have a variety of biomass, including roots, stems, as well as varying levels of organic matter within the sediment.

The samples (approx. 10 – 15 g) were taken from the orbital shaker and added to Sediment-Microplastic Isolation (SMI) units, ensuring all sediment was rinsed (using zinc chloride solution, density 1.54 g/cm^3) from the beaker. Beakers were then resealed and stored for usage later in the protocol. Using the salt solution zinc chloride (approx. 250 mL), the SMI's were filled up till the solutions level was 5-6 cm above the central valve (see Figure 3.4). The SMI's were sealed with parafilm and inverted several times to mix the solution and then allowed to separate for 24 hours. Within the separators the mud will have settled and there is a colour difference between the solutions in the top and bottom halves. Being lighter than the density of the zinc chloride solution, microplastics and other debris have floated to the top of the solution. The supernatant solution was then collected in the stored glass beakers, before rinsing and refilling the SMI with zinc chloride, repeating this extraction process for a total of three times per sample to increase recovery rates.

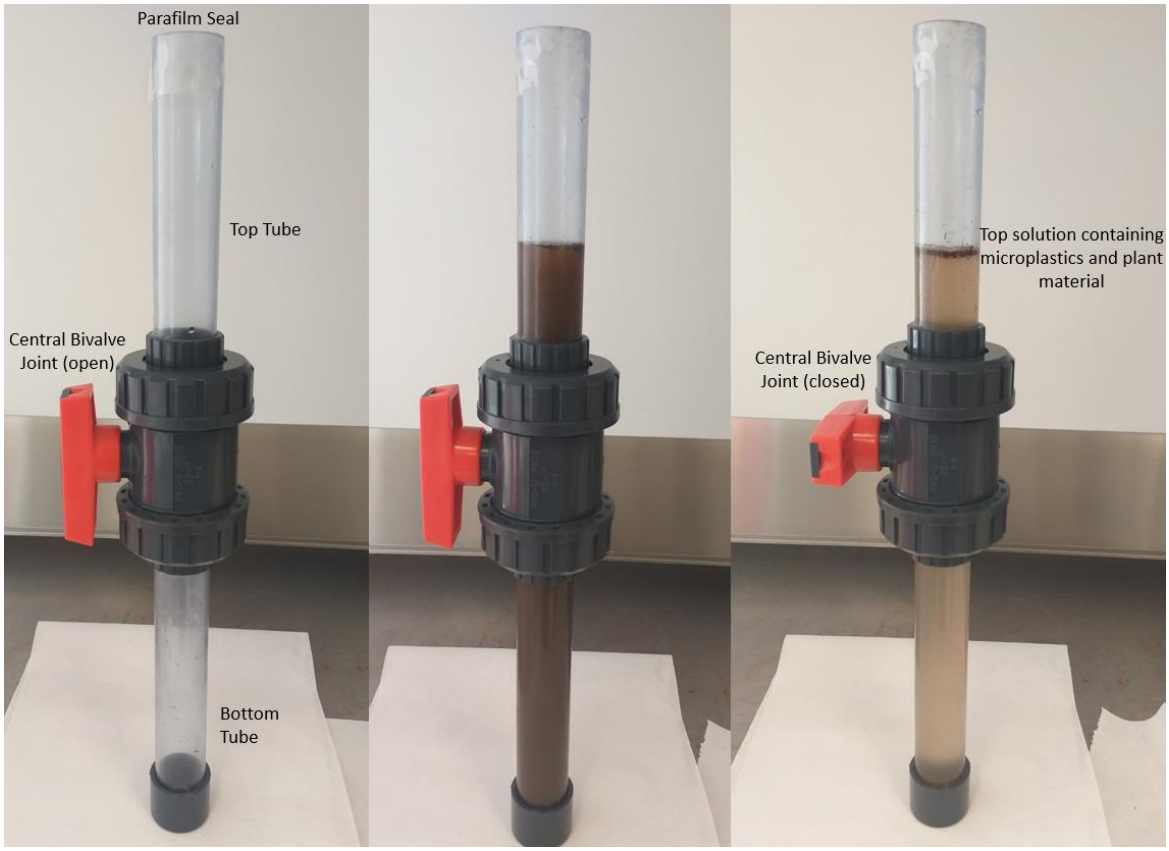

Figure 3.4 - Sediment Microplastic Isolation (SMI) units showing individual components, and salt marsh samples after mixing and after extraction: empty (left), after mixing, and after separation (right).

Filtration occurs at several stages within this method; however, they follow the same operating procedure. A glass conical flask, filter core and funnel piece are all assembled along with a membrane filter and held in place using a metal clamp. The filtration apparatus is then connected to a vacuum pump via a glass manifold (Figure 3.5) inside a laminar flow cabinet. Finally, a custom metal sieve is dropped into the funnel piece, and the apparatus is closed off by placing a glass petri dish on top. The supernatant solution is poured into the apparatus, sieving out the largest plant pieces (Figure 3.6). The solution is filtered onto a 0.45 μm pore-size 47 mm cellulose nitrate filter and rinsed three times to remove as much as possible of microplastics trapped in the vegetation. The filter is transferred to a clean beaker and immersed in 50 mL of 30% KOH:NaClO alkaline solution (15% KOH and 1% active chlorine after dilution) to digest organic material which may otherwise obscure or be mistaken for microplastics. The beakers were then placed in an Incubating Orbital Mini Shaker (VWR) at 50^{o}C, 120 rpm and left for 72 hours to digest.

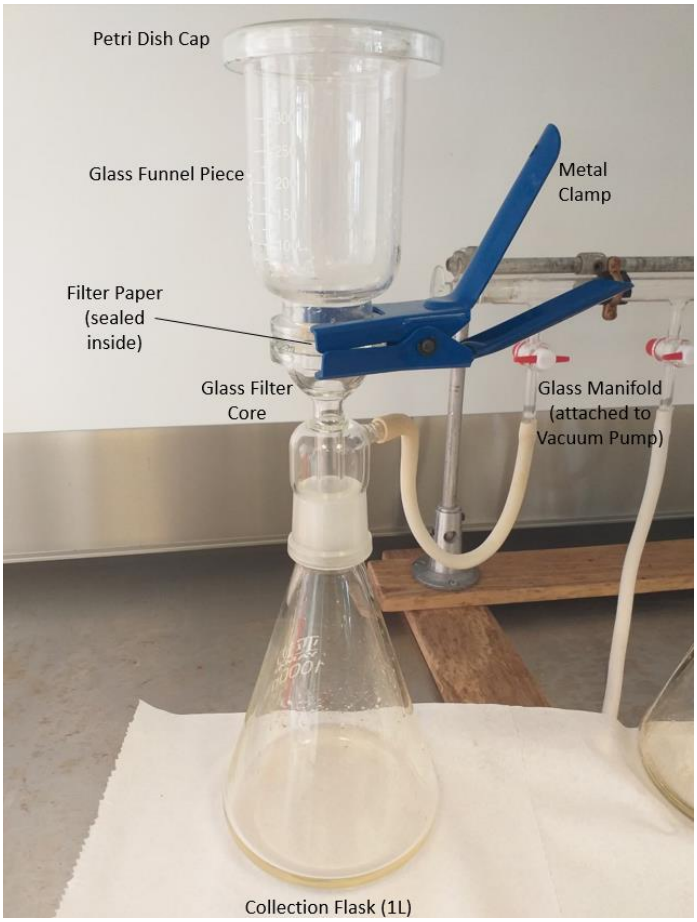

Figure 3.5 - The filtration apparatus set-up within the laminar flow cabinet, showing; individual components in final set-up, and attached glass manifold.

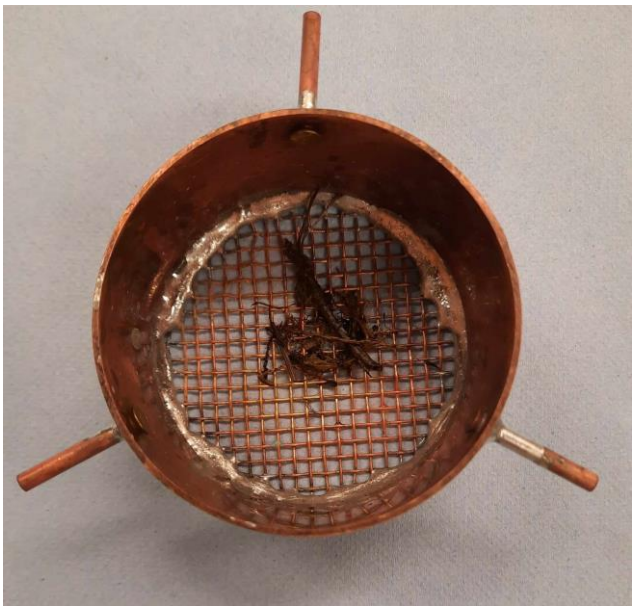

Figure 3.6 - Custom metal sieve piece, shown detached from glassware apparatus, containing larger vegetation pieces that have been sieved off.

After digestion the samples were filtered (using the previous apparatus set up) onto a 47 mm polycarbonate 2 μm pore size filters (Isopore) and rinsed will MilliQ water. The samples were then immersed with 5 mL of Nile Red solution (10 μg mL^{-1} in a 50:50 ethanol: water mix) and left for 30 minutes to ensure the complete staining of any plastics (Maes *et al.,* 2017). The filters are then removed from the apparatus, then either imaged immediately, or sealed in a glass petri dish and kept in the freezer (-20 ^{o}C) until analysed.

For each set of samples (4-5) a lab control (no sediment) was also carried following the exact same procedures.

3.3.9 Microplastic Imaging

Microplastics on the filters were counted using a UV fluorescence imaging technique (Maes *et al.,* 2017). Pre-stained filters were placed onto a motorised camera rig (see Figure 3.7), containing a UV torch (Crime-Lite 420-470 nm Blue) and a modified Canon EOS 6000 (with a Hoya 55mm Orange filter attached). By running a script in Mach 3 CNC, the camera and rig followed a programmed course taking a total of 24 photos (Shutter Speed: 1/20, Focal length: 5.6, Iso:800). The images are then stitched together using the program ImageJ to create an image of the fluorescing filter. Microplastics are then counted using an automated particle identification software on ImageJ, which measures the relative brightness of the particles in comparison to the background filter. In this final array, an individual pixel is approximately 1.5 μm, so by selecting a minimum particle size of 9 pixels (to avoid random bright spots on the camera), the minimum detection limit of microplastics was 15-20 μm. Microplastics were then categorized into size groups; <50 μm, 50-99 μm, 100-199 μm, 200-399 μm, 400-999 μm, 1000-5000 μm as recommended by Galgani (2023).

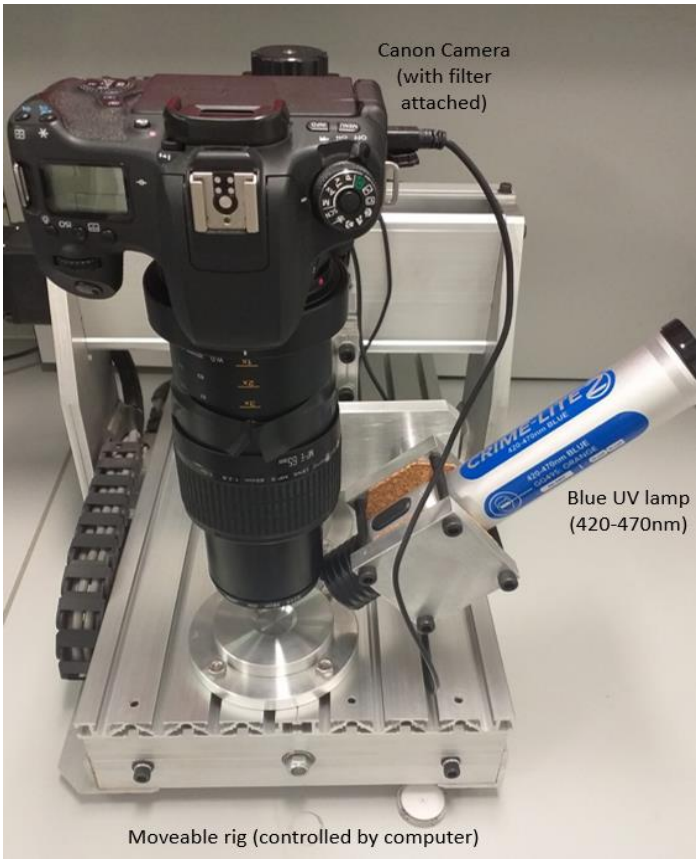

Figure 3.7 - Camera Rig used for imaging microplastic filters, showing labelled individual components.

3.3.10 Microplastic Analysis

A total of 107 particles were selected for analysis for their polymeric type to confirm detection using the Nile Red staining method. Microplastics were analysed using micro-ATR-FTIR Spectrometry to confirm particles as plastics and to gain more information regarding chemical composition. Using filters that had been sealed and stored, particles were tested using a Brooker Hyperion 2000 (20x ATR objective lens, resolution 4 cm^{-1}, 32-64 scans depending on the complexity of the sample). Filters were randomly selected, from which 3-5 particles were then tested (particles tested were based on particles that could be found using the instrument's built in fluorescent imaging – since this fluorescence was weaker than the Imaging phase it could be that some particles were missed due to a lower brightness). Of the 107 particles initially selected, a total of 83 were successfully analysed using micro-ATR-FTIR spectrometry. Spectra generated by the instrument were then compared to the online library Open Specy and analysed to find the best match (Figure 3.8).

To identify processed spectra, the hit quality was used only as a guideline, and spectra were manually reviewed with a focus on peak number alignment. These were then characterised as Known Plastic (>75% similarity), Potential Plastic (65>75% similarity), Non-plastics (>75% similarity with a non-plastic material) and Unidentifiable (no similarity with anything at least 65%). Potential plastics were then further scrutinised and based on the presence of indicator peaks/quality of the overall spectra, either accepted as plastics or not.

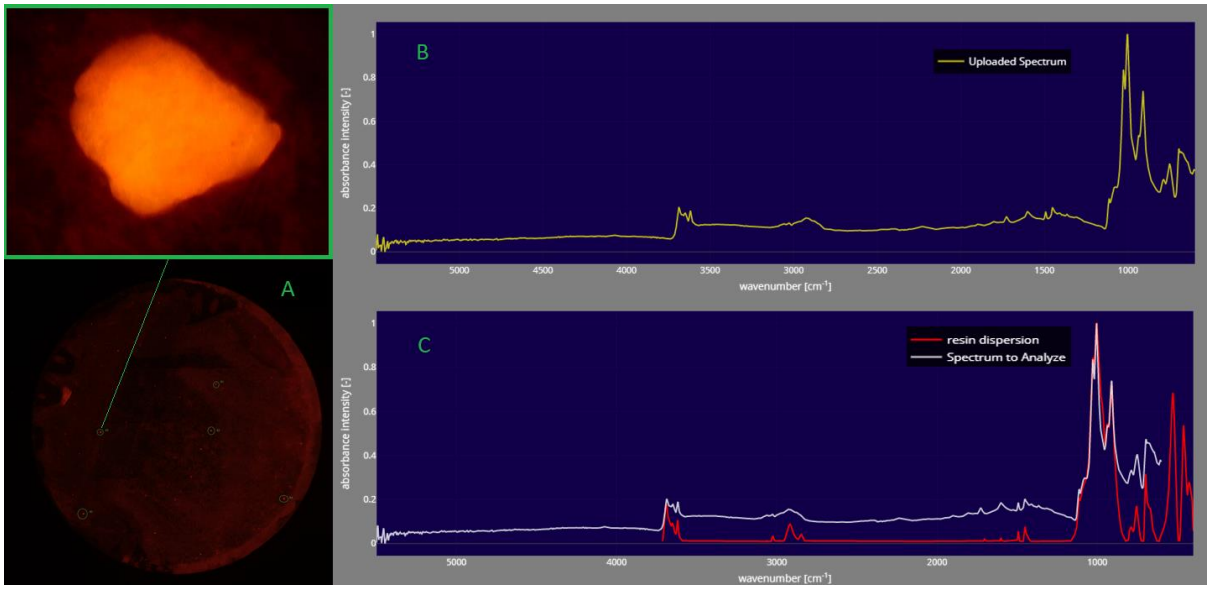

Figure 3.8 - Microplastic analysis outputs showing; A – Nile Red stained filter and particle, B – generated IR spectra for particle, and C – matched IR spectra on Open Specy.

3.3.11 Statistical Analysis

Correlation between all response and explanatory variables was done using Pearson Correlation Coefficient. Multiple correlations were detected (Figure 3.9, Tables 3.3 and 3.4) and therefore we proceeded to analyse the response of microplastic numbers to single explanatory variables instead of including multiple variables in a single model. Microplastic abundance (per area and per weight) in relation to the explanatory variables Vegetation Height, Elevation, and Distance from the edge of salt marsh, was initially analysed using linear regression models. Due to missing values for Elevation, we had to remove three observations from the Elevation models. Comparing microplastic abundance between different vegetation zones was initially done with ANOVA.

We detected heterogeneity of variance in all models with a greater spread of residuals in the low/mid marsh, and depending on Vegetation Height, Elevation, and Distance (Zuur *et al.,* 2009). Therefore, generalized least squares models (GLS) were applied using the 'nlme' package (Pinheiro *et al.,* 2017)and the appropriate variance structure was selected using the Akaike information criterion (AIC) following the protocol outlined in (Zuur *et al.,* 2009). For the explanatory variable Zone we applied the 'VarIdent' variance structure to allow for different variance between zones. The 'varExp' Variance Structure was used for the other three models (Vegetation Height, Elevation, and Distance). All statistical tests were carried out using 'R' version 4.4.3 (R Core Team, 2025).

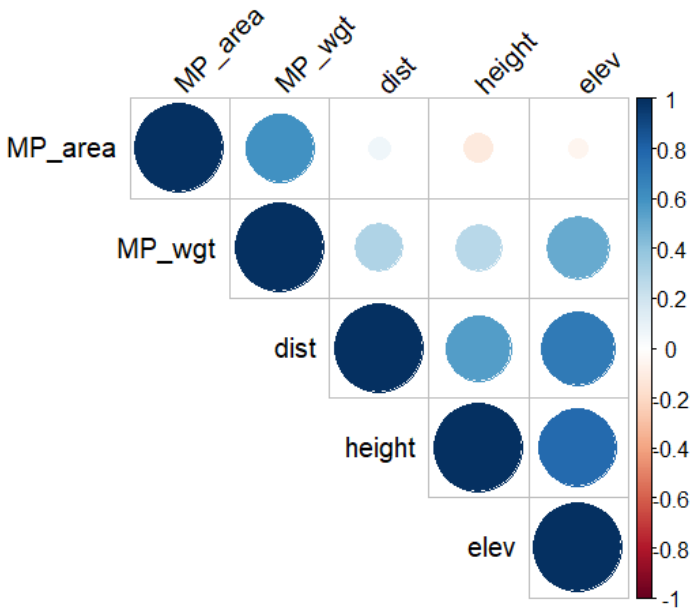

Figure 3.9 - Correlation plot of weight (kg) and area (m^2) based microplastic data with the factors distance from the edge of the salt marsh, vegetation height and elevation. Shows significant correlations as dots, with colour indicating the correlation coefficient.

Table 3.3 - Correlation coefficients between microplastic data per area (m^2) and per weight (kg) with the factors vegetation height, distance from the edge of the salt marsh and elevation

	MP_area	**MP_wgt**	**height**	**distance**	**elevation**
MP_area	1	0.62	-0.13	0.06	-0.05
MP_wgt	0.62	1	0.25	0.29	0.5
height	-0.13	0.25	1	0.54	0.78
distance	0.06	0.29	0.54	1	0.71
elevation	-0.05	0.5	0.78	0.71	1

Table 3.4 - P values of correlations between microplastic data per area (m^2) and per weight (kg) with the factors vegetation height, distance from the edge of the salt marsh and elevation

	MP_area	MP_wgt	height	distance	elevation
MP_area		0	0.2255	0.6816	0.7078
MP_wgt	0		0.0555	0.027	0
height	0.3355	0.0555		0	0
distance	0.6816	0.027	0		0
elevation	0.7078	0	0	0	

3.4 Results

3.4.1 Reporting Microplastic Numbers

Within current microplastic literature there is no definitive units for reporting microplastics, with sediment studies most commonly using; microplastics per weight (g – Kg, dry weight and wet weight), microplastics per area (cm^2 – m^2) or microplastics per volume (cm^3 – m^3). The units used are often dictated by the type of samples and research questions of the study.

For all data analysis and comparisons in this study, the number of microplastics was calculated per area (m^2) of sediment and per dry weight (kg) of sediment. Volume based measurements were not considered due to the study only focussing on the surface samples, and therefore results would not be representative for larger volumes of sediment with greater depths. Number of microplastics for each measurement are reported and tested against when considering the impact of different physical factors. However, when considering the discussion of the results, per area is primarily considered when comparing the impact of different marsh factors. This is due to sediment weight varying based on composition and therefore is not constant across the various cores sampled. Given the samples consist of only 1 cm depth, area-based measurements give the most evenly distributed microplastic data for when comparing samples from different zones. If trends observed differ between the area-based and weight-based microplastic abundance, this is highlighted in the discussion of those results.

3.4.2 Evaluation of Procedural Blanks

Alongside each set of three sediment samples, a procedural blank was also run to assess the contamination from the laboratory sources. An average of 14 ± 2.1 microplastics were found per control (see Appendix 1.3 for full control data). This contamination was anticipated, due to the use of plastic wash bottles and SMI units within the procedure, as well as potential air contamination, however was in line with control results reported in previous sediment and SMI studies (Hanvey *et al.,* 2017; Prata *et al.,* 2019). As recommended by Wright et al. (2021) this number of particles was subtracted from each sediment sample when calculating the final number of microplastics.

3.4.3 Abundance of Microplastics

Of the 58 sediment samples that were analysed, microplastics were found in 100% of them. This amounted to a combined total of 1817 individual microplastics, including fibres and fragments, and varying in size from 20 μm to 4000 μm. The abundance of microplastics varied considerably, ranging from a low of 873 (mudflat sample) to a high of 98,689 (lower/mid marsh) particles/m^2. The average number of particles across all samples was 21,000 ± 2,300 particles/m^2 in the size range of ~ 20 - 5000 μm.

3.4.4 Microplastic information

Of the microplastics imaged, nearly all were fragments with only four fibres and a single film detected. Due to the nature of the imaging protocol; being stained with Nile Red and then set against a cluttered background, colour of the microplastic particles was not able to be determined. The most dominant size fraction of microplastics was <50 μm (Table 3.5), accounting for 73% of the total particles. As size increases, the number of particles per fraction decreases, with particles in the >1 mm fraction making up the last 0.3% of the microplastics. These size-based trends are consistent across all the vegetation zones, with the high marsh showing a slight alteration with its larger population of 1000-5000 μm sized microplastics comparative to the other zones.

Table 3.5 - Total number of microplastics of different size fractions for each vegetation zone

	Marsh Zone - Total Number of Microplastics					
Microplastic Size Fraction	**Mudflat (n=12)**	**Pioneer (n=15)**	**Spartina (n=6)**	**Lower/Mid Marsh (n=15)**	**High Marsh (n=10)**	**Total Number of Microplastics per size fraction**
<50 µm	199 ± 2.76	269 ± 2.83	90 ± 2.57	620 ± 8.8	116 ± 2.75	1294
50-99 µm	43 ± 0.7	70 ± 0.98	14 ± 0.51	115 ± 2.18	32 ± 0.71	274
100-199 µm	34 ± 0.77	24 ± 0.58	5 ± 0.37	65 ± 1.13	10 ± 0.37	138
200-399 µm	6 ± 0.25	18 ± 0.33	1 ± 0.15	21 ± 0.37	5 ± 0.21	51
400-999 µm	5 ± 0.18	6 ± 0.18	2 ± 0.19	6 ± 0.16	2 ± 0.13	21
1000-5000 µm	1 ± 0.08	0	0	0	5 ± 0.29	6
Total Number of Microplastics per zone	288	387	112	827	170	1784

3.4.5 Confirmation of Microplastics

A total of 83 particles were successfully tested using micro-ATR FTIR spectroscopy. 43 particles were confirmed to be known plastics. Only one particle was confirmed to be non-plastic (chitin), whilst another 32 were found to be potential plastics and the remaining 10 were unidentifiable. Of the 32 potential plastics another 30 were concluded to be plastics after further consideration of the spectra. Over 10 different polymers were identified, with paint resin, Polypropylene and PVC being the most common plastics (Figure 3.10).

Whilst not all particles could be successfully identified, this is expected in environmental samples where damage and degradation of particle surfaces is common. However, as over 80% of particles were successfully identified, and this was in line with other environmental studies (Pinheiro *et al.,* 2022; Wu *et al.,* 2020), these results were considered as a successful chemical validation of microplastics from the samples.

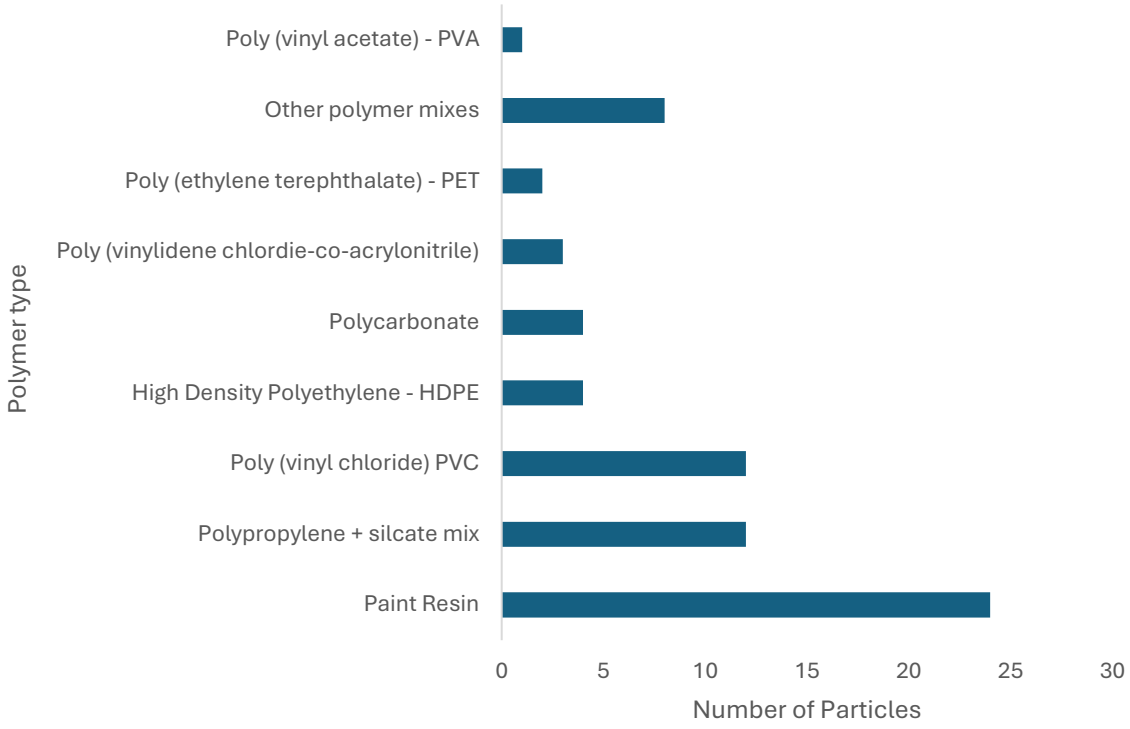

Figure 3.10 - Polymers successfully identified by FTIR, showing the number of particles for each polymer found.

3.4.6 Microplastic Distribution

Microplastics (per area and per weight) were compared against the physical factors measured, and the results plotted. Figure 3.11 shows the comparison of the number of microplastics with the first factor, vegetation height. Microplastics show a wide distribution, with a slight peak between 10-30 cm vegetation height. The GLS model indicates a significant negative correlation with vegetation height for area data, whilst a significant positive correlation for weight data (Table 3.6).

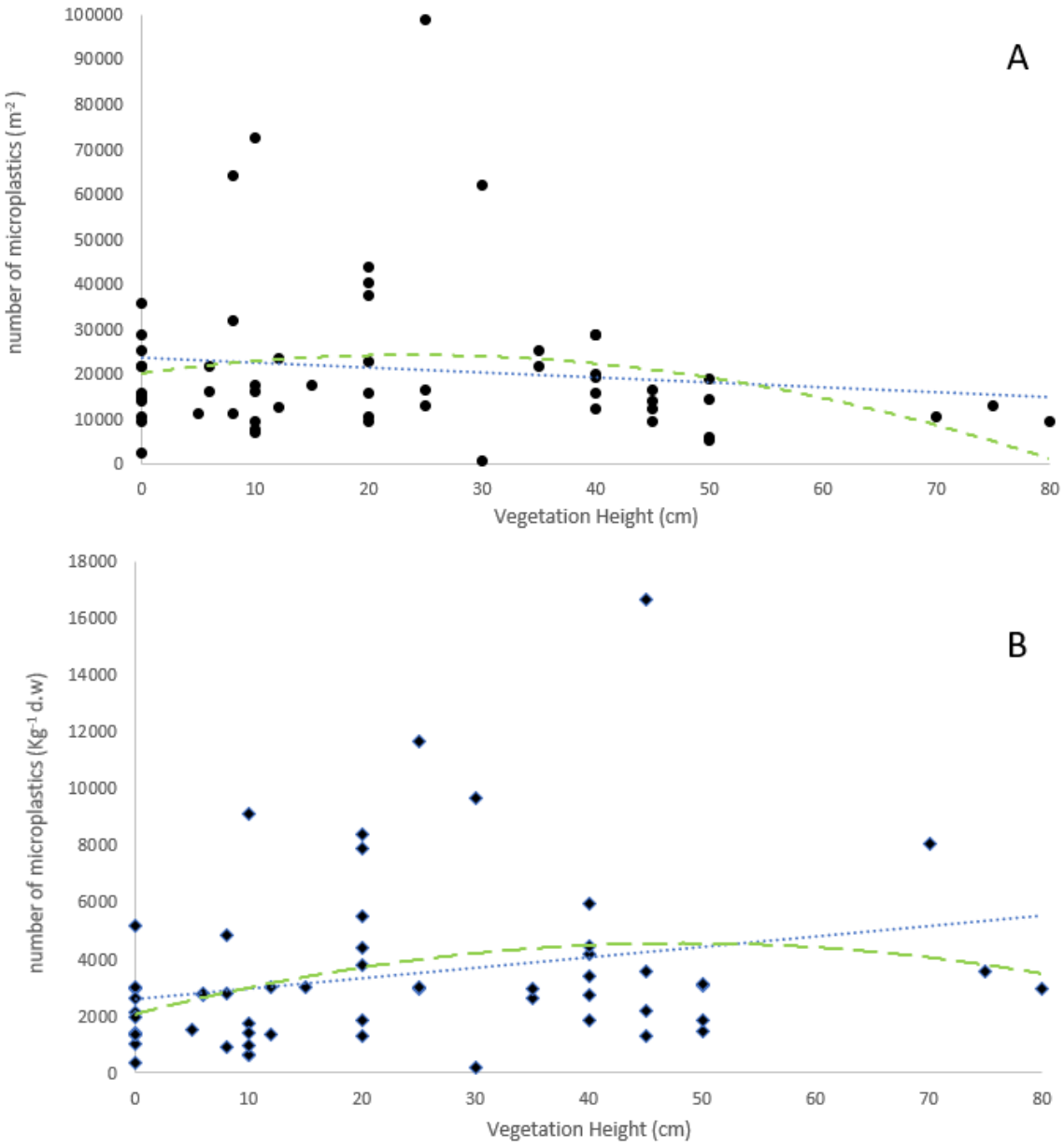

Figure 3.11 - The plot between number of microplastics and vegetation height; A – particles per area (m^2), B – particles per weight (kg d.w), showing linear (blue) and non-linear (polynomial - green) lines of best fit.

Table 3.6 – Results of the GLS models used to test marsh factors vegetation height, elevation and distance from the edge of the salt marsh with the microplastics per area and per weight datasets, showing degrees of freedom (DF), F values and p values

	area			**weight**		
	Df	**F**	**p**	**Df**	**F**	**p**
height	1	6.17	0.016	1	7.04	0.01
elevation	1	2.71	0.1054	1	11.45	0.0014
distance	1	1.72	0.1948	1	9.4	0.0034
zone	4	4.69	0.0026	4	5	0.0017

Figure 3.12 shows the results of number of microplastics plotted against the factor elevation. Microplastics show a wide variation of values, however have a distinct peak around 2.3m elevation. For the area data, the GLS model indicates no significance with microplastic abundance. For the weight data the model shows a significant correlation for elevation (Table 3.6).

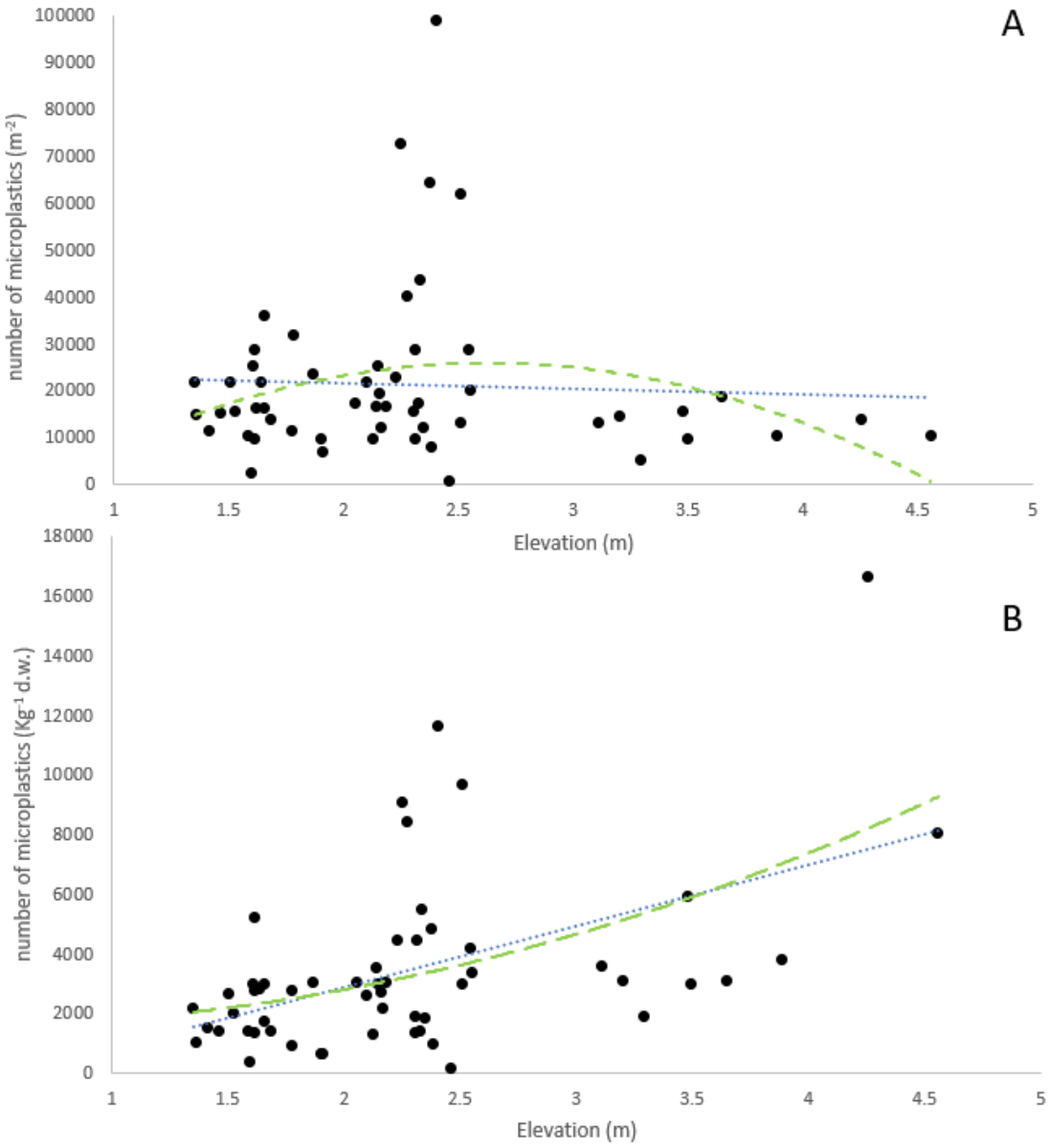

Figure 3.12 - The plot between number of microplastics and elevation: A – particles per area (m^2), B – particles per weight (kg), showing linear (blue) and non-linear (polynomial - green) lines of best fit.

Figure 3.13 shows the results of number of microplastics plotted against the final factor, distance from the salt marsh edge. The creek-side edge was designated as the starting point, with the start of the adjacent mudflat being 0 m and values increasing up towards the high marsh. Microplastics show a wide variation, with the highest values for both weight and area measurements occurring approximately 60 m from the salt marsh edge. The GLS model showed no significance for per area data, whilst the per weight data showed a significant positive correlation with distance (Table 3.6).

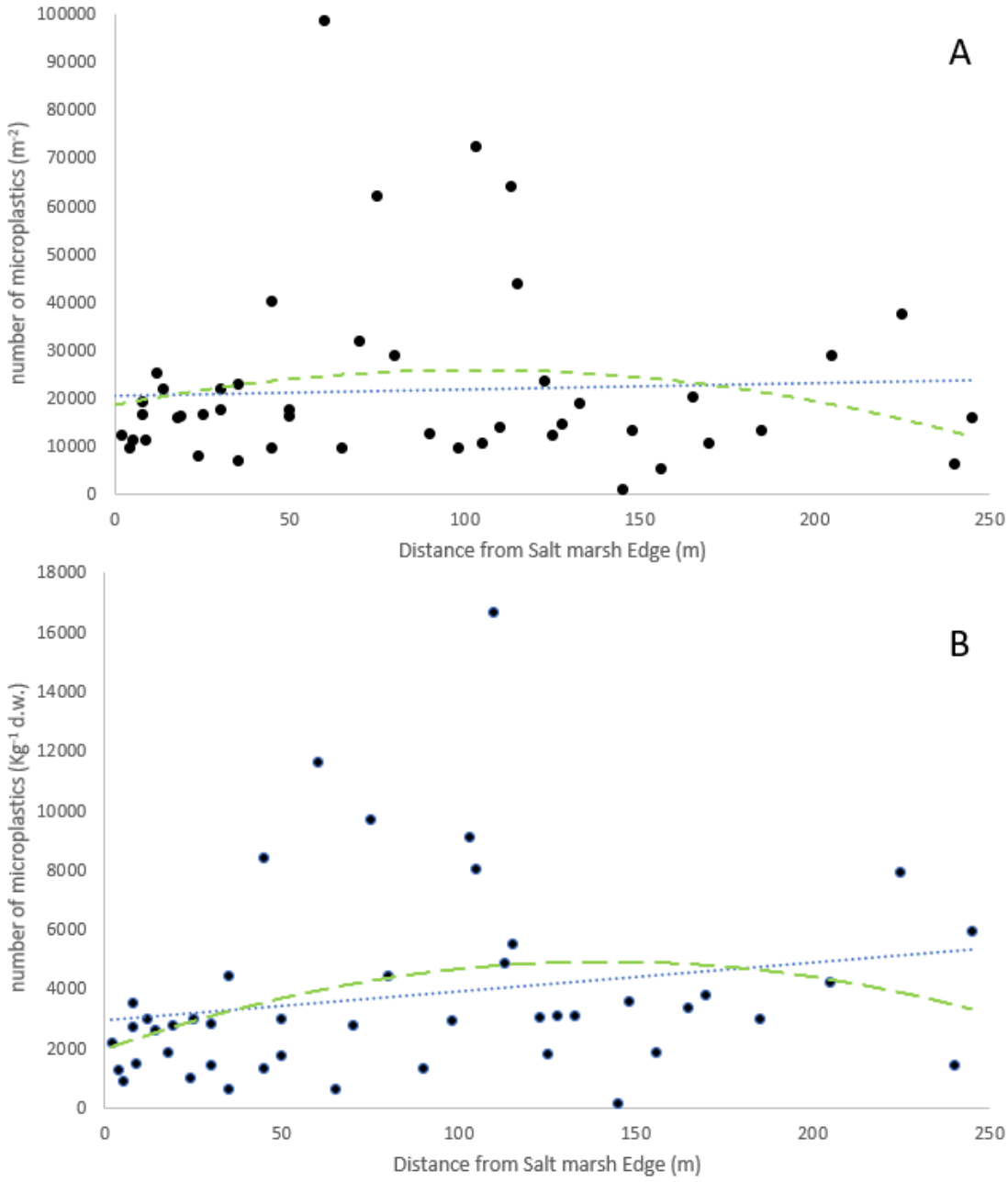

Figure 3.13 - The plot between number of microplastics and distance from the salt marsh edge: A – particles per area (m^2), B – particles per weight (kg), showing linear and non-linear (polynomial) lines of best fit.

Finally, we compared salt marsh zonation (Figure 3.14) and microplastic abundance. Whilst most of the zones show no visible difference between microplastic numbers, there is an increase in the Lower/Mid marsh zone, over twice the amount of microplastics found in the other zones. A significant effect of zone (Table 3.6) was found for the area data, showing the Lower/Mid marsh zone having significantly higher values. The weight data showed a

similar pattern, however with both the Lower/Mid and High marsh zones showing significantly higher values.

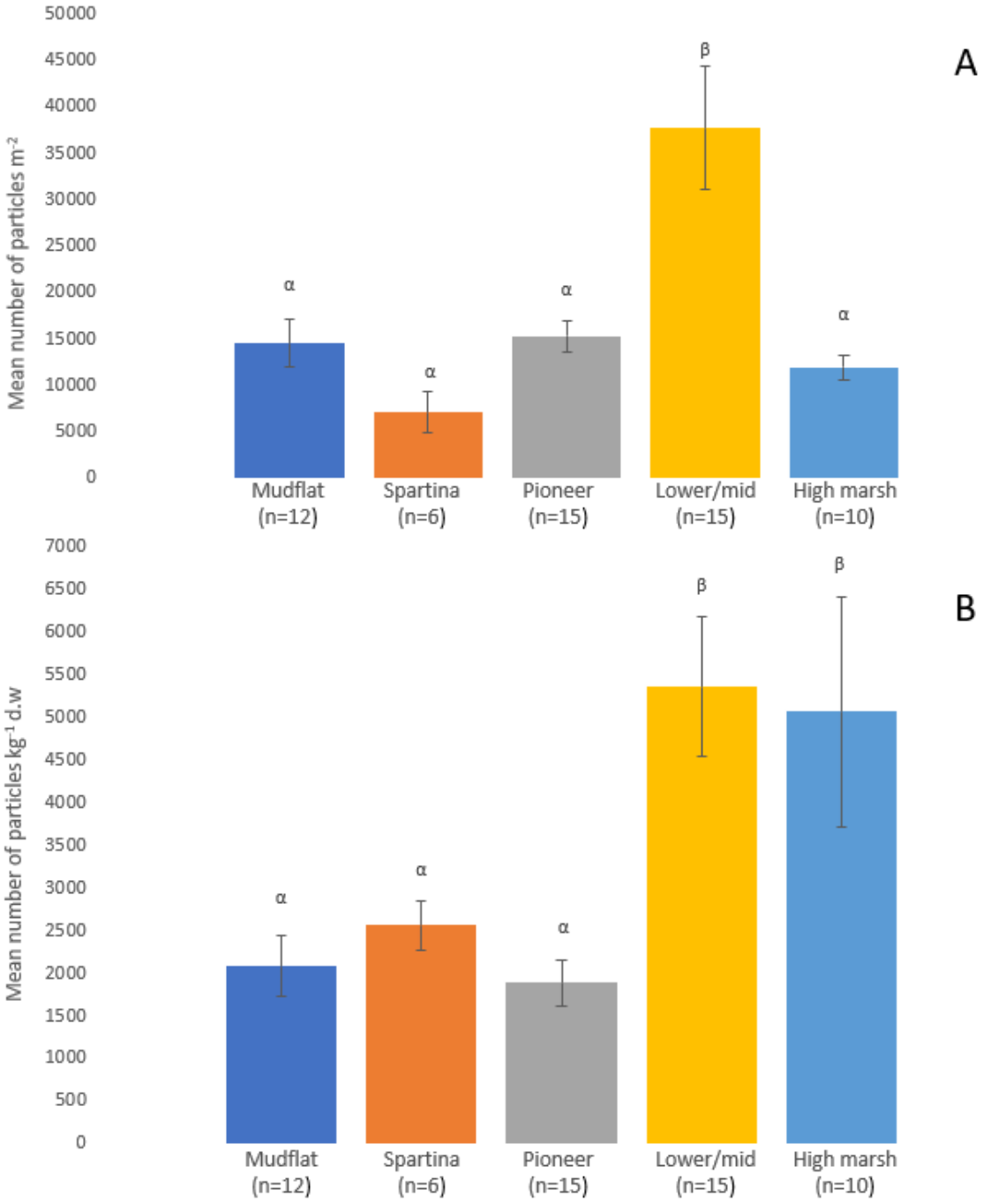

Figure 3.14 - Effect of vegetation zones on the average number of microplastics, A – particles per area (m^2), B – particles per weight (kg d.w.). Error bars represent standard error, letters (α, β) indicate significant differences between groups.

3.4.7 Sediment Analysis

Using the additional sediment cores collected (Section 3.3.2), sub-samples were tested and analysed using a variety of different techniques. These include grain size analysis and calculating bulk density (d.w.) (Table 3.7).

Table 3.7 - Mean bulk densities of the vegetation zones and adjacent mudflat

Vegetation Zone	**Mean Bulk Density (g/cm^3) (d.w.)**
Mudflat (n=4)	0.854
Spartina (n=2)	0.587
Pioneer (n=4)	0.826
Lower/Mid Marsh (n=6)	0.808
High Marsh (n=4)	0.320

The bulk densities show similarities between several zones, with the mudflat having the highest density, and the lowest density belonging to the high marsh.

Due to having a limited sample size, the grain size analysis data was not compared with any microplastic data, however, is included in Appendix 1.3.

3.5 Discussion

3.5.1 Presence of Microplastics

In this study we reported some of the first values for microplastics in UK salt marsh sediments. The concentrations varied across the marsh, ranging from 873 – 98,689 particles/m^2 (164 – 11684 particles/kg d.w.), with an average number of 21,000 ± 2,300 particles/m^2 (3400 ± 390 particles/kg d.w.) for particles in the size range ~ 20 – 5000 μm. When investigating the effect of different factors on microplastic abundance, it was found that elevation and distance from the salt marsh edge had no significance. Vegetation height was found to be significant however none of the factors showed any distribution trends. Zonation was found to have a significant effect on the number of microplastics per area and per weight-based data, with the lower-mid marsh having a significantly greater number of microplastics than the other zones.

Microplastic data are widely reported using either a per weight or per area system, as these are easily converted and compared across different studies. However, when comparing the findings of the different factors, we observe that the two datasets (per m^2 and per kg) have differing results. Considering the factors vegetation height, elevation, and distance, we observe that these factors are significant when tested against microplastic distribution for per kg dataset. Comparing this to per m^2, only vegetation height was found to be significant, and the correlation is now negative rather than positive as in the weight data. However, when plotting the data sets against each factor, the data points are widespread with poor trendlines and no observable patterns. From these results no single factor explains the microplastic distribution in both the weight and area data sets, suggesting that the unit has no real effect on our results. However, a further difference is noted in the effect of zonation on the number of microplastics. For microplastics per area, the Lower/Mid marsh zone is found to be statistically significant from all four other zones. Compared with microplastics per weight, both the Lower/Mid marsh and High marsh zones are found to be significantly different. This is due to the natural variation in bulk density of salt marsh sediments. Composition of the sediments can vary greatly depending where on the marsh the sediment was collected (Table 3.7). This is particularly noticeable in High marsh samples, where the sediment is largely comprised of leaf litter and sand, resulting in a lower bulk density than sediments in other zones. Having such a variation in sediment density means that reporting microplastics per weight of sediment can be skewed, and therefore when comparing these results with patterns observed in other studies, we shall focus on the microplastics per area dataset.

When comparing the microplastics at Blakeney Point to other salt marshes around the world, the abundance of microplastics reported from this study were quite substantial. Pinheiro et al. (2022) and Wu et al. (2020) reported average microplastic concentrations of 100 – 300 particles/kg d.w. sediment, with Gray et al. (2018) reporting a concentration of 413.8 ± 76.7 particles/m^2. The average number of microplastics found at Blakeney was 21,000 ± 2,300 particles/m^2 (3,400 ± 390 particles/kg d.w. sediment), several orders of magnitude greater than these other studies. However, the highest recorded microplastic concentrations were found in estuarine salt marshes with large catchment areas (>10,000 km^2) neighbouring waste disposal sites or major urbanisation. Such marshes, as studied by

Lautaro et al. (2023) and P. Yao et al. (2019), reported an abundance of microplastics in the range of 716 - 20,060 and 9,600 – 130,725 particles/m^2 respectively. Our numbers are more comparable to these results than other studies, however the Blakeney salt marshes have a much smaller catchment area (137.1 km^2 (Kershaw *et al.,* n.d.)) and very little anthropogenic footfall or urbanisation. The relatively high numbers recorded likely resulted from the differences in microplastic detection methods. Pinheiro et al. (2022), Wu et al. (2020) and Gray et al. (2018) all used visual detection with a microscope, resulting in a lower detection limit (50 – 100 μm) and lower accuracy of detection in smaller particle sizes. The Nile Red methodology in this study has a detection limit of about 10-20 μm (Kukkola *et al.,* 2022; Maes *et al.,* 2017), and thus with automated detection can detect a broader range of particles. The large microplastic concentration found may therefore come from measuring the broadest range of sizes, with over 70% of the total microplastics found being <50 μm. This fraction may be very important for salt marsh environments, where the natural conditions will accelerate microplastic breakdown (Weinstein *et al.,* 2016) and so we expect to observe a high percentage of the plastic population in these smallest size categories.

The global region of studies may also have an impact on the microplastics recorded, with most salt marsh studies taking place in Southeast Asia or South America, areas with very different approaches to handling plastic disposal than in the UK. When inspecting UK based studies, Nel et al. (2020) and Wilson et al. (2021) reported microplastics in UK beaches at concentrations of >700 particles/kg d.w. sediment and 132 ± 66 particles/m^2 respectively. Higgins and Turner (2023) investigated the occurrence and abundance of microplastics in marine surface waters in Plymouth, finding concentrations in the range of 0.26 to 0.68 particles/m^3. Finally, Gallagher et al. (2016) collected microplastics via plankton net trawling from several estuaries around the UK, finding a range of microplastics from 296 – 1155 particles per site. Whilst these studies featured a range of different sites and methodologies, they showed average numbers of microplastics around the UK to be relatively low. This study reports microplastic values much greater than otherwise observed around the UK, suggesting that salt marshes might be environments with high microplastic accumulation potential.

3.5.2 Comparison of Microplastic Distribution

Whilst microplastics were abundant across the whole of the salt marsh, there was a clear hotspot area in the data, the Lower/Mid marsh zone. Other zones (including the neighbouring mudflat) averaged between 6,986.9 – 15,196.51 particles/m^2, whereas the Lower/Mid marsh averaged 37,583.7 particles/m^2. This peak is generally consistent with data from other salt marsh studies, with Pinheiro et al. (2022), Wu et al. (2020) and W. Yao et al. (2019) finding a similar increase between vegetated environments and their neighbouring unvegetated mudflats. Whilst many studies did not consider zonation or were not conducted in large enough marshes to display a clear zonation, Mazarrasa et al. (2019) and Lautaro et al. (2023) both observed a steady increase in the number of microplastics heading inland, peaking in the strandline on the edge of the High marsh. Whilst our studies High marsh samples were significantly lower, this might be due to the topography of the marsh itself. Unlike the gradual incline up the marsh, the High marsh at Blakeney lies on a raised levee, thus quickly increases in elevation, being 1-2 m higher than the adjacent Lower/Mid marsh. This steep increase in elevation means the High marsh does not receive much inundation even at high tide, and thus the strandline lies on the edge of the Lower/Mid marsh, coinciding with the higher number of microplastics found there. There are also other factors that may be influencing microplastic distribution, as suggested by Qian et al. (2021), such as sediment properties and hydrodynamic conditions. Even within these vegetation zones, the coverage, density, and speciation of the vegetation likely varies between these globally different marsh sites (Yando *et al.,* 2023), thus resulting in unique local environments that trap microplastics in their own specific way. However, comparing to these other salt marsh sites confirms there is a trapping effect of vegetation when contrasted with mudflats, and that microplastic trapping may also be influenced by other marsh factors as well.

3.5.3 Parameters driving Microplastic Distribution

With marsh vegetation having a known trapping effect on sediment (Li and Yang, 2009), we hypothesized that it may therefore have a similar effect on microplastics as well. This has been observed by Cozzolino et al. (2020) who showed increasing leaf and frond area increased microplastic accumulation in coastal vegetated habitats. Vegetation effects were also shown in artificial flume studies, with de los Santos et al. (2021) showing the vegetated

canopy of a seagrass meadow effectively trapped microplastics, especially when compared to non-vegetated sand. McIlwraith et al. (2024) demonstrated that microplastics can be trapped on the vegetation blades and the presence of vegetation influenced microplastic depositional patterns, whilst Gallitelli et al. (2023) found an entrapment rate for microplastics in riverine vegetation of 44.3 – 55.7%. For our study we tested this influence using vegetation height. Taller vegetation has a larger surface area, providing greater surface to trap microplastics, but also slowing more water and encouraging more particle deposition than shorter vegetation (Cozzolino *et al.,* 2020). Whilst other vegetation metadata was recorded such as density and speciation, these were not quantitatively measured and were instead based on rough observation, therefore not suitable for comparison with our microplastic data. Initially our results suggested that the vegetation height was significant, however the correlation with our microplastic data was negative. This suggests microplastics decrease with increasing vegetation height, the is the opposite of what we expect. To observe whether this was the case, vegetation height was plotted against our number of microplastics in each sample (Figure 3.11). Despite the significant negative correlation from the GLC model, the plotted data was erratic and widespread, with no discernible trends and poor linear and non-linear lines of best fit. A slight peak between 10 – 30cm was observed, this is likely caused by the exceptionally high values of the lower-mid marsh, whose vegetation was predominantly within this height range. Initially, the negative correlation and lack of overall trends was thought to be due to differences in vegetation species in the different transects. Transects C and D feature a large Spartina zone, which deviate from the otherwise linear trend of increasing vegetation height along the transects. However, when plotted as individual transects (Appendix 2) the trendlines were still poor ($R^2 < 0.05$) and do not correlate with the microplastic abundance. This is likely due to vegetation only being partly responsible for microplastic trapping, with McIlwraith et al. (2024) and de los Santos et al. (2021) noting that particle shape and flow velocity also had the most significant influence over the microplastic distribution in their vegetation studies. Therefore, while the initial hypothesis (ii) is supported for vegetation height, the correlation is the opposite of what we anticipated. For the other factors this hypothesis was found to be incorrect. Hypothesis (iii) was also found to be incorrect, with each individual factor having no positive correlation on microplastic abundance and showing no observable effect on microplastic distribution.

The other factors plotted in this study were distance from the edge of the salt marsh (creek side edge, the mudflat was classified as 0m with values increasing up towards the high marsh), and elevation. We assume the primary source of microplastics in this study is the channel water, or plastic litter deposited from the channel (marshes sites were away from footpaths and so likely received little anthropogenic litter deposition). Initially we hypothesized that both these factors should negatively impact the amount of microplastics. Plotting both factors against the microplastic data (Figure 3.11 and Figure 3.12) revealed that neither factor has any significant correlation. Microplastics numbers seem to be consistent despite changes in both factors, resulting in very poor trendlines, with any slight peaks in the data being explained by the high microplastic numbers reported in lower-mid marsh samples. These poor fits continued even when we plotted individual transects and added mudflat data (Appendix 2), suggesting that neither distance nor elevation has any significant (p values > 0.7) influence over the deposition of microplastics. Whilst this is contrary to Temmerman et al. (2003) findings for sediment deposition in a marsh, it highlights an important difference in the behaviour of microplastics. There are several potential factors for this, such as the wider variety of morphologies observed in microplastics. Alternatively, it could be due to the influence of surface properties, with microplastics again showing great variety which could affect their deposition, retention and ability to aggregate. Finally, this difference could be due to influence of a more dominant, unmeasured factor. Many hydrological factors were considered for this study, including inundation time and microplastic concentration within the river, creek, and sea, all of which may influence the microplastic distribution. Due to limitations of time and cost this data was not collected, and therefore these factors, or even more complex hydrodynamic properties such as local flow velocity and patterns, could not be measured, and so effects of these factors may potentially be masking the influence of the physical marsh factors that we expected to see.

In contrast to the initial hypothesis (iii), the data suggested that none of the individual factors have any strong trend with the microplastic distribution. However, it needs to be considered that in this field study, many of the factors are not independent. With both positive and negative correlation found between all the factors (Tables 3.3 and 3.4), there was a need to consider the effects of multiple factors combined. To do this we looked at

zonation, which is influenced by the previously studied factors, but also factors such as inundation time and frequency.

When examining the effect of zonation, we found a significantly higher number of microplastics in the Lower/Mid marsh compared to all other zones. This could be due to the cumulative effects of the studied factors (and other unmeasured factors). The Mudflat, Pioneer and Spartina zones all showed very similar abundances of microplastics (Figure 3.14). As these zones are closest to the marsh edge (within 50 m) they receive the greatest amount of inundation and will be submerged with nearly every tide. Vegetation is limited, with bare mud representing 100%, 70% and 25% of each zone respectively. Despite the regular immersion allowing for potential microplastic deposition, the low vegetation coverage and overall short plant species (5 - 30 cm) do not provide much physical area for the trapping of microplastics. Furthermore, the combination of exposed mud and regular tidal motion means that many microplastics deposited on the incoming tide are potentially again resuspended as it recedes. The Lower/Mid marsh is the largest zone in the marsh and receives relatively frequent inundation. This is where small shrubs (*Halimone portulacoides)* start to dominate and therefore have good coverage of vegetation density (<5% exposed mud) and height to trap microplastics. Effectively, its sits in the area of optimal overlap between these several factors, receiving enough inundation to supply a source of microplastics, whilst having enough vegetation to be able to trap and retain them. As the High marsh is the furthest zone from the channel (100 – 200 m), it receives the least amount of inundation and is likely only fully submerged during storm flooding. Vegetation here is dense and dominated by larger shrubs (*Suada vera,* 50 – 100 cm high). However, since the inundation is so infrequent, microplastics in the water are rarely transported up into this zone to be deposited and trapped.

Another explanation for the significant increase in the Lower/Mid marsh zone, would be to consider the distribution of macroplastics. Whilst macroplastic litter was quite low in the Blakeney marsh, when it was recorded it was found in the Lower/Mid marsh zone. This is likely due to the strand line situated within this zone, and therefore macroplastics brought in with the tide are washed up here. These macroplastics would provide a secondary source of microplastics to the zone, via their rapid degradation. No macroplastics were recorded in the other zones, and so while the overall macroplastic influence is low, it was localised to

the Lower/Mid marsh and therefore potentially contributing to the significantly high microplastic numbers observed.

3.5.4 Microplastics Sizes

Microplastic size information is often limited by the detection methods used in a study, and so whilst the official definition includes the range of 1 μm – 5 mm, most reported microplastics are between 1 – 5 mm. Studies which use visual identification find most microplastics within this size category, with Lautaro et al. (2023) finding 80.9% of their plastic litter within this range, and Pinheiro et al. (2022) reporting similar size ranges. Where lower size detection is possible, there is a notable increase in microplastics as size decreases. Li et al. (2019) observed that over 80% of their particles were in the micro range (1-999 μm), whilst Gray et al. (2018) and P. Yao et al. (2019) found 150-499 μm and 50-100 μm to be the most prominent size fractions respectively. This matches the findings of this study, in which the <50 μm size fraction was most common, making up 72.5% of all microplastics. Numbers then decreased as size fraction increased, with the largest size fraction (1-5 mm) representing only 0.33% of the total amount. The dominance of lower size fractions is generally seen in environmental samples, due to natural degradation and breakdown of samples. Salt marshes provide ideal conditions for microplastic breakdown (Weinstein *et al.,* 2016), which may explain the trend in size distribution observed.

3.5.5 Microplastic Morphologies

In this study microplastics were dominated by fragments (>99%). In the 1817 particles recorded, 4 were identified as fibres, 1 as a film and the rest as fragments. This is contrary to most environmental studies, where fibres are the most dominant microplastic type. Values of fibres range from 60 – 90% prevalence (Cozzolino *et al.,* 2020; J. Li *et al.,* 2020; Lo *et al.,* 2018; Stead *et al.,* 2020; Wu *et al.,* 2020; Q. Zhou *et al.,* 2020), and this high percentage matches with global production and availability of fibre waste (Lima *et al.,* 2021). The sources of fibres are anthropogenic runoff and wastewater treatment (Boucher and Friot, n.d.); thus, they naturally accumulate in river systems before being washed out to coastal habitats (Barrows *et al.,* 2018). However, as previously discussed the Blakeney marsh has a very small catchment area (137.1 km^2) and only a single small river inflow, making the potential input of fibres quite low. Furthermore, the methodology of this study

is not conducive to fibre detection. The Nile Red staining has varied fluorescence intensity in fibres (Galvão *et al.,* 2023), which against the background fluorescence of the filter makes them harder to detect by automated means. It could also be the dynamics of salt marshes are not suited to trapping fibres. Fibres are generally lighter than fragments, and therefore are more easily resuspended by the tide. This is supported by fibre numbers in other salt marsh studies, as Lautaro et al. (2023) saw a dominance of films, and Pinheiro et al. (2022) found high prevalence of fragments in both the high and mid marsh zones (80%+). Finally, with the majority of microplastics in this study being <50 µm, this suggests they might be the result of natural plastic breakdown. Fibres and films would degrade into fragments, again contributing to their lack of representation.

3.5.6 Microplastic Types

In this study, over 10 different polymers were successfully identified using FTIR spectrometry, the most common being Paint Resin (resin dispersion (Primpke *et al.,* 2018), Polypropylene and PVC. This occurrence differed from other coastal studies, in which Polyethylene was either the most common polymer (varying from 25-67% prevalence), or at least in the top three. We would expect this sort of distribution, as it corresponds to the main types of polymers used in global plastic production (Geyer *et al.,* 2017). However, there is also local plastic variation to consider, with J. Li et al. (2020) finding Polystyrene most abundant (40.1%) and Wu et al. (2020) showing Rayon (45%) as the most prevalent polymer type. Here, the most dominant polymer, the paint resin, matches that of hydrophobic resins used in boat coatings, therefore the site being adjacent to a small harbour would explain this unusually high result in this study. Additionally, the samples tested with FTIR were primarily to test the efficiency of the Nile Red staining method and were selected randomly. The tested samples represented only 5% of the total microplastics found, and therefore may not provide an accurate description of the whole marsh.

3.5.7 Future Work

Whilst Blakeney Point is a good example of a remote and otherwise natural salt marsh, it is only one of many around the UK coast. As such the data represented in this study are extremely localised. Factors such as hydrology, vegetation coverage and plastic input, will

differ from site to site, and thus generalised conclusions about the impacts of certain factors are difficult to apply to all salt marshes. Furthermore, due to collecting only one batch of samples, the data only describes the marsh during the collection period, with no representation of seasonality. However, whilst seasonal variations have impacted microplastic abundance in previous coastal studies (Jiwarungrueangkul *et al.,* 2021), these reports come from regions with significant seasonal change (wet and dry seasons), therefore in a more temperate climate this factor is less of a concern. These data would benefit from a repeat study, which would both highlight whether microplastics are continuing to accumulate and also look at different factors that influence the microplastic accumulation. Whilst initially considered, carrying out similar studies around the UK was found to be too costly to include as part of our study. This should definitely be considered in future studies however, as it would give a better understanding of microplastic distribution when salt marshes have different environmental conditions. Regardless, Blakeney Point is representative of the salt marshes found on the North Norfolk coast and so acts as a good indicator for this region.

Due to time and funding constraints, hydrological data could not be collected as part of this study. This information could be very important and may have provided insight into the microplastic concentrations within channel waters, as well as allowing us to study the effects of inundation time. Despite this, we are still able to measure hydrological effects indirectly through their natural link to other factors, such as inundation time being linked to elevation and vegetation zonation (Bockelmann *et al.,* 2002). The effects of this factor are still therefore represented within the results even if a trend cannot be directly drawn from the data. However, other factors such as the flow velocity cannot be accounted for and therefore may be having an effect on microplastic distribution which we have not observed.

Finally, the methodology used within this study may be under-representing the total amount of microplastics found. The Nile Red staining technique has known limitations, mainly poor staining in fibres, and the complete lack of fluorescence in black particles. However, it remains a widely used method in microplastic identification studies (Shruti *et al.,* 2022). Since within our study we visually checked filters, fibres with poor staining that would have been missed in automated identification were caught. Whilst we did not report

colours nor were able to detect black particles, these represent approximately 7% of the total plastic litter in the ocean (Martí *et al.,* 2020) and so was an acceptable portion of the microplastic population to potentially miss. The possibility of missed microplastic must all be balanced against the possibility of false positives. While the IR studies confirmed a high proportion of stained particles were indeed plastics, there is always greater uncertainty and less checking as the sizes get smaller due to the practical challenge of the measurement itself, so the relative proportion of false positives could be higher in the small particle fraction. This simply has to be accepted within the Nile Red staining approach as an acceptable compromise between accuracy and ability to process and measure large numbers of samples.

3.6 Conclusion

This study highlighted the presence and distribution of microplastics in the Blakeney Point salt marsh. The first hypothesis was proven correct, with the salt marsh found to have contained microplastics in concentrations comparable to or exceeding other coastal environments. Microplastics were found to be present across the entirety of the marsh, being prevalent in every sample studied. Furthermore, variation in distribution was observed, with the abundance of microplastics in different samples ranging from 873 – 98,689 particles/m^2 (164 – 11684 particles/kg d.w.). With microplastics found in every region of the marsh, even the rarely inundated high marsh, we can therefore conclude that the Blakeney salt marsh is effectively trapping microplastics and retaining them in high concentrations. However, when considering physical marsh factors which might influence the distribution of microplastics in the salt marsh, the second hypothesis was found to be incorrect. Despite being factors which influence sediment deposition, elevation and distance from the edge of the salt marsh (Creekside) had no significant correlation with the abundance of microplastics across the salt marsh. Whilst vegetation height was significantly correlated with the number of microplastics, this correlation was negative and like the other factors it showed no discernible trends when plotted against microplastic abundance, having no effect on the microplastic distribution. Therefore, we can state that microplastics are not influenced by the same factors and sediments and thus behave and are distributed differently. Additionally, the distribution of microplastics cannot be linked to a single factor;

rather it may be influenced by other unmeasured factors such as hydrodynamics, or the combination of several factors at once. The final hypothesis supports this, for when considering the impact of vegetation zonation, which represents changes in numerous physical factors, a significant correlation was observed with the distribution of microplastics. A spatial distribution pattern was found, with the Lower/Mid marsh zone showing concentrations of microplastics up to twice as high as other marsh zones. This not only demonstrates the effect of vegetation on microplastic trapping, but also how this distribution is likely the result of the combined influence of multiple factors. Zonation represents changes in vegetation height, density, speciation, and changes in inundation rates, and as such the Lower/Mid marsh represents the optimal overlap between all of these factors.

From the plastics found, both the large presence of fragments and high percentage of paint resin identified suggest that many of the microplastics come from the nearby harbour. However, the presence of macroplastics in the marsh may also indicate that degradation is occurring and potentially the cause of the large amounts of microplastics observed. Regardless, Blakeney salt marsh has a considerable microplastic presence, and with microplastic hotspots in the lower/mid marsh identified, this warrants further studies into the uptake and potential impacts of microplastics upon the salt marsh habitat.

Chapter 4 - Temporal Distribution of Microplastics in a Wadden Sea Salt Marsh

Abstract

The scientific study of microplastics is a relatively recent field, with increasing awareness and understanding being gained over the past two decades. However, the history of microplastics is much older than that, dating back to the production of plastics in the 1950s. Temporal studies have confirmed the presence of microplastics dating back to this time, showing microplastics in deep sediments in a variety of different environments. This study aims to provide temporal information of microplastics in two sediment cores from a Wadden Sea salt marsh, and using sediment properties, vegetation, and land management information, to explain the temporal distribution of microplastics. Microplastics were found at all depths studied, with abundance values ranging from 8,486.56 – 74,257.43 particles/m^2 (472.44 – 9,615.39 particles/kg d.w.), and 1,414.43 – 20,509.19 particles/m^2 (160.64 – 1,836.16 particles/kg d.w.) in the two cores. A general trend of microplastics decreasing with depth was observed, aligning with the globally predicted behaviour of microplastics over time. However, unexpected values were found at certain depths in each core, perhaps relating to a change in anthropogenic management or the surface vegetation over time. When trying to explain the temporal distribution patterns observed, microplastics and sediments shared a significant relationship based on their different size fractions. Despite this, end-member determined sediment depositional processes could not be used to explain the distribution of microplastics.

4.1 Introduction

4.1.1 The Wadden Sea

With an area of over 1 million ha, the Wadden Sea is the largest connected environment of intertidal sand and mudflats on the globe (Figure 4.1). Stretching along the Danish, German, and Dutch coastlines, this UNESCO World Heritage site features a wide variety of different coastal habitats, including seagrass meadows, mudflats, salt marshes, estuaries, and beaches. This results in a rich biodiversity. Its vegetated ecosystems are host to over 6000 different floral and faunal species, with a significant portion of the biomass being fish, shellfish, and birds. For the latter, the Wadden Sea is considered a critical area for migratory birds around the world, with approximately 10-12 million individual birds passing through the Wadden Sea every year (Unesco World Heritage Convention, n.d.).

Figure 4.1 - Map of the Wadden Sea, showing its extent along the western European coast.

Plastic pollution is a global problem, and the Wadden Sea is no exception to that. Whilst most of the area is well managed to minimise certain anthropogenic impacts, plastic debris is still prevalent from riverine input, and from plastics in the adjacent North Sea. Fortunately, plastics have been identified as a potential hazard to the Wadden Sea, and so monitoring studies have been carried out to gauge the levels of plastic litter in this area as summarised in the Wadden Sea Quality Status report (Fleet, 2017). These include data from wider OSPAR programs, as well as individual research projects inspecting the distribution of plastic litter in different organisms. The OSPAR beach litter surveys (2000-2014) show plastic as the most common litter material on Wadden Sea beaches, with a wide range of different items being recorded. This widespread plastic occurrence is mirrored in the studies which looked at different organisms within and around the Wadden Sea. Plastics were found to be present at every stage of the food web, including benthic worms, shellfish, fish, seabirds, seals, and many, other different species (Bravo Rebolledo *et al.,* 2013; Collard *et al.,* 2015; De Witte *et al.,* 2014; Lusher *et al.,* 2013; Rummel *et al.,* 2016; Van Cauwenberghe *et al.,* 2015; Van Franeker *et al.,* 2011; Van Franeker and Law, 2015).

4.1.2 Microplastics in Wadden Sea sediments

Burial of microplastics in sediments is a potentially important sink (Rillig, 2012), yet the rates of microplastic trapping are still largely unknown (Bancone *et al.,* 2020). Studies have found microplastics in all studied types of Wadden Sea sediments, from dunes and beaches (Liebezeit and Dubaish, 2012), to tidal flats (Polt *et al.,* 2023), and the subtidal seafloor (Leslie *et al.,* 2013). A wide range of abundances were reported, from as little as 100 particles/kg (Strand and Bioscience, n.d.), up to as many as 62,100 particles/kg (Liebezeit and Dubaish, 2012). However, the abundance of microplastics within Wadden Sea salt marshes remains unexplored. As a habitat already known to accumulate microplastics (W. Yao *et al.,* 2019), they could hold a significant portion of the plastics within the Wadden Sea. There is, therefore, a need to understand the abundance of microplastics within Wadden Sea salt marshes, and how they have accumulated over depth and time.

4.1.3 Temporal Distribution of Microplastics in Sediments

Deep sediment cores represent an archive of information, telling the history of sediment/soil and can record the accumulation of pollutants such as microplastics over time. Whilst we expect to discover most microplastics nearest the surface, they can be found much deeper. Major plastic production started in the 1950s (Geyer *et al.,* 2017), and so microplastics have been accumulating since then. However, there are several processes by which microplastics can move once deposited in the sediment, including anthropogenic soil management (e.g. digging and dredging) (Matsuguma *et al.,* 2017) and bioturbation (Näkki *et al.,* 2017). Several studies, which will be reviewed here, have carried out microplastic research focusing on depth and temporal variation. To represent microplastic trends across several decades, temporal studies with cores < 20 cm were removed from this review as they on average represent a shorter time scale (Appendix 3).

4.1.3.1 Overview of Core Studies

Sampling techniques were varied in both method and core length. In partially submerged areas, shorter cores (<60 cm) were preferred, using manual corers or augers to collect sediment (Y.-L. Cheng *et al.,* 2021; Liu *et al.,* 2022; Mao *et al.,* 2021; Zhou *et al.,* 2021). In fully submerged sites, gravity corers (Belivermiş *et al.,* 2021; M. Dong *et al.,* 2020; Fan *et al.,* 2019), box corers (Corcoran *et al.,* 2015) and grab samplers (Xue *et al.,* 2020) were used, whereas longer cores (up to 2m) were extracted through either drilling a core or by pile driving (Weber *et al.,* 2022; Weber and Opp, 2020). Lengths of the cores ranged from 30 cm (M. L. H. Cheng *et al.,* 2021; Corcoran *et al.,* 2015a; Lloret *et al.,* 2021; Mao *et al.,* 2021; Paes *et al.,* 2022) up to 200 cm (Weber *et al.,* 2022; Weber and Opp 2020), with 53% of all cores being shorter than 50 cm. In many studies rates of sedimentation were also given, showing the relative time captured within each core length. Cores also varied in width, and in depth of sub-sampled layers. This means the amount of sediment per sample analysed for microplastics was different in each study, ranging from 10 cm^3 - 0.7 m^3 in volume and 5 – 3500 g in weight (Yuan *et al.,* 2023). Finally, in some studies PVC or other plastic piping was used as a core to collect their sample (Martin *et al.,* 2021; Niu *et al.,* 2021; Viet Dung *et al.,* 2021; Zheng *et al.,* 2020). This represents a significant potential for contamination in the sample, however in many cases cores were subsampled, or the outer layers removed to try and alleviate this issue. Despite the considerable variation between habitats studied,

core depths, amounts of sediments and methodologies in reporting microplastics, there was a general trend observed in the data, with 83.33% reporting a decreasing trend of microplastic abundance with depth/time. This trend was reported in numerous ecosystems, including rivers (Mani *et al.,* 2019), lakes (Abbasi and Turner, 2022) and floodplains (Weber and Opp 2020). Within coastal environments such as estuaries (Culligan *et al.,* 2022), tidal flats (J. Li *et al.,* 2020) and mangroves (Martin *et al.,* 2021), microplastics were observed to decrease with depth as well. However, this trend is not reported for all studies, with many reporting varied microplastic distribution throughout the core (Niu *et al.,* 2021; Paes *et al.,* 2022; Weber *et al.,* 2022). The latter studies note disturbances to the sediments in their site, through both natural (bioturbation) and anthropogenic (dredging) means (Matsuguma *et al.,* 2017), which may have affected microplastic distribution. Nonetheless, without disturbance, a decreasing trend of microplastic abundance with depth would generally be the expectation in salt marshes as well, as microplastic concentration should increase in more recent time periods due to the widespread increase in global plastic use.

4.1.4 Temporal Distribution in Salt Marshes

Lately there has been an increased awareness for the ability of salt marshes to trap and accumulate microplastics (Almeida *et al.,* 2023; Lautaro *et al.,* 2023). Yet, there are currently only a few studies which have measured microplastics in deeper salt marsh cores. However, for the studies which have, both (Lloret *et al.,* 2021) and (J. Li *et al.,* 2020) recorded microplastics from as early as 1980s. These studies agreed with the general trend of microplastics decreasing with depth, however both recorded outlier points, and generally had quite low amounts of microplastics present. As salt marshes are dynamic environments, they can change greatly over the space of just a few years. Changes to the sediment with depth in cores can be used to shed light on aspects such as sediment deposition type, vegetative succession and land use changes of the salt marsh. This can be particularly noticeable in managed sites, where anthropogenic activities represent a marked disturbance. Salt marshes naturally develop in low-energy intertidal environments (Hofstede, 1996; Plater, 1994) however they can also be created for land reclamation purposes through various management techniques. Common techniques include drainage, groynes, dams and livestock grazing (Hofstede, 2003). Drainage involves the creation of artificial channels (also known as ditches) to increase outflow in an area and promote the

establishment of pioneer plant species (Houwing *et al.,* 1999), and was a technique used extensively when managing the Wadden Sea marshes (Figure 4.2). Unlike natural channels, these often follow very linear patterns and so alter the structure of the salt marsh. Sediment dug out of these ditches is often moved to other areas of the marsh, artificially changing the topography to enhance salt marsh formation (Vincent *et al.,* 2013). To ensure drainage is sustained, these artificial channels are regularly maintained, using milling and dredging machines (Hofstede, 2003). These processes of ditching and dredging disrupt the natural deposition of sediment and can be identified in the sediment profile when studying sediment cores.

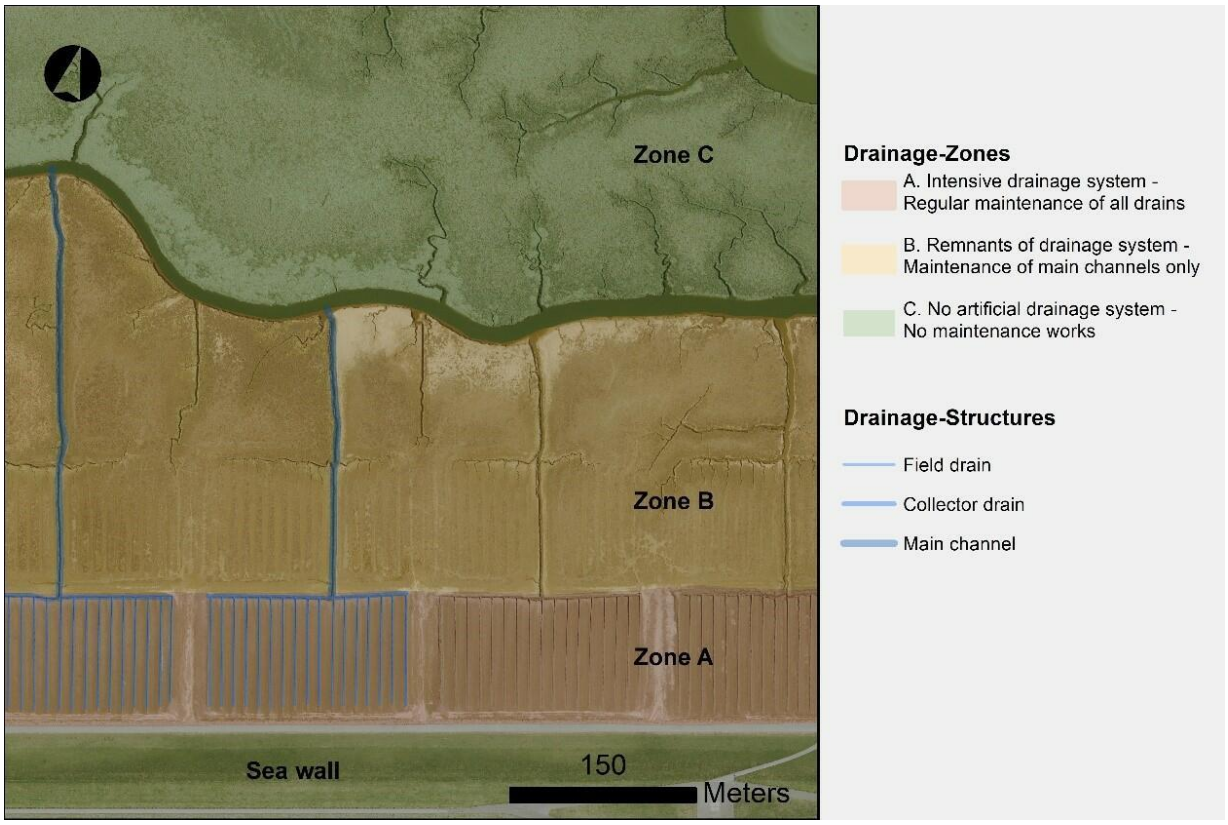

Figure 4.2 - The system of management from the Schleswig-Holstein marshes, showing artificial drainage pattern on the salt marsh (Zones A and B) compared with natural salt marsh channelling (Zone C) (Elschot K., 2024).

Radiometric data for dating, alongside properties such as grain-size and element composition, offers insight into the sedimentary history of a site. This, combined with a knowledge of the management history of a marsh, or monitoring of its vegetation, can lead to an understanding of how natural and anthropogenic processes may have influenced

sediments across a marsh. Such information may also be applied to temporal microplastic studies, to investigate how factors such as vegetation zonation and artificial drainage may affect the deposition of microplastics together with sediments.

4.1.5 Relationship between Sediments and Microplastics

As small particles with overlapping size and density ranges, it is easy to see why microplastics are often assumed to behave like sediment particles (Harris, 2020; Lofty *et al.,* 2023; Malli *et al.,* 2022; Nizzetto *et al.,* 2016). Sediment behaviour in both transport and deposition can be influenced by a variety of different factors, including geometric, chemical and physical properties, as well as external environmental pressures (Howe and Rouse, 1943). Particle size is one such important factor, representing changes in mineralogy and geochemistry, as well as changes in surface area, cation exchange capacity and various other properties which affect how particles interact with each other and the environment (Ersahin *et al.,* 2006; Walling and Moorehead, 1989). Particle density is also crucial when considering particle behaviour, strongly influencing settling velocity and advection (Hostache *et al.,* 2014; Lepesqueur *et al.,* 2019). These two factors are widely recognised as critical physical properties in particle motion, and as such their impacts on particle deposition and transport is described in various studies in different environments (Hostache *et al.,* 2014; Lepesqueur *et al.,* 2019; McCave, 2008; Rubin and Topping, 2001; Shi *et al.,* 2003) .

Recent studies on transport mechanisms show that microplastics and sediment have strong similarities when it comes to size dependent transport modes. Flume experiments carried out by Lofty et al. (2023) tested microplastics and sediments of varying size and density, measuring their effect on the saltation, transport velocity, and collision angles. Their results showed that the saltation trajectories for microplastics were parallel to those of sediments with similar properties, with only 1.4% of cases showing different results. Whilst this showed a strong correlation in microplastic and sediment behaviours, the study was completed under lab conditions and only considered spherical particles, thus does not represent the diversity of microplastic shapes expected in environmental studies. Nonetheless, real world studies have still provided data that continue to support this trend. Vianello et al. (2013) showed that microplastic distribution correlated with the mud fraction (1-63 μm) of sampling sites, finding microplastic concentrations highest in areas of fine

sediment deposition. These areas were characterised by low velocity water currents, suggesting that microplastics were being deposited in the same way as fine sediments. This study focussed on a lagoon habitat, however similar trends have also been seen in rivers (Horton and Dixon, 2018) and estuaries. Here, Lourenço et al. (2017) found microplastic concentration in both sediment and bivalves to be related to the accumulation of fine sediments at those sampling sites. This corresponded to lower velocity environments, suggesting that hydrodynamics can have a comparable effect on similar sized microplastics and sediments.

However, not all research shows a size-based correlation between microplastics and sediments. Mohamed Nor and Obbard (2014) compared the distribution of microplastics of different sizes and morphology to sediment properties in mangroves, none of which showed a clear relationship with the grain-size distribution of the sediments. Mathalon and Hill (2014) compared microplastics to sediment grain-size in three different beach sites, however no correlations between the two parameters were observed in any site. This is further supported by Cluzard et al. (2015), who compared both grain-size and % organic matter with the concentration and spatial distribution of microplastics in intertidal sediments. Microplastic abundance and distribution was found to be independent of these two factors, suggesting that the components which influence sediment distribution do not necessarily govern microplastic fate.

These studies demonstrate both a negative and positive correlation between microplastics and sediment properties such as size and density. However, amongst the various coastal ecosystems which have been studied, salt marshes are markedly absent. Studying salt marshes provides an opportunity to research the effect of sediment properties such as size on microplastic accumulation, whilst also representing environments with more complex vegetation and hydrodynamics. These ecosystems may present new ways to consider how microplastics are being accumulated and stored within the sediment.

4.1.6 Particle Deposition and Transport Processes

Sediment cores can also be an archive to document different types of sedimentation processes over time. Salt marshes are submitted to regular inundation by tidal waters, providing a constant supply of sediment, resulting in the gradual vertical accretion of salt

marshes, with their surface elevation rising to keep pace with sea-level rise (Allen, 1990; French, 1993). Sedimentation within salt marshes is complex and is the result of various interactions between the sediment, vegetation, and hydrodynamics. This can lead to variation in sediment transport and deposition modes, as well as the spatial distribution of the sediment within a marsh site (Temmerman *et al.,* 2005; van Proosdij *et al.,* 2006).

4.1.6.1 Transport

Sediment transport in salt marshes is dominated by two main processes (Rahman and Plater, 2014). Firstly, "traction load" represents the combined effects of particles sliding or rolling along the sea floor, and incorporates particles transported by similar saltation or surface creep processes (Visher, 1969; Yang, 1986). This mechanism tends to result in the deposition of larger, coarser sediments, the size limits of which depend on the flow velocity of the water which transports them (Rahman and Plater, 2014). Traction load in salt marshes is most prevalent during high energy tides, or during storm surge events. Microplastics have also been observed to show this mode of transportation. Polymers such as polyvinylchloride (PVC), polyethylene terephthalate (PET), and nylon, all have densities greater than fresh and saline water, therefore naturally sink down the water column (Horton and Dixon, 2018; Malli *et al.,* 2022). If the velocity of water flow is high enough then microplastics can also be transported through bedload traction as observed by Lofty et al. (2023).

The second mode "suspension load" involves the velocity of the current being sufficient to keep particles floating within the water column, most commonly small fine sediments. When the flow velocity decreases below the threshold for transport, these particles start to sink to the bottom of the water column and can be deposited (Rahman and Plater, 2014). This mechanism can also include flocculation, in which particles aggregate together resulting in more rapid settling and deposition (Pejrup, 1988). Suspension load is widely observed in different marshes due to the reduction of flow velocity of tidal water through vegetation. For buoyant microplastics, this suspension within the water column is observed (Nizzetto *et al.,* 2016; Zhang, 2017), and through this process they can be carried great distances, circulating from river systems into the open ocean and being transported with the currents to environments around the world (Horton and Dixon, 2018). However, this process is similarly dependent on the energy within the system, and thus in areas of low

water flow suspended microplastics can be deposited, something that is particularly prevalent in estuarine environments (Malli *et al.,* 2022).

4.1.6.2 Microplastic Modes

Since microplastics have various sizes, shapes and other physical characteristics, the modes of transport and deposition are often dictated by their physical properties. While studies carried out on both riverine (Nizzetto *et al.,* 2016) and estuarine (Malli *et al.,* 2022) habitats, as well as in flume settings (Lofty *et al.,* 2023) demonstrate the processes of microplastic transport through suspension and traction, microplastics also exhibit another unique mode of transport.

For the lightest and most buoyant microplastics, flotation at the water's surface leads to a different transport mode than that in regular, suspended particles. These floating particles are directly influenced by the wind which plays a crucial role in the movement of the surface layer of waters, and thus have unique transport patterns when compared to microplastics in suspension (Zhang *et al.,* 2020). This has been observed in estuaries, where a study by (Browne, 2015) found that the deposition of macroplastic and large PVC microplastics were strongly correlated with wind-blown distribution.

4.1.6.3 Sedimentation

In general, sediment deposition in salt marshes occurs during inundation, whereby the vegetation canopy acts as a barrier for the incoming tide, slowing the waves and currents to create a low velocity environment which encourages particles to settle (Bouma *et al.,* 2005; Leonard and Luther, 1995; Möller *et al.,* 1999; Neumeier and Amos, 2006; Stumpf, 1983). This process also allows for the settling of microplastics, which share many of the same settling behaviours as sediments (Khatmullina and Isachenko, 2017). In addition to indirectly influencing particle deposition through drag force, salt marsh vegetation has also been found to directly trap sediments (Chen *et al.,* 2016; S. Temmerman *et al.,* 2005), the extent of which is determined by the density, height and stiffness of the vegetation present (Temmerman *et al.,* 2005). Vegetation has also been shown to have a trapping effect on microplastics, with studies demonstrating this effect in seagrass meadows (Huang *et al.,* 2020) and flume studies (de los Santos *et al.,* 2021).

Sediment particles may also be deposited through anthropogenic means. During the process of artificial drainage, sediments that are dug out to create ditches or dredged to clear channels, are redistributed to other areas within the marsh (Hofstede, 2003). Whilst only present in artificially created or heavily managed salt marshes, nonetheless anthropogenic effects can also be detected from sediment properties and therefore should be considered a deposition mode for sediment within salt marshes. Whilst no studies have directly studied this, (Matsuguma *et al.,* 2017) found unexpected microplastic temporal distribution in heavily dredged canal samples, compared to the expected linear trend observed in less managed sites. Therefore, it is possible that microplastics already present in the sediment will be redistributed through these processes, potentially disrupting the microplastic depth profile within the sediment.

4.1.6.4 End-Member Modelling

Sediment depositional processes can be determined using End-member modelling (Lenz *et al.,* 2023). This analysis works to breakdown various grain-size distributions into separated sub-populations, each representing sediment carried by a different depositional process. End-member models can be used to determine the importance and relative contribution of different members throughout a sample, allowing for a historical profile of sedimentary deposition processes. Therefore, there is the opportunity to compare microplastic distribution with sediment depositional types to determine whether microplastics are deposited in the same way as salt marsh sediments, or in fact behave as unique particles which deposit in different ways.

4.2 Aims

This study aims to address some of the many knowledge gaps of microplastics within salt marshes on a temporal scale of several decades. The main objectives are: i) to investigate the abundance of microplastics over depth (and so time) in a Wadden Sea salt marsh and suggest whether environmental factors influence this abundance, ii) to explore the relationship between sediment and microplastics based on their size, and iii) to study whether sediment deposition processes influence microplastic distribution and can suggest whether microplastics behave like sediments.

We hypothesise that i) microplastic distribution on a temporal scale should be negatively correlated, and hence microplastic abundance decreases with depth. Regarding sediment properties, microplastics and sediments are expected to behave similarly, and so it is hypothesized that ii) microplastic abundance in different size fractions will correlate with the sediment composition (and by extension grain size). Finally, we hypothesise iii) that the abundance of microplastics is affected by the sediment deposition process as indicated by end-member modelling.

4.3 Methodology

4.3.1 Study Site

The study was conducted in a salt marsh in the Wadden Sea National Park on the German North Sea coast. Located in the Bay of Tümlau, the site is a formerly managed salt marsh within a tidal basin on the Eiderstedt peninsula (Figure 4.3)

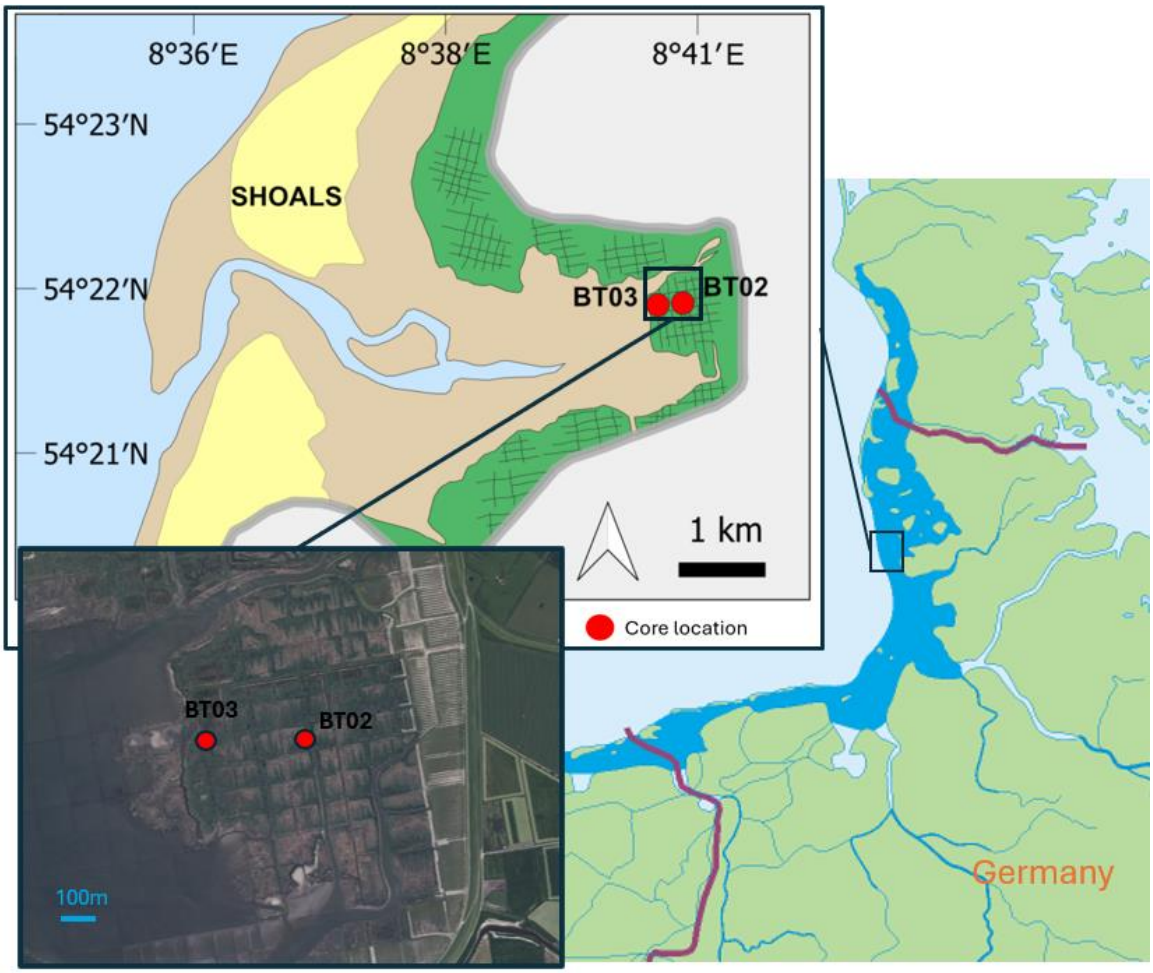

Figure 4.3 - Map of the Bay of Tümlau, showing location within the Wadden Sea and core sampling sites.

The Bay of Tümlau features both suspension and traction load as depositional processes due to its exposed nature and high-energy setting (Lenz *et al.,* 2023). Sediment is sourced from the adjacent tidal flat, in addition to particles eroded from the marsh cliff (Schuerch *et al.,* 2019). However, the site also has a history of anthropogenic management. Artificial land reclamation of the area began as early as the 12th century (Meier, 2004), followed by the construction of artificial drainage systems which fostered marsh formation (Stock, 2011). The mainland was then diked in 1933, with continuous ditching and dredging of the marsh occurring every three to seven years (Müller-Navarra *et al.,* 2016). Dredging was halted with the foundation of the Wadden Sea National Park in 1985, and with the designation to nature reserve in 1998, ditching ended as well (Stock, 2005). Since then,

natural tidal channels have developed, even though the pattern of former drainage channels is still visible throughout the marsh.

Elevation across the marsh is relatively flat, ranging from 1.3 – 1.9 m in relation to the German Ordnance datum (NHN), with the highest point being at the cliff on the seaward edge (Figure 4.4). Vegetation in the salt marsh of the Bay of Tümlau is predominantly low marsh plants, comprising of *Triglochin maritimum*, *Atriplex portulacoides* and *Agrostis stolonifera*. Other vegetation zones are present, with scattered patches of high marsh with *Artemisa maritima* and *Halimione portulacoides*. On the seaward edge pioneer marsh zones are indicated by the presence of *Spartina anglica.*

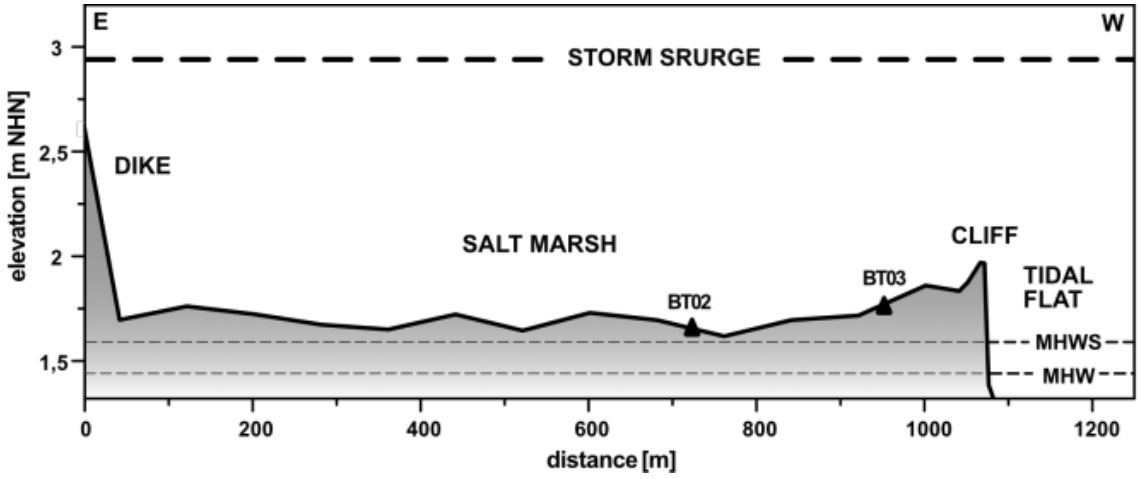

Figure 4.4 - Topographic profile over the salt marsh study site showing locations of core BT02 and BT03. Dashed lines represent levels of mean high water (MHW), mean high water springs (MHWS) and storm surges (Lenz *et al.,* 2023).

Past vegetation cover in the Bay of Tümlau was determined from digital vegetation maps provided by the administration of the Wadden Sea National Park Schleswig-Holstein created in the framework of the Trilateral Monit(*TMAP Typology of Coastal Vegetation in the Wadden Sea Area* n.d.) (Table 4.1).

Table 4.1 Vegetation zonation determined from TMAP vegetation maps (LM: Low Marsh zone, PIO: Pioneer zone)

Core	Year	1988	1996	2001	2006	2011
BT02	Vegetation	LM	LM	PIO	LM	LM
BT03	Vegetation	LM	LM	PIO	LM	LM

4.3.2 Core Collection and Sampling

The cores were collected within the framework of the SEASTORM project, for a full description see (Lenz *et al.,* 2023). In short, the cores BT02 and BT03 were taken 400 m and 120 m from the seaward edge, in the low marsh vegetation zone. Each core recovered approximately 0.92m of sediment. Cores were collected in PVC tubes (diameter 125 mm and length 1.5 m) with a steel core catcher on the bottom end. The tubes were manually hammered into the marsh and then recovered using a piston and car lift.

To access the sediment, each core was cut in half down the length. One half of both cores was analysed for sediment properties, while the other half was wrapped and sealed, and transported to the University of East Anglia, where the microplastic extraction and analysis was carried out. At this point the cores were renamed Landward Side Core (LWC – previously BT02) and Seaward Side Core (SWC – previously BT03) to better represent their location and make the results clearer and thus are named as such in this work.

4.3.3 Sediment Analysis

Sediment analysis was performed by Nina Lenz and Sebastian Lindhorst at the University of Hamburg and detailed information can be found in (Lenz *et al.,* 2023).

For grain-size analysis, both cores were sub-sampled for the entire length of the core (1 cm^3 every 1 cm depth) and analysis was carried out using a laser-diffraction particle-sizer (Sympatec HELOS/KF Magic, measuring range 0.5/18 – 3200 μm). Composition of the sediment fractions was also recorded, showing the % representation of the different sediment fractions, mud (1 – 63 μm), sand (64-1000 μm) and gravel (1001 – 16000 μm). In every sample tested, gravel was found to contribute 0% of the make-up, and thus for analysis % Mud is used to represent sediment composition.

To provide highly detailed chemical make-up of the sediment, X-Ray Fluorescence Scanning (XRF) was carried out. The cores were scanned at the Leibniz Institute for Baltic Sea Research Warnemünde (IOW) using an ITRAX XRF core scanner with a high-power Chromium XRF source.

An age model for both cores was determined using ^{210}Pb and ^{137}Cs radionuclide distributions. ^{210}Pb analysis was carried out at Flett Research Ltd (Canada) using alpha spectrometry of the isotope ^{209}Po. ^{209}Po and ^{210}Po were measured at 15 intervals in core LWC (0-90 cm) and 14 intervals in SWC (0-92 cm), with sampling resolution decreasing with increased depth. ^{137}Cs activity measurements were carried out across 17 intervals in LWC (6-62 cm) and 14 intervals in SWC (18-60 cm). Sedimentation rates were calculated using the constant rate of supply model (CRS). The CRS model calculates sediment age from ^{210}Pb profiles, where assuming constant atmospheric ^{210}Pb input, changes in accumulation rates will affect ^{210}Pb concentration. A linear regression model was used to estimate the average sediment accumulation rate using the ^{137}Cs measurements (high values are assigned dates of known historical nuclear tests), which was then used to calibrate the CRS model.

4.3.3.1 End-member Modelling

End-member modelling of each core was carried out at the University of Hamburg by Nina Lenz and Sebastian Lindhorst (Lenz *et al.,* 2023), using the grain-size data and the R package EMMAgeo. The three end-members determined were: suspension load, flood deposition, and dredging/ditching. Scores were obtained which represented the contribution of each end-member to the sample (average per 10 mm layers), with then the dominant end-member for each layer reported (Appendix 4.2).

4.3.4 Microplastic Analysis

For microplastic sampling, extraction and analysis, the method established in Chapter 2 was used (SOP in Appendix 5). However, as the sediment cores were not taken with microplastics in mind, some adjustments were made to the protocol, listed here.

4.3.4.1 Microplastic Sampling

A sample consisted of a 1 cm sediment slice, taken every other centimetre depth down the core. This was repeated down to a depth of 40 cm, where further samples were then taken in 5 cm intervals. In core LWC 20 samples were taken, with the lowest depth being 40 cm. In core SWC 25 samples were taken, with the lowest depth being 60 cm. The lowest depths in each core were both found to be deposited in 1960 according to the age model, representing the industrial production of plastic thus the earliest time that microplastics

could be deposited. This was therefore used as the cut-off date for sampling within each core. Additional slices were taken from the 80-90 cm depth to be used as control samples.

4.3.4.2 Digestion of Organic Matter

The digestion step of the protocol used the same volumes and solutions as given in Chapter 3; however, heating and agitation was provided using a mini orbital shaker incubator (VWR). For runs with more than 6 samples, 2 such instruments were used in conjunction.

4.3.4.3 Minimising Contamination

In addition to the protocols in the SOP, further control measures were carried out given the high likelihood of contamination within these samples. For each slice, 1 cm from the edge was cut off to remove any sediment which had been in direct contact with the PVC pipe. From the centre of each slice a circular sub sample was cut (diameter 4.5 cm) and transferred to a clean petri dish. The samples were then weighed, dried, and extracted using following the steps in the SOP (Appendix 5).

4.3.4.5 Microplastic Identification

Extracted microplastics were analysed at the Centre of Environment Fisheries and Aquaculture (CEFAS), Lowestoft. Filters were stored in clean petri dishes in a freezer at -20 ^{o}C until taken out for analysis. Filters were stained with Nile Red solution (in 100% ethanol) and then imaged using the same imaging rig set-up as in the SOP. Images were stitched and particles counted using ImageJ as per the SOP.

Microplastics sizes were determined, and split into 2 size fractions: 10-100 μm and 100 – 5000 μm.

Particles were identified under a stereomicroscope whilst exposed to fluorescent light. Particles were visually studied under white light, details recorded, and then transferred to a 25 mm anodisc filter. A random selection of particles (<10%) were then analysed using micro-ATR FTIR using a Bruker Lumos II spectrometer to confirm they were plastics.

4.3.5 Statistical Analysis

Microplastic abundance in relation to the factors; depth and % Mud, was analysed using both linear and non-linear regression models. Regression was calculated and used to determine any significance between the datasets of microplastic abundance and each factor. Finally, comparing microplastic abundance between different sediment depositional processes derived from end-member modelling was achieved with an ANOVA. All statistical tests were carried out using Microsoft Excel's Data Analysis plug-in.

4.4 Results

4.4.1 Reporting Microplastic Numbers

For all data analysis and comparisons, the number of microplastics was calculated per area (m^2) of sediment and per dry weight (kg) of sediment. Values are reported for both units when discussing results, however for analysis and graphical representations of the data, only per area (m^2) is reported. This is due to sediment weight varying based on composition and therefore is not constant throughout the entire core. Given the samples consist of only 1 cm depth, area-based measurements give the most evenly distributed microplastic data for when comparing the impacts of different environmental and physical factors. Furthermore, this study focusses on temporal differences, and thus reporting microplastics per volume (m^3) for each sample may misrepresent the total amount of microplastics actually found in a m^3 worth of sediment. If trends observed differ between the area-based and weight-based microplastic abundance, this is highlighted in the discussion of those results.

4.4.2 Evaluation of Procedural Blanks

Alongside each set of five sediment samples, a procedural blank was also run to assess the contamination from the laboratory sources. An average of 9 ± 1.7 microplastics were found per control (see Appendix 4.3 for full control data). This contamination is to be expected, due to the use of plastic wash bottles and SMI units within the procedure, as well as potential air contamination. This number of particles was subtracted from each sediment sample when calculating the final number of microplastics.

4.4.3 Microplastic Distribution

Microplastics were found to be present in every layer of sediment, in both cores (Figure 4.5). The average number of microplastics in each core was, for LWC; 30,100 ± 980 particles/m^2 (3,300.16 ± 110 particles/kg d.w.), for SWC; 8,400 ± 200 particles/m^2 (720 ± 23 particles/kg d.w.). In LWC microplastic abundance ranges in values from 8,486.56 – 74,257.43 particles/m^2 (472.44 – 9615.39 particles/kg d.w.), whilst in core SWC they varied between 1,414.43 – 20,509.19 particles/m^2 (160.64 – 1,836.16 particles/kg d.w.).

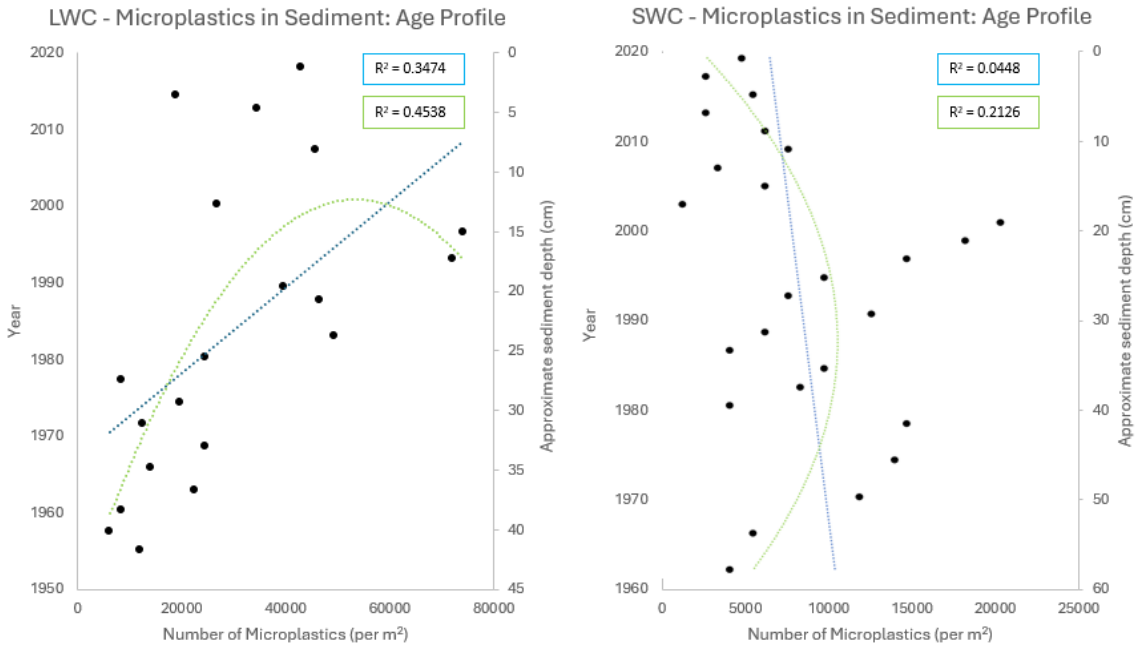

Figure 4.5 - Microplastic abundance plotted against depth of sediment (y-axis showing year in which the respective layer of sediment was deposited, year calculated using CRS and linear regression models) for cores LWC (Landward Core) and SWC (Seaward Core), showing linear (blue) and non-linear polynomial (green) regression models.

Simple linear and non-linear regression was used to investigate whether the depth of deposition significantly affected the abundance of microplastics in core LWC and SWC (Appendix 4.4.1). In LWC, we observe the significant decline of microplastic abundance with depth, while in SWC this pattern is not observed (Figure 4.5).

Whilst LWC showed the decreasing trend of microplastics with depth, the highest microplastic concentration was not observed in the most recent sediments, but in 1996 and 2002 for LWC and SWC respectively. When considering anthropogenic management, we can observe that the peak microplastic counts, then subsequent drop off in microplastic abundance in the following years, occurs within a couple of years of the ditching ending (1998) (Figure 4.6a and 4.7a). Both cores also show a substantial increase in microplastics between the end of dredging (1985) and end of ditching.

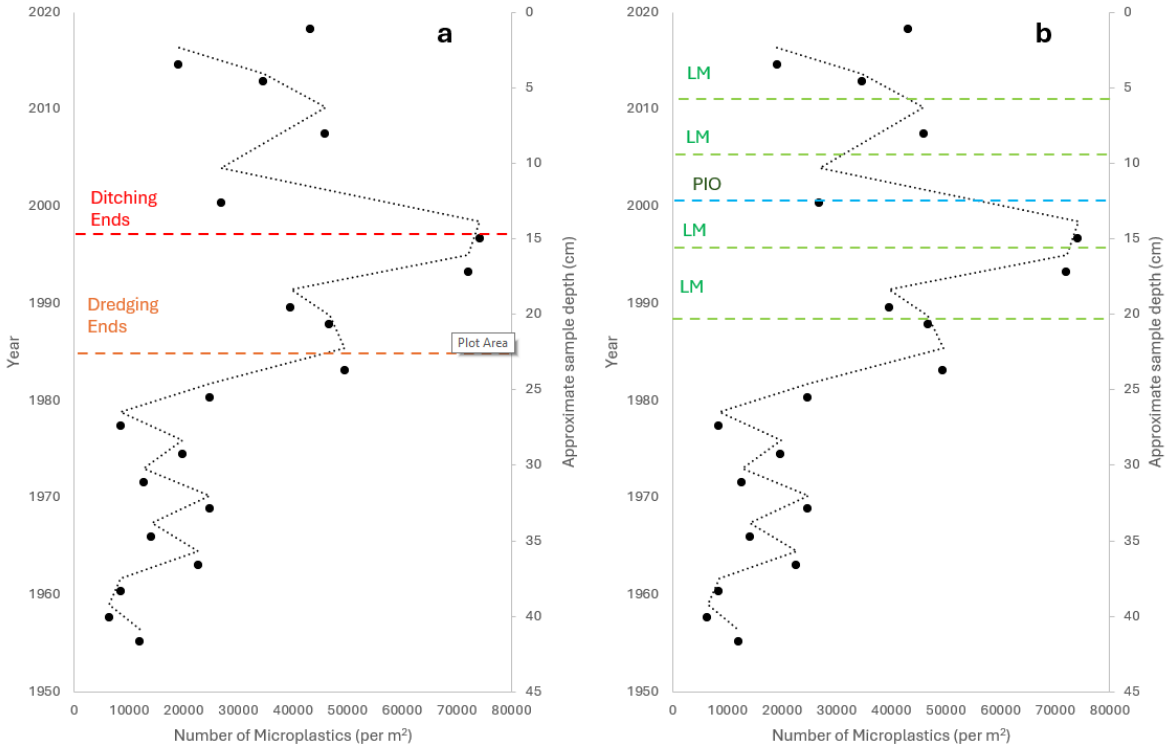

Figure 4.6 - LWC Microplastic abundance with depth, showing; (**a**) anthropogenic management, and (**b**) vegetation zonation (LM – Low Marsh, PIO – Pioneer).

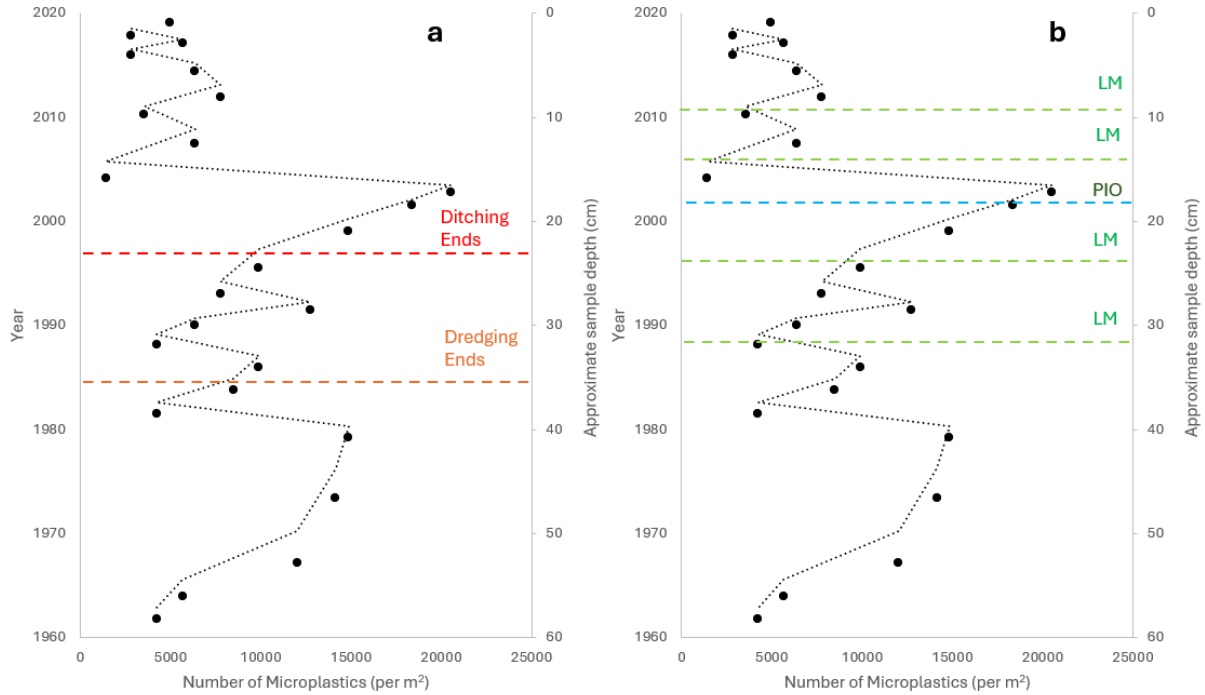

Figure 4.7 - SWC Microplastic abundance with depth, showing; (**a**) anthropogenic management, and (**b**) vegetation zonation (LM – Low Marsh, PIO – Pioneer).

Examining the vegetation zonation over time (Figure 4.6b and 4.7b), evidence for this was limited and so only 5 years with known zonation are shown. For the time period known (present day – 1988), both of the cores are predominantly in the low marsh zone. However, both cores also show a period in which the marsh enters a pioneer stage in 2001. Following this pioneer zone though, there is no significant trend in the abundance of microplastics as the low marsh re-establishes.

4.4.4 Sediment composition

Sediment composition (% Mud) was compared against different size fractions of microplastics to determine if microplastic and sediment particles of a similar size were being deposited alongside one another. Linear and non-linear regression was performed to determine significance (Appendix 4.4.2).

Within LWC it was found that the abundance of microplastics in size fraction (10 – 100 μm) shared a polynomial relationship with % Mud, with more microplastics being deposited in

samples with higher mud content. In SWC the opposite is found with the small microplastic size fraction decreasing as % of mud increases (Figure 4.8).

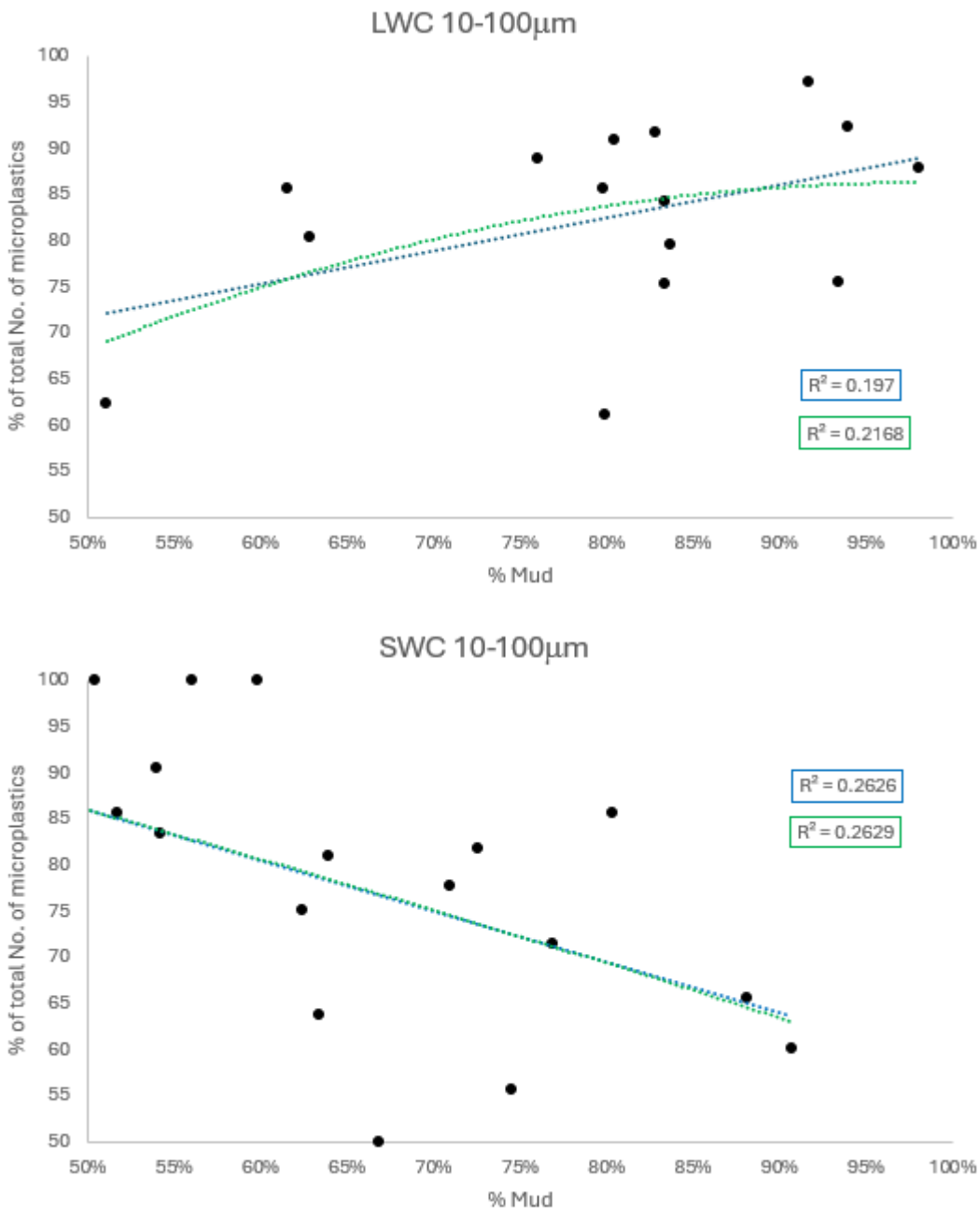

Figure 4.8 - % of total microplastics (10-100 µm size fraction) vs % Mud in sediment, showing linear (blue) and polynomial (green) models.

For the larger microplastic size fraction (101 – 5000 μm) both cores were again found to have a significant relationship with % Mud. In LWC the percentage of microplastics in this size fraction decreased with increasing mud. SWC shows the opposite, with more of the larger microplastics being found in samples with higher mud content (Figure 4.9).

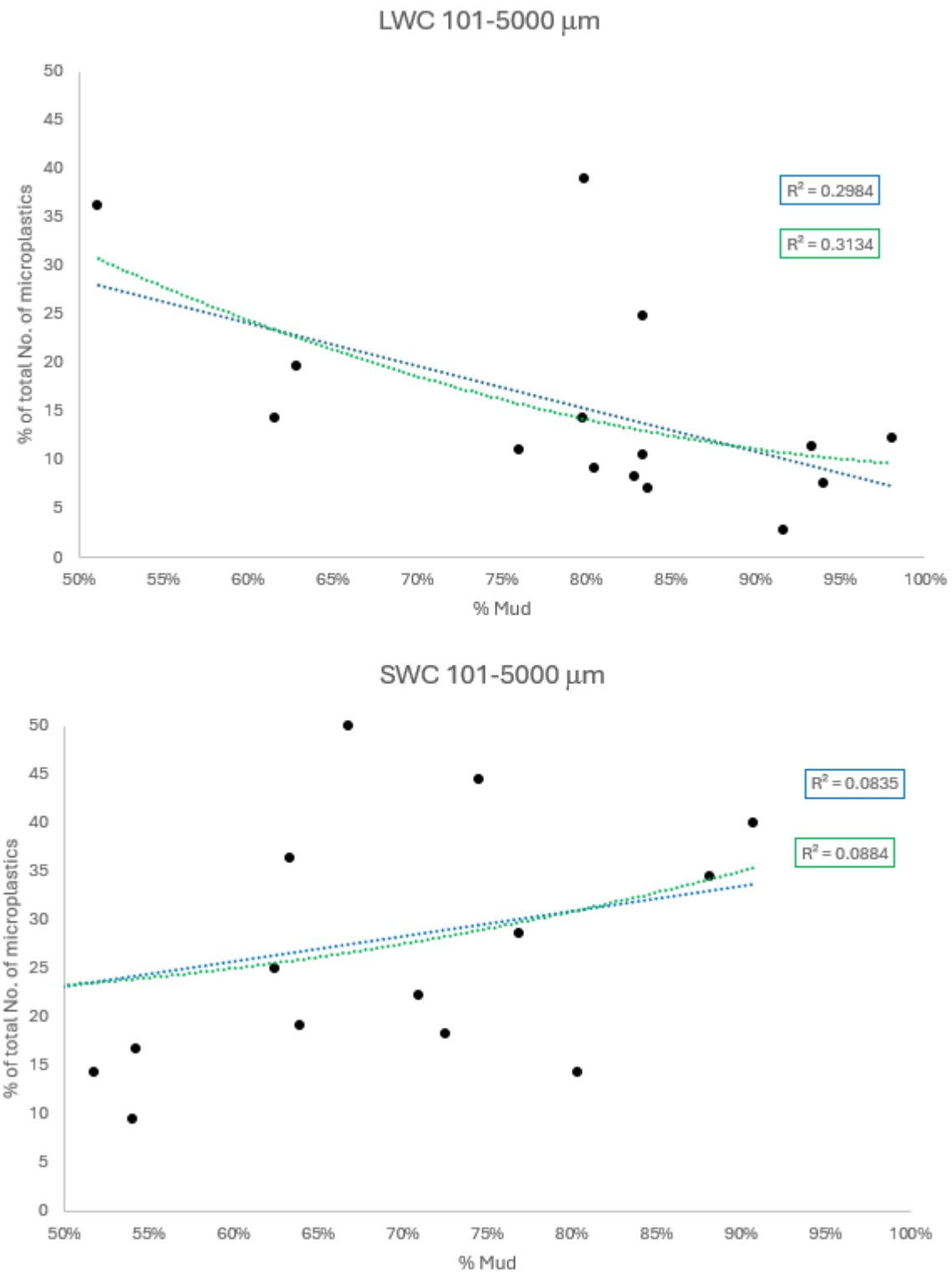

Figure 4.9 - % of total microplastics (101-5000 µm size fraction) vs % Mud in sediment, showing linear (blue) and polynomial (green) models.

4.4.5 Depositional Processes

To compare the effect of different depositional processes on microplastic abundance, the previously collected end-member modelling data (Appendix 4.2) was used to determine the dominant depositional process for each sample using their respective sediment depths. The samples were then grouped using the dominant depositional process, and the impact on microplastic numbers was analysed using a single factor ANOVA (Figure 4.10). For LWC, the ANOVA revealed that there was no statistically significant effect of depositional process on number of microplastics ($F(2, 17) = 0.154$, $p = 0.858$). In SWC the result of the ANOVA also did not show a significant effect of the depositional process on number of microplastics ($F(2, 22) = 0.317$, $p = 0.731$).

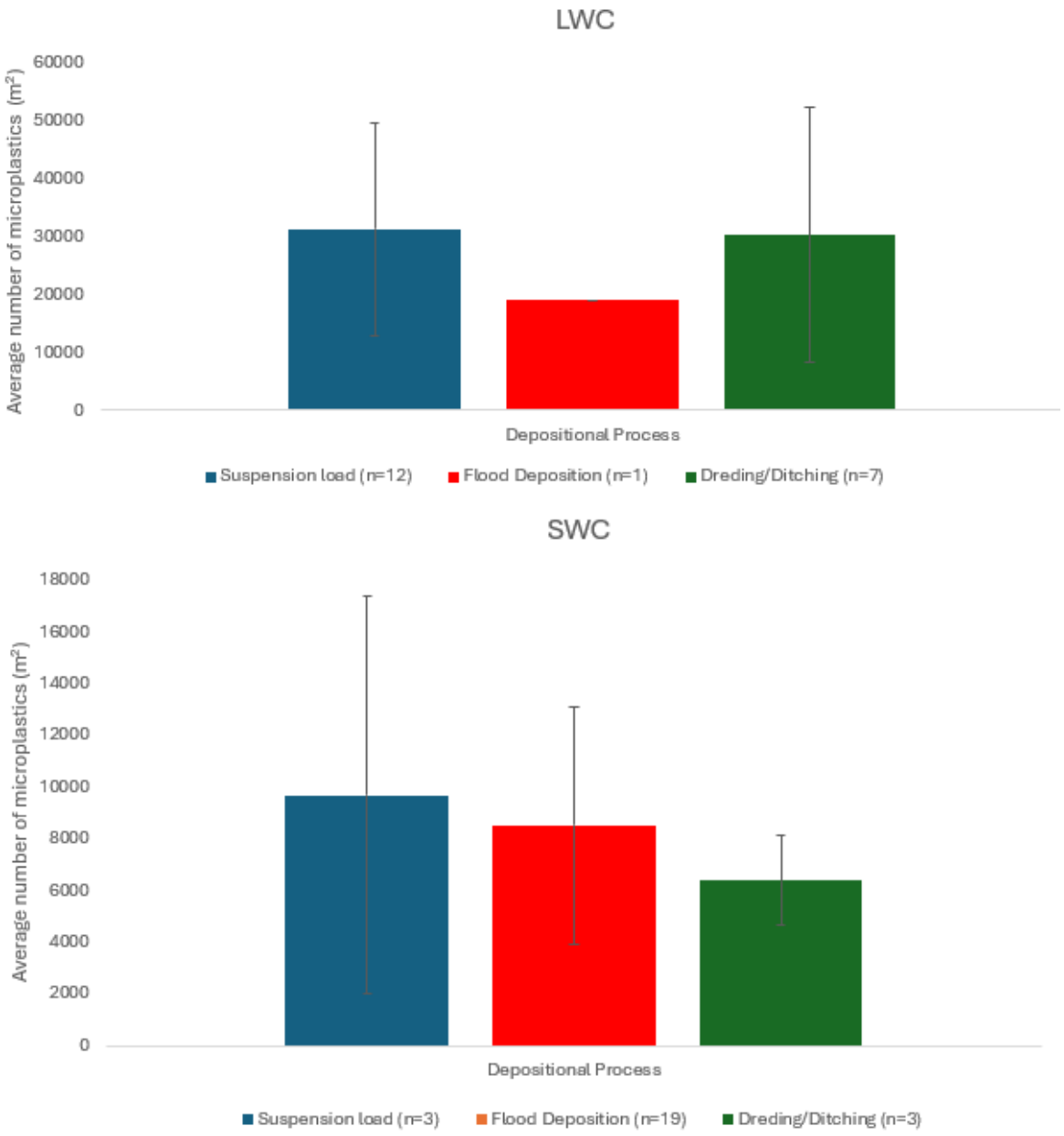

Figure 4.10 - Abundance of microplastics with different sediment deposition processes, samples grouped by the dominant depositional process (suspension load, flood deposition or degrading/ditching), error bars represent standard deviation.

4.5 Discussion

4.5.1 Microplastics

4.5.1.1 Abundance

Between the two cores, LWC had a substantially higher abundance than SWC with the average microplastic values being over three times larger. The cores vary slightly in location, with LWC being more landward (400 m from the seaward marsh edge) and SWC being more seaward (120 m from the marsh edge). However, both cores still lie within the lower marsh vegetation zone (*Triglochin maritima*, *Atriplex portulacoides* and *Agrostis stolonifera).* Despite being more landward, LWC is at a lower elevation than SWC and probably spends more time submerged. This means there is greater opportunities for microplastics to be trapped at LWC, since it is exposed to microplastics in the tidal waters for longer. Examining the sediment deposition, the data support this with the main modes found being suspension load and flood deposition in LWC and SWC respectively. This suggests that particles are only deposited at SWC during high energy events such as high spring tides and storm surge events, and thus much less frequently than compared to LWC. However, there are other factors that could be influencing the microplastic accumulation, such as differences in the local hydrodynamics caused by the previous established ditching channels, or the relative microplastic concentration during submerged periods.

Comparing the values of LWC and SWC to other Wadden Sea studies, we can discern that the samples have relatively average microplastic numbers. Bäuerlein et al. (2023), Leslie et al. (2013) and Strand (2013) report microplastic concentrations in the range of 100 – 785 particles/kg d.w. of sediment, values that are similar to those found in core SWC. Examining intertidal studies, Liebezeit and Dubaish (2012) reported an average microplastic count of 3,800 particles/kg d.w. for dunes, and 8,600 particle/kg d.w. for tidal flats. Polt et al. (2023) reports similar values, with a range of 0 – 8128 particles/kg d.w. within a tidal flat. These values are very similar to what we report in LWC (472.44 – 9615.39 particles/kg d.w.), suggesting similar levels of microplastic concentration in tidal habitats within the Wadden Sea.

Collating these values with other salt marsh studies, we find that the Wadden Sea is proportional to marshes around the world. The lowest values (160 and 472 particles/kg d.w.

SWC and LWC respectively), are still equal to values found by Pinheiro et al. (2022), Wu et al. (2020) and Gray et al. (2018), all of which report average microplastic concentrations of less than 300 particles/kg d.w. Meanwhile, the highest concentrations in LWC (74,257 particles/m^2) are closely related to those found by Lautaro et al. (2023) and P. Yao et al. (2019), with averages of 10,388 and 70,162 particles/m^2 respectively. Whilst this suggests that the Wadden Sea salt marshes are trapping microplastics similarly to other sites, this data covers a large range of values. Furthermore, differences in the site's inputs and study methods means no two salt marsh studies are alike.

4.5.1.2 Temporal Distribution

Core LWC shows a significant trend of decreasing microplastics with depth, in line with hypothesis one. Similar patterns were observed in most temporal microplastic studies, in which 72% of the reviewed papers reported a decreasing microplastic concentration with increasing depth. However, LWC still showed several outlier points which had higher concentrations than we would have expected for that period.

In contrast, core SWC did not show this trend of decreasing microplastic abundance with depth. Yet not all the literature supports this hypothesis, with several studies finding no trend (Paes *et al.,* 2022) or heterogeneous distribution (Weber *et al.,* 2022). Matsuguma et al. (2017) noted in their study that the samples in which this trend was not observed come from sediments which have heavy amounts of disturbance, either anthropogenic or biological. Anthropogenic disturbance in the Bay of Tümlau could similarly have altered the levels of microplastics in the sediment. With this source of microplastics potentially disrupting the microplastic abundance, the lack of any trends in SWC is not unexpected.

4.5.1.3 Factors Affecting Microplastic Temporal Variation

Whilst the initial hypothesis of decreasing microplastic abundance with depth and time is supported by LWC, there is still some unexplained variability, a pattern which is shared in SWC. Unexpectedly, we observe the peak microplastic abundance for each core being at 13 cm (~1996) and 19 cm (~2002) for LWC and SWC respectively. In the years following this peak, microplastic abundance suddenly drops off for both cores, before starting to rise again. One potential explanation for this is the impact of anthropogenic management. Whilst dredging halted in 1985, ditching continued till 1998. Following the end of this

management, microplastics concentrations suddenly decreased by over 60% in the early 2000s for both cores. Ditching involves taking sediment from the marsh to create channels and then redistributing that sediment elsewhere on the marsh. As such, these other areas of the marsh receive not only microplastics from regular tidal trapping, but also any microplastics already trapped in the ditch sediment. Combining these effects could result in the unexpected increase in microplastic abundance. Then when ditching stops microplastic input returns to predominantly tidal trapping, thus causing the sudden decrease observed in the profile.

Another potential explanation for this disturbance is the change in vegetation zone observed in the marsh. The earliest recorded plant communities (1998) for the marsh show it to be dominated by lower marsh vegetation. This remains consistent for many years, till in 2001 the vegetation is now predominantly that of a pioneer marsh. This change in zonation is probably also due to the halting of ditching, which caused reduced drainage and therefore conditions which are favourable for pioneer vegetation. This secondary pioneer zone eventually becomes low marsh once more as natural succession occurs. Based on previous studies (Cozzolino *et al.,* 2020; de los Santos *et al.,* 2021; Li and Yang, 2009), we know that vegetation plays an important role in the trapping of microplastics. We demonstrated the difference of microplastics trapped between various vegetation zones, with the low marsh showing significantly more microplastics than the pioneer in Chapter 3. The sudden decrease in microplastics in both cores could be due to the establishment of the pioneer zone, which would not trap microplastics as effectively as the prior lower marsh zone. As such, the eventual natural re-establishment of the low marsh would lead to gradual increase in microplastic trapping and abundance once again.

4.5.2 Sediment and Microplastic Shared Properties

In this study particle size was used to measure variability of microplastic abundance within the two cores. Since microplastics are assumed to behave as sediments, we hypothesize that microplastics will accumulate alongside sediments of similar size fractions (Lofty *et al.,* 2023). With microplastics being measured and counted individually, for each sample they can be split into different size fractions (10 – 100 μm and 100 – 5000 μm), and the representation of that fraction within the total population of microplastics calculated. As gravel was absent from all samples, % Mud (1-63 μm) can be used to represent % Sand (64-

1000 μm) with an inverse relationship. We study this size-based relationship between microplastics and sediment by comparing the % Mud in the sediment with the % fraction of microplastics for each size categories. For large microplastics (100 – 5000 μm) we expect to find them in samples with highest sand content, thus have a negative relationship with % Mud. This relationship was observed in core LWC and found to be significant. However, the inverse was found in SWC with large microplastics increasing with % Mud, suggesting that this relationship is not always present. When we consider small microplastics (10 - 100 μm), based on the hypothesis we expect to find them in samples with similar size particles, so high mud content. Whilst LWC supports this with a positive trend between microplastics and increasing % Mud, SWC once again shows a significant negative relationship. Therefore, data from core LWC supports the hypothesis in both size fractions, whereas SWC contradicts this.

There are several potential reasons as to why these various relationships are being observed. First is the disparity between microplastics and sediment measurements. Sediment composition was determined from grain size, taken from sub samples in 1 cm layers from one half of a core. Microplastics measurements were taken every other centimetre and analysed independently using separate sub-samples to the grain size. This means there is variation in both the vertical and lateral displacements of samples for the two measurements, and despite minimising the effects of this where possible, the data may not be fully homogenised. Furthermore, the methods used for both sets of particles create disparity as well. Sediment particle size is measured by laser diffraction and therefore represents an average of millions of particles. Microplastics however are measured individually and so constitute a much more precise set of measurements. Secondly, the conflicting patterns may be influenced by the difference in microplastic populations between the two cores. SWC features a lower number of total microplastics than LWC, thus is more susceptible to variation from different size fractions. This is particularly noticeable in large microplastics, in which despite LWC having four times the number of microplastics sized 1000 – 5000 μm, this size fraction only represents 9% of the total microplastics within the core. In SWC this same size fraction however makes up almost twice the amount of the total microplastic population. A final explanation is that microplastics do not have linear relationships with sediment composition, and the hypothesis was incorrect. Whilst studies

have observed correlations between particle size, sediment type and microplastic distribution (Horton and Dixon, 2018; Vianello *et al.,* 2013), they are not conclusive. Furthermore, studies by Mohamed Nor and Obbard (2014) and Cluzard et al. (2015) compared microplastics against grain-size, % organic matter and sediment morphology, finding no pattern with any of the factors. The contradictory relationships between sediment composition and microplastic distribution could therefore be due to microplastics not always behaving like sediment particles, and so should not be expected to follow the same size distribution patterns.

4.5.3 Depositional Processes

Sediment deposition is a complex process, however one of the dominant factors that influence the depositional process is particle size. As particles with similar size ranges, we hypothesized that microplastics can be deposited via the same processes. These sediment deposition processes were determined using end-member modelling, a process which deconstructs sediment data using grain-size characteristics into different end-members, each representing a different mode of deposition.

Variation between the different processes was tested using an ANOVA, testing microplastic abundance with the dominant end-member (the most common depositional process in that sample) per 1 cm slice. Contrary to the hypothesis, the results showed no patterns between microplastics and the different processes in either core. Furthermore, no differences between the individual end-members and their effect on microplastic abundance was observed. Despite microplastics and sediments sharing a relationship based on different size fractions, microplastics are not evenly deposited by these modes observed for sediment. Thus, this data does not support the assumption that they share the same behaviour as sediments.

There are several potential explanations for this observed deviation from the depositional processes. Despite being on a similar scale, microplastics have many different properties compared to sediment particles, including a larger spread of densities and entirely different morphology types such as fibres and films. Alongside size, shape is also an important factor in determining sediment behaviour, with differences between smooth spherical grains and coarse aspherical grains shown to impact sediment transport (Deal *et al.,* 2023). Whilst

some evidence shows spherical microplastics behaving similarly to sediments (Lofty *et al.,* 2023), there are several studies which report different effects on sinking and settling velocity caused by irregular morphologies (Kaiser *et al.,* 2019; Wang *et al.,* 2021). In addition, microplastic behaviours can be further dictated by their polymer composition, resulting in different surface properties. This can influence how they interact with other particles, such as flocculating with other microplastics and sediments, or being covered by biofilms. Therefore, microplastics represent a much more diverse range of particles than sediments. This varied difference in morphology, density and composition will influence how microplastics are deposited, especially when considering microplastics suspended in the water column. Finally, considering the deposition processes observed in this study, both cores were dominated by a singular process, suspension load in LWC and flood deposition in SWC. This resulted in the populations of the other processes being very small, and therefore hard to draw any reliable conclusions for. Nonetheless, with no variation between any process in either core, sediment depositional processes cannot be used to predict microplastic abundance, and therefore microplastics should be considered as separate particles with unique behaviours in future studies.

4.5.4 Summary

This study highlighted the presence and temporal distribution of microplastics within a Wadden Sea salt marsh. In support of the first hypothesis, core LWC showed a significant trend ($p = 0.006$) of microplastic abundance decreasing with depth, however SWC showed a more variable distribution. Whilst this supports data from other temporal studies that microplastics decrease with depth/time, it also suggests that other factors may contribute to microplastic distribution in the sediment, particularly in areas with previous management. Concerning the second hypothesis, both cores support the theory of microplastics being influenced by the sediment composition. Significant relationships are found between the different size fractions of microplastics and sediment; however in LWC large microplastics (100 – 5000 μm) decrease as % Mud increases ($p = 0.035$), whilst in SWC small microplastics (10 – 100 μm) decrease with increasing mud ($p = 0.018$), and large microplastics increase with increasing mud ($p = 0.018$). Finally, considering the different

sediment depositional processes, the hypothesis was not supported, with the dominant depositional processes over time showing no relationship with microplastic distribution.

4.6 Future Work

The samples used in this study represented a great opportunity to research microplastics in an otherwise unstudied area, the Wadden Sea salt marshes. By collaborating with the University of Hamburg, we had the opportunity to not only measure microplastics in this area but combine this knowledge with their sedimentary data to explore questions around sediment effects on temporal distribution and microplastic deposition. However, a significant limitation of this work was that the initial sediment study was not designed with microplastics in mind. Therefore, field and lab sampling for the sediment cores followed protocols that differed from how microplastic samples are normally collected. The most noticeable impact is the cores themselves, in which large PVC pipes were used to collect the sediment samples. This represents a potential large source of microplastic contamination. Whilst steps were taken to try and minimise this contamination, as shown by the results of the control samples (Appendix 4.3) a considerable number of microplastics were still observed.

Another constraint in this study was the number of cores, limited to two. Whilst each core was effectively sub-sampled over 20 times to provide accurate depth profiles, the disparity in microplastic populations between both cores makes comparing the patterns observed challenging. In particular, the data from LWC would often support the hypotheses, whilst SWC would demonstrate the opposite. Having additional cores to draw data from would allow for a better comparison of the various hypotheses and would create stronger conclusions regarding the different temporal trends.

For future work, this research would benefit from studies that are able to sample from marshes across the Wadden Sea. Not only would this provide more data to compare with the current observations but would further build up understanding of microplastics in European marshes. These studies should look to focus on marshes with either a documented history of management, or if possible, sites which have little to no ditching/dredging. Whilst all sites will have anthropogenic impacts of some kind, these

digging processes disrupt the stratigraphy of the sediment, and so represent a potential disturbance to the accumulation of microplastics over time. Furthermore, studies could look to incorporate a more spatial focus, sampling across a wider area of marsh. By covering more of the marsh, this will show the difference to microplastic trapping caused by different vegetation spatially but also demonstrate whether this difference is observed on a temporal scale as well. This will allow for a better understanding of the amount of microplastics in all salt marsh sediments, and not just the topsoil.

4.7 Conclusion

This study highlights the temporal distribution of microplastics in a salt marsh from the Bay of Tümlau. The salt marsh was found to be an effective sink for microplastics throughout time, showing their presence in all samples and depths as early as the 1950s. A temporal trend was observed in the core LWC, with microplastic abundance found to decrease with increasing depth and time. This supported the hypothesis that microplastic abundance should correlate to global plastic usage, and therefore be steadily increasing in more recent years/depths. However, it was observed that this trend with depth is not linear, and unexpectedly high microplastic concentrations occur at certain depths. This could be due to anthropogenic management of the site disrupting the accumulation of microplastics or be the influence of other changes in the marsh such as vegetation succession and coverage. When studying the effects of sediment properties on this temporal pattern, it was found that microplastic distribution can be related to particle size. In LWC large microplastics (100 – 5000 μm) were found to be present alongside sandier sediments (64 – 1000 μm), whilst smaller microplastics are most prevalent in the finer, muddier (1 – 63 μm) sediments. A similar size-based pattern is observed for SWC; however, the trends are inverted. This suggests that microplastics of different size fractions may behave correspondingly to sediments of similar scale, however it is not the only factor that influences their temporal distribution. Finally, we showed that despite these size-based similarities, microplastic abundance was not correlated with sediment depositional processes. This indicates that microplastics are being accumulated separately to sediments and may have their own unique depositional modes.

The results of this study match that of other temporal studies around the world, showing that even in a widespread range of different habitats, microplastic abundance is generally increasing in more recent years (P. Yao *et al.,* 2019). With this observation matching the trend of increasing global plastic production and usage, we expect microplastic abundance to continue to increase in the coming years. However, it is also important to highlight that variation in microplastic abundance with depth was observed in this study. In particular, the highest microplastic concentrations did not occur in the topsoil as was expected. When considering other microplastic studies within salt marshes, the majority focus on either topsoil or shallow sediment cores, with only a few reporting any temporal studies of significant depth (J. Li *et al.,* 2020; Lloret *et al.,* 2021). While such studies are effective in reporting surface microplastic concentrations, it could be that an even greater microplastic population remains buried within deeper sediments, especially within heavily trafficked and managed sites. Therefore, this study highlights the need to include temporal based studies when assessing the levels of microplastic in salt marshes, to provide a complete understanding of microplastic abundance within these habitats.

Chapter 5 - Synthesis

5.1 Overview

Since being termed microplastics in 2004 (Thompson *et al.,* 2004), research into microplastics has continued to grow over the last 20 years. There are now hundreds of publications regarding microplastics each year, coming from all around the world. In a 2022 review of current microplastic literature, the marine environment is the most frequent keyword used when regarding research topics (M. Li *et al.,* 2022). Accumulation and sediment also rank as 5th and 7th most recurring, demonstrating that measuring microplastic abundance in coastal sediments has been a key focus of microplastic work to date. As coastal environments in which sedimentation plays an important role, salt marshes are no exception to the increasing microplastic scrutiny, with several new studies coming out in the past few years (Almeida *et al.,* 2023; Lautaro *et al.,* 2023; Pinheiro *et al.,* 2022). This dissertation adds to the growing body of literature by focussing on various salt marshes to expand the understanding of microplastic spatial and temporal distribution throughout a salt marsh ecosystem.

The main aim of this research was to study the presence and distribution of microplastics across a set of salt marshes. By selecting remote marshes in previously unstudied areas, this would report the abundance of microplastics for these areas, as well as providing more baseline data for comparison with different salt marshes globally. In addition to measuring abundance, this study focussed on measuring and explaining the distribution of microplastics across the salt marsh. This included the horizontal distribution of microplastics across the length of a salt marsh, as well as their dispersal throughout the sediment depth profile. Various factors would be considered for the potential effects on the distributions observed, including the effects of factors such as vegetation, elevation, and even specific sediment properties like grain-size and depositional modes. This should provide a wide array of data in which to compare the distribution of microplastics, and hopefully provide some explanation as to how and why they have accumulated across the salt marsh.

Before sampling began, there was the need to develop a robust and effective methodology for studying microplastics in the complex salt marshes sediments (**Chapter 2**). This involved optimising an existing microplastic extraction protocol, and testing several steps including density separation, chemical digestion, sieving and staining, to ensure the protocol was suitable for dealing with the high level of vegetative biomass expected in salt marsh sediments. Following the development of this methodology, the initial goals of this thesis were to record and report microplastic numbers across previously unstudied marshes.

Chapter 3 begins this work, focussing on a local salt marsh on the North Norfolk coastline, Blakeney Point. From over 100 sediment samples collected, microplastics were detected in all of them, with an average of 21,000 ± 2,300 particles/m^2 (3,400 ± 390 particles/kg d.w.) found per sample. Upon finding an abundance of microplastics, the data were used to study the effects of different marsh factors on the distribution of microplastics. The results showed that microplastic abundance only correlated with vegetation height, but plotting this, elevation, and distance from the salt marsh edge revealed no trends regarding distribution. However, a relationship was found between microplastics and the vegetative zonation of the salt marsh, with the Lower/Mid marsh zone showing significantly ($p = 0.0003$) more microplastics than any others. This work demonstrated the effectiveness of the Blakeney salt marsh in trapping microplastics, whilst also showing that distribution patterns are occurring in relation to the interplay of several marsh factors, represented by zonation.

In **Chapter 4**, a different salt marsh was sampled, this time from within the Wadden Sea, Germany, and the research now focussed on the abundance of microplastics on a temporal scale. This site once again reported microplastics, found at every depth within the two cores LWC and SWC, with average values of 30,100 ± 990 particles/m^2 (3,300 ± 110 particles/kg d.w.) and 8,400 ± 200. particles/m^2 (720 ± 23 particles/kg d.w.) respectively. Regarding distribution on a temporal scale, in core LWC the abundance of microplastics decreased as age increased, however SWC shows no overall trend. Examining the impact of environmental factors, both cores were found to experience microplastic abundance changes, potentially in relation to either anthropogenic management or changes in vegetation zone. Within this study we also explored the relationship between microplastic and sediment sizes. When comparing microplastic and sediment types of similar size

fractions, LWC showed larger microplastics (100 – 5000 μm) were more abundant in samples with a higher sand content (64 – 1000 μm). Meanwhile, in SWC a relationship was found between both large and small microplastics (10 – 100 μm), with large microplastic most prevalent in samples with high mud content, and smaller microplastics most abundant in samples with high sand content. Finally, the effect of sediment depositional processes on microplastic abundance was studied. In both cores the different sediment deposition processes of suspension load, traction load and deposition from ditching/dredging, were found to have no significant ($p = 0.858$) impact on the abundance of microplastics detected. This work showed the occurrence of temporal trends for microplastics within salt marshes. Whilst a relationship between microplastic and sediment size was observed, it could not conclusively explain microplastic distribution. The lack of trends found when comparing sediment depositional processes suggested that microplastics can behave independently of sediments.

5.2 General Findings

5.2.1 Abundance of Microplastics in Salt marshes

For both marshes selected in this study, microplastics were abundant in all samples taken. Microplastic abundance varied greatly within the sites, with Blakeney Point ranging from 873.36 – 98,689.96 particles/m^2 (58 samples), highlighting the importance of having a wide spread of samples in these distribution studies. The Wadden sea samples had a combined range of 1,414.43 – 74,257.43 particles/m^2 (45 samples) down the two cores. The two sites are comparable, showing similar values of microplastics across a wide range of samples. This is not unexpected, as the marshes share the same temperate climate, both found in western Europe bordering the North Sea. Each site is quite remote with no major ports or centres of urbanisation within 75 km, and show characteristic vegetation befitting, including *Spartina anglica* and *Halimone portulacoides*. The marshes are still distinctive however, with Blakeney Point being shorter (150 m) with a steep elevational gradient (1.4 – 3.9 m), whilst the Wadden Sea marsh is much longer (1000 m) and has a reverse elevation gradient (1.3-1.9 m) running from a cliff and levee on the seaward edge (Figure 5.1). Both salt marshes show high concentrations of microplastic present, with the average values being

21,000 ± 2,300 particles/m^2 (3,400 ± 390 particles/kg d.w.) for Blakeney Point, and 18,000 ± 2,600 (2,000 ± 68 particles/kg d.w.) for the Wadden Sea salt marsh. At each site, microplastics were found in every sample taken, demonstrating microplastics being present both spatially and temporally. Therefore, we can conclude that each marsh is an effective sink for microplastics, indicating that European salt marshes are habitats where we would expect to find high microplastic concentrations.

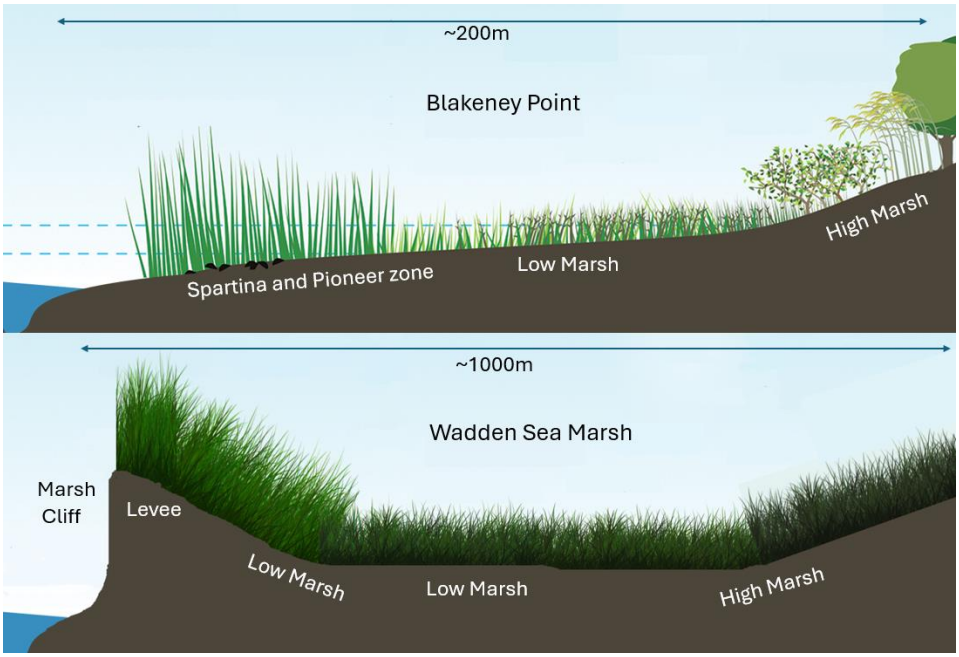

Figure 5.1 - Comparison of topography of Blakeney Point and Wadden Sea marshes (not to scale).

5.2.2 Comparison with other Studies

The microplastic abundance reported in the present study is addressing an important knowledge gap in salt marsh studies globally. Most of the previous research comes from salt marshes in China (J. Li *et al.,* 2020; Wu *et al.,* 2020; W. Yao *et al.,* 2019), or the Americas (Gray *et al.,* 2018; Lautaro *et al.,* 2023; Lloret *et al.,* 2021; Pinheiro *et al.,* 2021). Europe is an under-represented region for which this study can provide some local context. Compared to other marshes around the world, these European marshes show similar abundances of microplastics. Whilst having a range of values, the highest concentrations reported in this study are of similar magnitude to largest currently reported values in salt marshes, 20,060 and 130,725 particles/m^2 by Lautaro et al. (2023) and Yao et al. (2019), respectively. However, the values reported in the Blakeney and Wadden Sea sites become

more significant when we consider the levels of urbanisation reported in other studies. Both the Lautaro et al. (2023) and Yao et al. (2019) studies come from salt marshes neighbouring the port cities of Bahia Blanca and Wenzhou, respectively. The presence of urbanisation is not uncommon in salt marsh studies, with most sites being near coastal cities (Pinheiro *et al.,* 2022) or on large rivers with heavy traffic (J. Li *et al.,* 2020). These sites are therefore in areas that already receive high inputs of anthropogenic pollution, and as such being exposed to such high amounts of waste will likely lead to an influx in the amount of trapped macro and microplastic. This effect of urbanisation has been observed in other habitats, with increasing urbanisation having a noticeable impact on microplastics in both coastlines (X. Yu *et al.,* 2018) and wetlands (Townsend *et al.,* 2019). However, the two sites in this research were selected in part due to their remote locations, with both being over 80 km from the nearest port or major town. Furthermore, both marshes are part of reserve protection schemes, with Blakeney being part of a National Trust Nature reserve, and the Wadden Sea marsh being in the Schleswig-Holstein Wadden Sea National Park. This means both sites receive limited footfall, further reducing the impact of anthropogenic pollution on these marshes. These marshes still receive pollution in the form of local tourism, boat traffic and materials washed in with the tide, however they represent much more remote and clean marshes than previously studied. Therefore, to discover microplastics values like those reported in more urbanised sites suggests that even when anthropogenic input is low salt marshes are effectively able to collect microplastics, and as such are habitats with high microplastic levels.

This trapping efficiency is most apparent when we compare results to those of microplastic studies in other coastal environments. Focusing on coastal wetlands, these habitats include mangroves (Liu *et al.,* 2022; Mohamed Nor and Obbard, 2014; Q. Zhou *et al.,* 2020), tidal flats (Wu *et al.,* 2020), lagoons (Martins *et al.,* n.d.; Vianello *et al.,* 2013) and seagrass meadows (Balestri *et al.,* 2017; Huang *et al.,* 2020; Unsworth *et al.,* 2021). Mangroves are the most studied habitat, with almost 300 sites globally reporting microplastics within their sediments and water column (Ouyang *et al.,* 2022). Tidal flats are second most abundant with over 100 studies, whilst seagrass meadows and lagoons and marshes have considerably fewer studies. Consequently, a wide range of values have been observed. Zhou et al. (2020) reported a maximum of 2,310 ± 29 particles/kg d.w. in a mangrove, whilst

lagoons and tidal flats have recorded values as high as 2,175 (Vianello *et al.,* 2013) and 2,116 (Lo *et al.,* 2018) particles/kg d.w. respectively. However, average values are much lower, with the combined means for plastic abundance in mangroves, marshes, tidal flats and sea grasses being 209, 176.2, 166.4 and 46 particles/kg d.w., respectively (Ouyang *et al.,* 2022). The average values reported in this research were 3,400 ± 390 particles/kg d.w. for Blakeney Point, and 2000 ± 68 particles/kg d.w. for the Wadden Sea salt marsh. These values are larger than both the average and maximum microplastic abundances reported in different coastal wetlands, suggesting that these salt marshes are particularly prone to trapping microplastics.

5.2.2 The Effect of Trapping by Vegetation

Within salt marshes, vegetation is widely considered to contribute to the sediment deposition process that results in the gradual vertical accretion of the marsh (Silva *et al.,* 2009). This occurs because vegetation reduces water flow velocity, creating a low energy environment that encourages sediment particles to drop out of suspension. Vegetation also then helps to prevent resuspension of sediments, effectively trapping them. These processes are theorised to be equally effective in trapping microplastics (Vianello *et al.,* 2013), and the importance of vegetation in microplastic deposition has been demonstrated in several flume studies (Gallitelli *et al.,* 2023; McIlwraith *et al.,* 2024).

Within European salt marshes, the vegetation changes along a topographical gradient. Known as zonation, this creates communities of different plant species based on how much tidal inundation they receive. Each zone has a community of characteristic species, along with varying vegetation heights and densities. With variability and spatial patterns of vegetation often not considered, this was something that was investigated in this research.

In **Chapter 3**, alongside microplastic sampling, also recorded data on vegetation surrounding each sample, including species composition, percentage coverage and vegetation height. These data were then used to determine the zonation across the marsh, with samples being sorted into one of five different zones. Initially we compared the microplastic abundance to the factor vegetation height, as we hypothesized that taller vegetation should be able to trap more microplastics. However, this was found to be

significantly negatively correlated and upon plotting the datasets no visual trends could be observed within the distribution of microplastics. This suggested that whilst vegetation might influence the trapping of microplastics, it is not enough to explain their distribution across the salt marsh. To further study this, we compared the microplastic distribution to the vegetation zone in which the sample was taken, from which a significant trend was observed. Samples taken from the Lower-Mid marsh zone had over twice the number of microplastics recorded than any of the other four zones. This was theorised to be due to this zone representing a combination of factors, including enough vegetation to be able to trap microplastics, but also receiving enough inundation to supply microplastics (Figure 5.2). These results from Blakeney Point (**Chapter 3**) were supported by the findings in **Chapter 4**, when inspecting the Wadden Sea marsh. Whilst both cores here were taken from the same vegetation zones, the lower-mid marsh, they would not have always been in this zone, and vegetation monitoring data from 1996, 2001 and 2006 show the vegetation zone to be lower-mid marsh, pioneer marsh, and then lower-mid marsh again. With anthropogenic ditching ending in 1998, this gave way for natural marsh processes to take over, leading to the re-establishment of the pioneer zone. In the microplastic data, this resulted in a sudden drop in microplastic abundance with both cores trapping over 60% less plastic than before. Following this sudden drop, microplastic values begin to slowly increase, matching the natural succession of the marsh from pioneer back into lower-mid vegetation again. This sudden change in microplastic abundance as the vegetation zone changes suggests that the pioneer zone is not nearly as effective as trapping microplastics as the lower-mid zone, perhaps due to reduced vegetation density and volume. Nonetheless, it shows that vegetation zone, as a combination of multiple environmental factors, influences the effectiveness of microplastic trapping and can perhaps be used to predict microplastic distribution in other marshes.

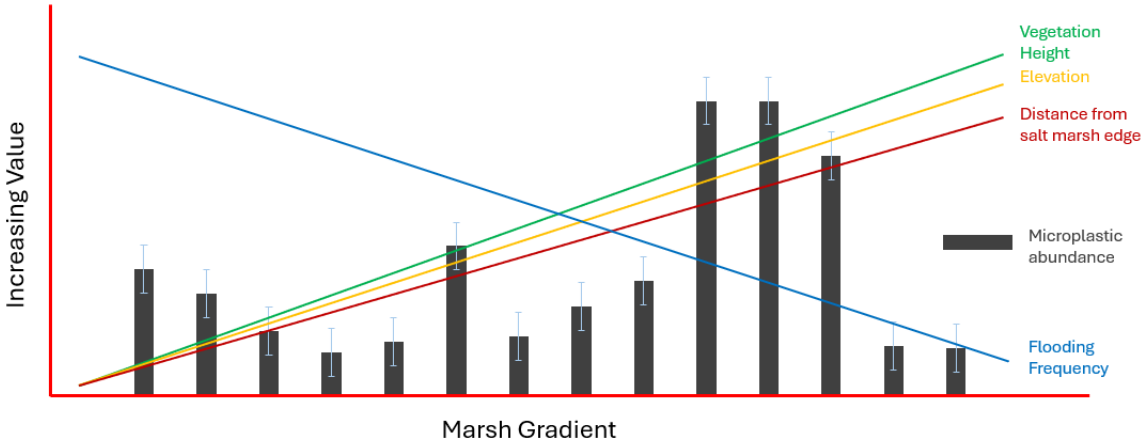

Figure 5.2 - Conceptual graph showing the various factors influencing microplastic distribution along the marsh gradient.

Vegetation has previously been observed to play an important role in the trapping of microplastics (Cozzolino *et al.,* 2020; de los Santos *et al.,* 2021; Li and Yang, 2009). Within this study we found an effect of vegetation zones on microplastics abundance. However, not all salt marshes show such distinctive vegetation patterns, or often such a factor was not considered in previous microplastic research. Despite this, other studies support the importance of vegetation in microplastic accumulation. Li and Yang (2009) reported that microplastics in vegetated areas of the marsh were higher than the adjacent, non-vegetated mudflats suggesting vegetation promotes trapping. In larger marshes, both Mazarrasa et al. (2019) and Lautaro et al. (2023) observed an increase of the abundance of microplastics from mudflat inland to vegetated marsh, with the highest microplastics counts being found around the strandline. With different marshes from around the world reporting this trapping effect by vegetation, we conclude that salt marsh plants play a key role in microplastic accumulation and distribution.

5.2.4 Microplastics are not Sediments!

From the onset of microplastic research, they have generally been compared to sediment particles (Browne *et al.,* 2010). As particles with similar size ranges and comparable densities it is easy to assume that microplastics will behave like sediments in the environment, particularly when it comes to their transportation in aqueous media and

subsequent eventual deposition (Harris, 2020). These assumptions often occur simply because there is a lack of data for microplastics, whereas sediment research has been ongoing for decades (Wadell, 1933). Microplastic research is still relatively new, and therefore insufficient studies have been carried out to properly understand their particle properties (Rochman *et al.,* 2019) and transport and deposition behaviours (Hoellein *et al.,* 2019; Petersen and Hubbart, 2021). Some studies have indeed demonstrated that microplastics show particle transport processes like suspension and traction (Lofty *et al.,* 2023; Nizzetto *et al.,* 2016), and will deposit alongside similarly sized and weighted sediments (Harris, 2020). Furthermore, several environmental studies have demonstrated positive depositional relationships between microplastics and sediments based on their sizes and densities (Horton and Dixon, 2018; Lourenço *et al.,* 2017; Vianello *et al.,* 2013). Based on this evidence, we hypothesised that microplastics would behave like sediments in salt marshes in **Chapters 3** and **4**.

In **Chapter 3**, we studied the impact of different physical marsh factors on the spatial distribution of microplastics. Sedimentation in salt marshes is a complex process dictated by multiple factors including vegetation, surface elevation and distance from the edge of the salt marsh (Silva *et al.,* 2009; Temmerman *et al.,* 2003). We selected these factors (using height for vegetation) on the basis that they should share a similar relationship with microplastics, hypothesizing that microplastics should have an observable trend with each factor, whether negative or positive. However, analysis of the microplastic distribution against each factor showed only vegetation height had a significant correlation, and none of the factors had any visually distinguishable trends when plotted. This suggests that microplastics are behaving differently from sediments in salt marshes.

We used similar assumptions to derive the hypotheses in **Chapter 4**. Whilst previous studies reported a relationship between sediments and microplastics of similar sizes, these comparisons have not been made within a salt marsh environment. However, contradictory results were found when comparing microplastic size fractions to similar sediment fractions (sand and mud, represented by % Mud composition within the sediment). Where LWC matched the hypothesis and showed smaller microplastics most dominant in sediments with highest mud content (and vice versa for large particles and sand content), SWC showed a significant trend in the opposite direction. This suggests that microplastics and sediments

can share similar behaviours, but this is not always the case and there may be more dominant factors influencing microplastic accumulation than just their physical properties. Lenz et al. (2023) applied end-member models and allowed for a detailed understanding of the various processes in which sediments in these cores were deposited. Following the assumption that microplastics behave like sediments, we would therefore expect these processes that cause sedimentation, should similarly result in the deposition of microplastics. We compared microplastic abundance with the dominant sediment depositional process (suspension load, traction load and dredging/ditching) for each sample depth. For all processes in both cores there was no significant relationship with the microplastic abundance, nor any variation between the different processes. This suggests that individual depositional processes were not dictating microplastic accumulation. Whilst this did not prove that microplastics cannot be deposited in this manner like sediments, such processes are not the main driving factor in microplastic accumulation within salt marshes. Some of the results in this chapter support the hypothesis and demonstrate similar behaviour between microplastics and sediments, however, overall, they suggest microplastics also have their own unique patterns when it comes to deposition and distribution.

This is because, despite similarities in many physical properties, there are differences as well. In general, microplastics are much more diverse particles. Sediments are generalised to be spherical with commonly uniform size distribution. Microplastics however have much greater shape diversity, including fibres, films and irregular fragments. Microplastics also have different chemical properties depending on their polymer structure, which alongside affecting their density, can result in a variety of different surface properties. This can influence how microplastics interact with other particles, including flocculation and retention by sediments after deposition. Furthermore, microplastic properties are not static, and can change over time as a microplastic is degraded or biofouled. Consequently, these differences between sediments and microplastic affect ways in which they can be distributed. Sediment transport is well documented, and can occur through aqueous mediums, aeolian transport and managed anthropogenic deposition. Microplastics can share these pathways, however, they also have their own unique pathways resulting from their different properties, such as surface transport (van Sebille *et al.,* 2020). Possibly the

greatest difference though is in unmanaged anthropogenic deposition or littering of larger plastics. Globally plastic pollution is a major issue, and macroplastic is present in many of the different environments where microplastics are studied. As macroplastics degrade in the environment, they generate secondary microplastics, thus providing a direct pathway for microplastics into a given environment (Julienne *et al.,* 2019; Weinstein *et al.,* 2016). Therefore, whilst sediment research is useful for creating a baseline for the expected microplastic behaviour (Waldschläger *et al.,* 2022), the results presented here show that microplastics behave differently to sediments when it comes to deposition. Whilst they may share properties and behaviours with sediments, in salt marshes microplastic distribution was not correlated with sediment properties or deposition methods, therefore we can conclude that microplastics do not behave as sediments and cannot use the same assumptions when trying to predict and understand how they will accumulate.

5.3 Research Implications

The results of this thesis demonstrated the widespread nature of microplastics over space and time. Microplastics should be considered as a potential pollutant to salt marshes, with their full effects not yet understood.

The studies in this thesis demonstrated the ability of salt marshes to effectively trap microplastics, resulting in concentrations within sediments that match or exceed that of other reported coastal ecosystems globally (Dalvand and Hamidian, 2023). These high levels of microplastic make them readily available to the various fauna and flora in the salt marsh ecosystem. Whilst the effects on the consumption of microplastics are still unknown for various organisms, evidence shows that microorganisms in other studies can be severely impacted (Cole *et al.,* 2015, 2013). With salt marsh organisms shown to have consumed microplastics (Piarulli *et al.,* 2020), there is the potential for harmful effects of salt marsh detritivores and other microorganisms, the impacts of which could affect further trophic levels. A similar threat exists for salt marsh plant communities. With high concentrations of microplastics being stored within sediments, there is the potential for various direct and indirect effects on the plants and sediments. Much of this is still unknown, however this study demonstrated microplastic abundance being greatest in the lower-mid marsh zone,

meaning vegetation there is at the greatest risk. Any impacts to plant communities will affect the whole marsh, including impacts on biodiversity, soil stability, and the potential for increased erosion and loss of salt marsh habitat.

Another indirect effect of microplastics is the potential social and economic impacts to salt marshes. Microplastics are known to be a potential human health risk through direct ingestion, with concerns in immune function disruption and neurotoxicity (Prata *et al.,* 2020). Salt marshes provide good staging grounds for young fish and molluscs, and so often have fisheries and oyster farms alongside them. With both fish and oysters having been found to ingest microplastics (Guilhermino *et al.,* 2021; Rivoira *et al.,* 2020), an increased exposure caused by the microplastic retention in salt marshes could lead to potentially more plastic ingested by consumers of this produce. Other social impacts include the visual affront that plastics cause both locals and tourists. Salt marshes are often part of protected areas (Blakeney Point resides in both a wildlife reserve and an AONB (Area of Natural Beauty), Bay of Tümlau sits within a UNESCO World Heritage site) and as such receive lots of tourism for their natural landscape (Fries, 2020). If microplastics are damaging the aesthetics of a salt marsh, the area could lose income through reduced tourism or having to fund clean-up operations. Finally, if microplastics affect salt marsh erosion and plant communities, there is the potential cost of marsh restoration initiatives. Damage to the salt marsh will affect all their ecosystem services, leading to potential impacts not just for the marsh, but the surrounding areas as well.

However, currently the harmful effects of microplastics are still being studied, and salt marshes initially seem to have had minimal impacts. Therefore, as effective microplastic trappers, salt marshes could be ideal ecosystems for the removal and storage of microplastics from waters. In doing so, they could effectively act as microplastic sinks, retaining microplastics in the sediment and removing them from more susceptible habitats such as rivers and seas. Whilst measures have not been developed to carry out the widescale removal of microplastics from sediments, the harsh conditions of salt marshes might be ideal in breaking down microplastics to a scale in which they are no longer harmful to the ecosystem.

As such, a complete understanding of microplastics within salt marshes needs to consider both their spatial and temporal distribution within sediments. In **Chapter 3** we

demonstrated variability across the length of a marsh, and in **Chapter 4** we showed the non-linear relationship of microplastics with depth. This research demonstrates the need to consider both space and time when trying to measure microplastics within a salt marsh. In doing so, an effective "stock" of microplastics can be measured. This will not only more effectively represent the potential threat and impact of microplastics by more accurately quantifying them but also allow for better comparison between different salt marshes. In doing so this should help to bring together the existing salt marsh research, as well as providing an effective strategy for future studies to follow.

5.4 Research Gaps and Future Questions

This thesis provides a greater understanding of microplastics in salt marsh ecosystems, as well as new insights into topics such as microplastic spatial and temporal distribution. However, with new knowledge comes new gaps, and thus there are several questions that should be explored in future salt marsh studies.

Firstly, regarding the spatial distribution across a salt marsh, this is the first study to consider the vegetation zonation in such detail. Therefore, it would benefit from other research which studies vegetation, sedimentation, and microplastic distribution in parallel, particularly in larger marshes with distinctive zones, or marshes from other global regions where the vegetation communities are markedly different. Following on from this, whilst this and other research suggest vegetation plays a crucial role in trapping microplastics, evidence stems from sediment or flume-based studies. To further understand the role vegetation plays in microplastic trapping, plants themselves need to be sampled and studied for microplastics, both in the above and below ground biomass. Finally, vegetation is still only a single factor, and so to fully understand the process of microplastic accumulation in salt marshes, the hydrodynamic factors need to be properly studied as well. Furthering knowledge by inspecting factors such as inundation time with microplastic distribution, or microplastic concentration in the tidal water combined with current-based hydrological models, will help to explain how and where microplastics are being deposited on a salt marsh.

The research presented in this study in the Wadden Sea has demonstrated that whilst microplastic abundance decreases with time, it does not do so linearly and there are several potential factors which can alter this distribution pattern. When considering the future directions of temporal microplastic distribution, more studies are needed to completely understand how microplastics accumulate over time. Salt marshes in NW Europe for example, studying a site with no history of anthropogenic management in the last 100 years would allow for an unaltered profile of microplastic accumulation through time. This would reveal the rate at which microplastics have accumulated in the past years, as well as showing that microplastics may still be mobile in the sediment through other means. When considering this deposition throughout time, factors other than sediment properties should be the key focus (such as anthropogenic, hydrodynamic or biotic factors). For example, sites with records of coverage or historical hydrological data such an inundation, such as the marshes at Terschelling and Schiermonnikoog (Netherlands) with their long-term monitoring scheme and Trilateral Monitoring and Assessment Programme (TMAP) (Bakker, 2014). This would allow for a greater understanding of what factors are influencing the microplastic accumulation over time other than the increase in plastic production.

Finally, another important follow-on question is the impact of microplastics on salt marshes. A targeted study by Piarulli et al. (2020) looked at six bivalve and crab species, each representing the most common organism within a different feeding zone within the salt marsh. Of the microplastics found, the majority came from crab species, with some individuals having over 100 MPs in their digestive system. This suggests some species may be more vulnerable than others depending on their location within the marsh. However, a keystone species that has not been considered in previous studies are detritivorous amphipods. These species play a key role within salt marshes, helping to breakdown organic matter and cycle nutrients, therefore any impact on them will have effects on the entire salt marsh community (Gracë *et al.,* n.d.). Such organisms have been shown to increase the biofouling of microplastics (Hodgson *et al.,* 2018), and therefore, studies are needed to show whether they directly consume microplastics and are as affected as similar organisms from other marine studies (Cole *et al.,* 2015, 2013) (Figure 5.3). Another area of concern are the plant species of salt marshes. The vegetation communities are balanced through competition and stressors such as flooding and salinity levels (Bertness, 2001), however

microplastics represent a potential new stressor. This research showed the highest accumulation in the Lower/Mid marsh zone, so plant species here are most vulnerable, and negative impacts could lead to changes in plant community and loss of biodiversity. Therefore, there is a need to understand the direct and indirect effects microplastics have on outcomes such as plant growth and sustainability, uptake of nutrients, and germination rates (Rillig *et al.,* 2019). This should be accompanied by studies that consider the effect on the sediment. Microplastics are known to sorb and concentrate a wide range of contaminants from their surrounding environment with potential for transport and transfer to biota following ingestion (Bakir *et al.,* 2014; Brennecke *et al.,* 2016; Tumwesigye *et al.,* 2023), and within salt marshes have been shown to aid the uptake of metals such as Cd and Cu (Almeida *et al.,* 2020). Microplastics have also been shown to change the biophysical properties of sediments, such as bulky density and water holding capacity (De Souza MacHado *et al.,* 2018; Wan *et al.,* 2019). If present in high enough concentrations, these could lead to changes in sediment stability, perhaps resulting in increased erosion of salt marsh sediments. Finally, future studies should not only study impacts in current salt marshes but also consider the effects of what an oversaturation of microplastics could cause. In doing so, we may be able to predict the outcomes caused by the ever-increasing microplastic accumulation in these environments.

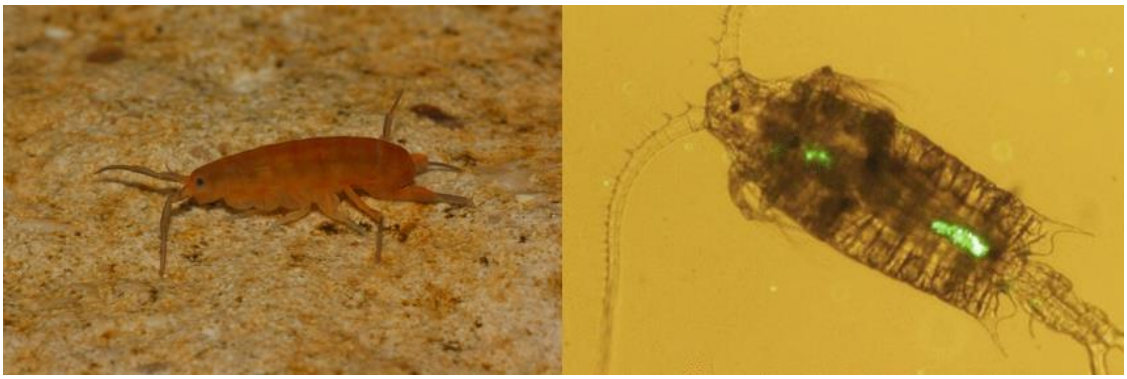

Figure 5.3 - Salt marsh detritivore (*Orchestia sp.*) alongside copepod *Centropages typicus* with microplastic fluorescing in digestive tract (Cole *et al.,* 2013).

5.5 Overall Conclusions

This thesis provides results detailing an advancement in the understanding of the spatial and temporal distribution of microplastics within salt marsh ecosystems. With the goal of establishing a method that could handle a variety of different sediments and vegetation loads, in **Chapter 2** we developed a protocol for extracting and analysing microplastics from salt marsh sediments. This was written up as the Standard Operating Procedure (SOP) and subsequently used as the method in the following chapters. This demonstrated the effectiveness of steps used in other microplastic protocols such as density separation and chemical digestion, whilst the addition of Nile Red staining helped bridge the gap between visual and chemical identification and verification of microplastics. Therefore, we concluded that a methodology suitable for extracting, counting, and analysing microplastics was indeed possible for salt marsh samples, and could be used for a wide range of different sediment samples.

In **Chapter 3** we worked on local salt marsh samples, focussing on the presence and spatial distribution of microplastics within a selected salt marsh. The results showed a large abundance of microplastics across the marsh, with particularly high concentrations located within the lower-mid marsh zone. When trying to understand this distribution, we observed that no singular factor had any distribution trend with microplastic abundance. However, vegetation zonation was found to be significantly correlated with the number of microplastics, suggesting that microplastic distribution may result from the combined influence of several different marsh factors. Thus, we can say that the Blakeney salt marsh has an abundance of microplastics widespread across the salt marsh, and a spatial distribution pattern can be observed, with the lower-mid marsh having significantly more microplastics than any other area of the salt marsh.

Chapter 4 similarly reported high abundances of microplastics in the Wadden Sea sediments, comparable to other reported studies and confirming the ability of salt marshes to effectively trap and retain microplastics. The results show a trend of microplastics generally decreasing with depth that, however, highlights the impact of anthropogenic management on salt marshes, as it can significantly affect the temporal distribution. This work also showed a relationship between the sizes of different microplastics and sediments (Figure 5.4). Whilst this suggested microplastics may be accumulated in a similar manner to

sediments, the results of end-member modelling show that microplastics are not being deposited by the same processes as sediments. Therefore, we are able to conclude that microplastics are again present in large concentrations within salt marshes and are still found when considering deeper samples. There is a temporal distribution pattern, with microplastics decreasing overall with depth and age. However, whilst we might be able to show microplastics are accumulating over time, we were unable to explain this distribution using sediment-based properties such as grain size and deposition method. We can conclude that microplastics do not behave in the same way as sediment particles, and thus accumulate in marshes in their own unique behaviours.

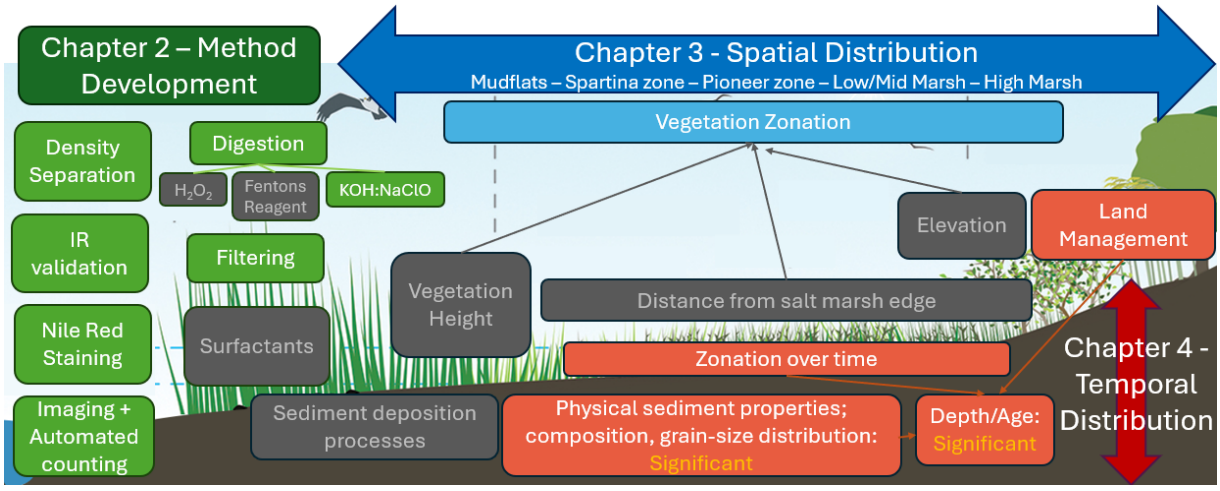

Figure 5.4 - Main findings of Chapters 2, 3, and 4, showing interlinked and significant factors affecting microplastic distribution.

Whilst many of the potential harmful effects of microplastics still need further research to fully understand, this study confirmed the ubiquity of microplastics in salt marsh environments. The combination of trapping by vegetation, suspension from the water and the degradation of macroplastics has resulted in a significant microplastic abundance caught within the sediment. Regardless of their current impacts, microplastics will continue to accumulate within salt marshes. Unless global plastic usage is reduced, these ecosystems will become saturated, and the natural beauty of salt marshes may be permanently tainted by our anthropogenic pollution.

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Appendices

Appendix 1 – Blakeney Data

1.1 Blakeney Microplastic Data

Table 0.1 - Raw data collected from the samples at Blakeney Point, showing; number of microplastics per sample, area (cm^2) and dry weight (g) of laboratory subsamples, and final calculated microplastics per area (m^2) and dry weight (kg)

Sample	No. of MPs	Area (cm^2)	Dry Wgt (g)	MPs/Wgt (Kg) (d.w)	MPs/Area (m^2)
AM1	34	22.9	33.1161	1026.690945	331161
AM2	50	22.9	23.362	2140.22772	233620
AM3	35	22.9	24.8077	1410.852276	248077
AMC	4	NA	NA	NA	NA
AP1	26	22.9	17.272	1505.32654	172720
AP2	37	22.9	13.418	2757.489939	134180
AP3	50	22.9	17.776	2812.781278	177760
APC	4	NA	NA	NA	NA
AP5	37	22.9	21.3	1737.089202	213000
AP6	73	22.9	26.26	2779.893374	262600
AP7	29	22.9	21.82	1329.055912	218200
APC 2	NA	NA	NA	NA	NA
AL/M1	166	22.9	18.254	9093.897228	182540
AL/M2	147	22.9	30.324	4847.645429	303240
AL/M3	54	22.9	17.742	3043.625296	177420
AL/MC	24	NA	NA	NA	NA
AH1	33	22.9	10.648	3099.173554	106480
AH2	43	22.9	13.871	3099.992791	138710
AH3	NA	22.9	3.66	NA	NA
AHC	12	NA	NA	NA	NA
BM1	41	11.45	13.88	2953.89049	138800
BM2	12	11.45	8.7	1379.310345	87000
BM3	3	11.45	8.75	342.8571429	87500
BMC	23	NA	NA	NA	NA
BP1	13	11.45	14.6	890.4109589	146000
BP2	8	11.45	12.44	643.0868167	124400
BP3	11	11.45	16.67	659.8680264	166700
BPC	3	NA	NA	NA	NA
BL/M5	14	11.45	7.64	1832.460733	76400
BL/M6	1	11.45	6.08	164.4736842	60800
BL/M7	23	11.45	6.81	3377.386197	68100
BL/MC2	20	NA	NA	NA	NA
BL/M1	15	11.45	5.05	2970.29703	50500
BL/M2	33	11.45	7.85	4203.821656	78500
BL/M3	43	11.45	5.44	7904.411765	54400
BL/MC	26	NA	NA	NA	NA

BH1	7	11.45	4.84	1446.280992	48400
BH2	18	11.45	3.03	5940.594059	30300
BH3	NA	11.45	2.14	NA	NA
BHC	NA	NA	NA	NA	NA
CM1	33	11.45	6.34	5205.047319	63400
CM2	29	11.45	9.65	3005.181347	96500
CM3	16	11.45	11.63	1375.752365	116300
CMC	6	NA	NA	NA	NA
CS1	14	11.45	6.45	2170.542636	64500
CS2	19	11.45	5.35	3551.401869	53500
CS3	25	11.45	9.54	2620.545073	95400
CSC	6	NA	NA	NA	NA
CP1	19	11.45	6.31	3011.093502	63100
CP2	26	11.45	5.87	4429.301533	58700
CP3	11	11.45	8.3	1325.301205	83000
CPC	4	NA	NA	NA	NA
CL/M1	20	11.45	6.64	3012.048193	66400
CL/M2	33	11.45	7.44	4435.483871	74400
CL/M3	50	11.45	9.1	5494.505495	91000
CL/MC	17	NA	NA	NA	NA
CH1	15	11.45	4.2	3571.428571	42000
CH2	6	11.45	3.2	1875	32000
CH3	12	11.45	3.15	3809.52381	31500
CHC	19	NA	NA	NA	NA
DM1	25	11.45	9.46	2642.706131	94600
DM2	18	11.45	9.1	1978.021978	91000
DM3	11	11.45	8.03	1369.863014	80300
DMC	21	NA	NA	NA	NA
DS1	11	11.45	8.65	1271.676301	86500
DS2	22	11.45	8.09	2719.406675	80900
DS3	29	11.45	9.75	2974.358974	97500
DSC	7	NA	NA	NA	NA
DP1	18	11.45	9.61	1873.048907	96100
DP2	9	11.45	9.15	983.6065574	91500
DP3	20	11.45	14.08	1420.454545	140800
DPC	22	NA	NA	NA	NA
DL/M1	46	11.45	5.47	8409.506399	54700
DL/M2	113	11.45	9.71	11637.48713	97100
DL/M3	71	11.45	7.33	9686.22101	73300
DL/MC	11	NA	NA	NA	NA
DH1	11	11.45	3.71	2964.959569	37100
DH2	12	11.45	1.49	8053.691275	14900
DH3	16	11.45	0.96	16666.66667	9600
DHC	5	NA	NA	NA	NA

1.2 Blakeney Meta Data

Table 0.2 - Metadata collected alongside Blakeney samples, showing; sample, transect collected in, zone collected in, dominant vegetation surrounding, vegetation height (cm), distance from edge of marsh (m), and elevation (m)

Sample	Transect	Zone	Dom Veg	Veg Hgt (cm)	Dist (m)	Elev (m)
AM1	A	Mud	Mud	0	-30	1.36
AM2	A	Mud	Mud	0	-20	1.35
AM3	A	Mud	Mud	0	-10	1.46
AMC	A	Mud	Mud	0	-20	1.35
AP1	A	Pio	Diatoms	5	9	1.4125
AP2	A	Pio	Diatoms	6	19	1.615
AP3	A	Pio	Mud	6	30	1.6375
APC	A	Pio	Diatoms	6	19	1.615
AP5	A	Pio	Mud	10	50	1.6525
AP6	A	Pio	Mud	8	70	1.7775
AP7	A	Pio	Mud	12	90	NA
APC 2	A	Pio	Mud	8	70	1.7775
AL/M1	A	LMM	Suaeda	10	103	2.2475
AL/M2	A	LMM	Hal	8	113	2.3725
AL/M3	A	LMM	Hal	12	123	1.8675
AL/MC	A	LMM	Hal	8	113	2.3725
AH1	A	HM	Sua ver	50	128	3.2
AH2	A	HM	Sua ver	50	133	3.6425
AH3	A	HM	Ely	15	138	3.88
AHC	A	HM	Sua ver	50	133	3.6425
BM1	B	Mud	Diatoms	0	-10	1.6525
BM2	B	Mud	Diatoms	0	-7	1.585
BM3	B	Mud	Diatoms	0	-4	1.595
BMC	B	Mud	Diatoms	0	-7	1.585
BP1	B	Pio	Mud	8	5	1.775
BP2	B	Pio	Mud	10	35	1.9075
BP3	B	Pio	Mud	10	65	1.9
BPC	B	Pio	Mud	10	35	1.9075
BL/M5	B	LMM	Spartina	40	125	2.345
BL/M6	B	LMM	Atr	30	145	2.4575
BL/M7	B	LMM	Spartina	40	165	2.55
BL/MC2	B	LMM	Atr	30	145	2.4575
BL/M1	B	LMM	Atr	25	185	2.51
BL/M2	B	LMM	Atr	40	205	2.545
BL/M3	B	LMM	Atr	20	225	NA
BL/MC	B	LMM	Atr	40	205	2.545
BH1	B	HM	Sua ver	50	240	NA
BH2	B	HM	Sua ver	40	245	3.4775
BH3	B	HM	Sua ver	80	250	3.9075

BHC	B	HM	Sua ver	40	245	3.4775
CM1	C	Mud	Mud	0	-18	1.6125
CM2	C	Mud	Mud	0	-12	1.605
CM3	C	Mud	Mud	0	-6	1.685
CMC	C	Mud	Mud	0	-12	1.605
CS1	C	Spa	Spartina	45	2	2.165
CS2	C	Spa	Spartina	45	8	2.14
CS3	C	Spa	Spartina	35	14	2.095
CSC	C	Spa	Spartina	45	8	2.14
CP1	C	Pio	Ast	25	25	2.1825
CP2	C	Pio	Ast	20	35	2.225
CP3	C	Pio	Ast	20	45	2.3075
CPC	C	Pio	Ast	20	35	2.225
CL/M1	C	LMM	Mud	15	50	2.05
CL/M2	C	LMM	Atr	40	80	2.3125
CL/M3	C	LMM	Ast	20	115	2.335
CL/MC	C	LMM	Atr	40	80	2.3125
CH1	C	HM	Sua ver	75	148	3.1075
CH2	C	HM	Sua ver	50	156	3.293
CH3	C	HM	Ely	20	170	3.8825
CHC	C	HM	Sua ver	50	156	3.293
DM1	D	Mud	Mud	0	-18	1.505
DM2	D	Mud	Mud	0	-12	1.525
DM3	D	Mud	Mud	0	-6	1.6125
DMC	D	Mud	Mud	0	-12	1.525
DS1	D	Spa	Spartina	45	4	2.125
DS2	D	Spa	Spartina	40	8	2.1575
DS3	D	Spa	Spartina	35	12	2.1475
DSC	D	Spa	Spartina	40	8	2.1575
DP1	D	Pio	Mud	20	18	2.3025
DP2	D	Pio	Mud	10	24	2.38
DP3	D	Pio	Mud	10	30	2.3225
DPC	D	Pio	Mud	10	24	2.38
DL/M1	D	LMM	Ast	20	45	2.2725
DL/M2	D	LMM	Ast	25	60	2.405
DL/M3	D	LMM	Atr	30	75	2.5075
DL/MC	D	LMM	Atr	25	60	2.405
DH1	D	HM	Sua ver	80	98	3.495
DH2	D	HM	Sua ver	70	105	4.555
DH3	D	HM	Ely	45	110	4.2525
DHC	D	HM	Sua ver	70	105	4.555

Table 0.3 - Blakeney Data and MetaData information Key, showing; zonation shorthand, measurement headings and vegetation species

Zone shorthand	Headings	Vegetation Species
Mud = Mudflat Pio = Pioneer zone LMM = Low/Mid Marsh HM = High Marsh Spa = Spartina Zone	Transect - which transect the core comes from (A,B, C or D) Zone - Which vegetation zone the core comes from Dom Veg - Dominant Vegetation species Veg Hgt (cm) - the height of the surface vegetation at the core Dist (m) - distance from 0 (creek side edge of the marsh) Elev (m) - Elevation Area (cm^2) - Area of the sample (in lab subsample) Dry Wgt (g) - Dry Weight of sample (lab subsample) No. of MPs - Number of Microplastics MPs/Weight (Kg) (d.w) - Number of Microplastics per Dry Weight (kg) MPs/Area (m^2) - Number of Microplastics per Volume (m^2)	Mud - mudflat Diatom - diatoms and algea Suada - Suade linearis (annual sea-blite) Hal - Halimione portulacoides (sea purslane) Sua ver - Suaeda vera (shrubby sea-blite) Ely - Elytrigia atherica (sea couch) Atr Ast - aster triploium (sea aster) Sali - Salicornia sp (Glasswort)

Table 0.4 - Blakeney samples grain size analysis raw data (Transects A-D, M = mudflat, P = pioneer, S = spartina, LM = lower/mid marsh, H = high marsh)

	Sediment Type (%)			
Sample	**Clay and Fine silt (<6.3um)**	**Medium Silt (6.3-20um)**	**Coarse silt (20-63um)**	**Sand (>63um)**
AM4 - Average	12.261699	20.39134	22.80152	44.54545
AP4 - Average	14.358988	24.67117	38.47107	22.49877
AP8 - Average	25.097519	34.1191	29.69226	11.09112
ALM4 - Average	17.492964	27.03802	31.43707	24.03194
AH4 - Average	1.70627	3.36676	7.337987	87.58898
BM4 - Average	21.596206	29.49806	32.29532	16.61042
BP4 - Average	23.528771	34.56519	30.95831	10.94773
BLM4 - Average	24.182107	34.09221	28.9417	12.78397
BLM8 - Average	21.441984	34.07561	30.45269	14.02971
BH4 - Average	5.709491	10.97915	28.63146	54.6799
CM4 - Average	18.707551	31.12704	33.81379	16.35162
CS4 - Average	21.19321	32.19266	37.80343	8.810699
CP4 - Average	26.232299	40.43952	32.78571	0.54247

CLM4 - Average	17.704416	30.56587	32.9255	18.80421
CH4 - Average	11.11089	18.98553	33.39315	36.51044
DM4 - Average	23.519051	32.59188	26.66622	17.22285
DS4 - Average	19.672045	28.00292	28.01303	24.31202
DP4 - Average	21.88476	30.0567	32.01873	16.03982
DLM4 - Average	21.286787	31.88253	27.98704	18.84365
DH4 - Average	2.238444	4.794237	10.64266	82.32466

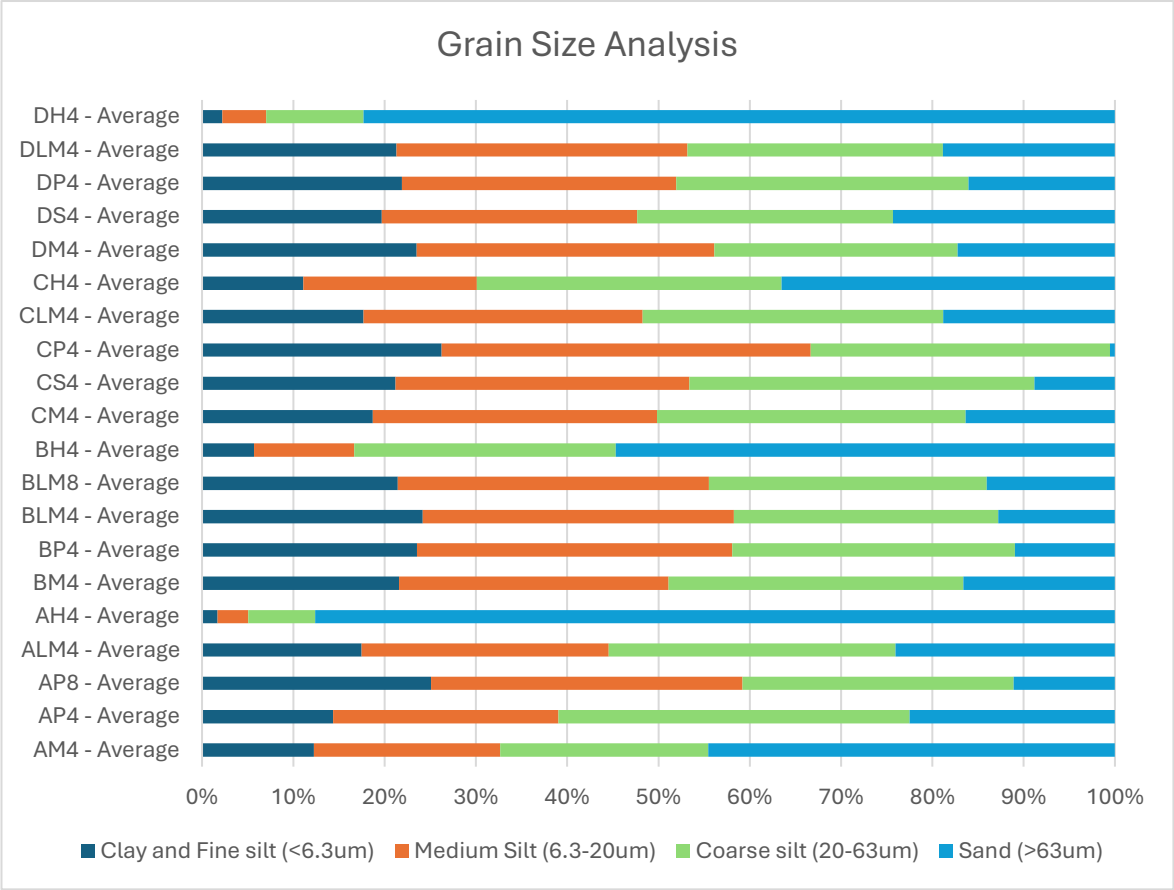


Figure 0.1– Grain size analysis of additional sediment samples, showing % composition of clay, silt and sand

1.3 – Control Data for Blakeney Chapter

Alongside each set of 3 sediment samples, a procedural blank was also run to assess the contamination from the laboratory sources (Table 0.5).

Table 0.5 Control data for samples from Blakeney Salt Marsh (Chapter 3)

Samples	Control	Number of Microplastics
AM1-3	AMC	4
AP1-3, AP5-7	APC	4
AL/M1-3	AL/MC	24

AH1-2	AHC	12
BM1-3	BMC	23
BP1-3	BPC	3
BL/M1-3	BL/MC	26
BL/M5-7	BL/MC2	20
BH1-3	BHC	Filter Damaged
CM1-3	CMC	6
CS1-3	CSC	6
CP1-3	CPC	4
CL/M1-3	CL/MC	17
CH1-3	CHC	19
DM1-3	DMC	21
DS1-3	DSC	7
DP1-3	DPC	22
DL/M1-3	DL/MC	11
DH1-3	DHC	5

An average of 14 ± 2.1 microplastics were found per control. This contamination is to be expected, due to the use of plastic wash bottles and SMI units within the procedure, as well as potential air contamination. This number of particles was subtracted from each sediment sample when calculating the final number of microplastics.

Appendix 2 – Microplastic Data showing Individual Transects

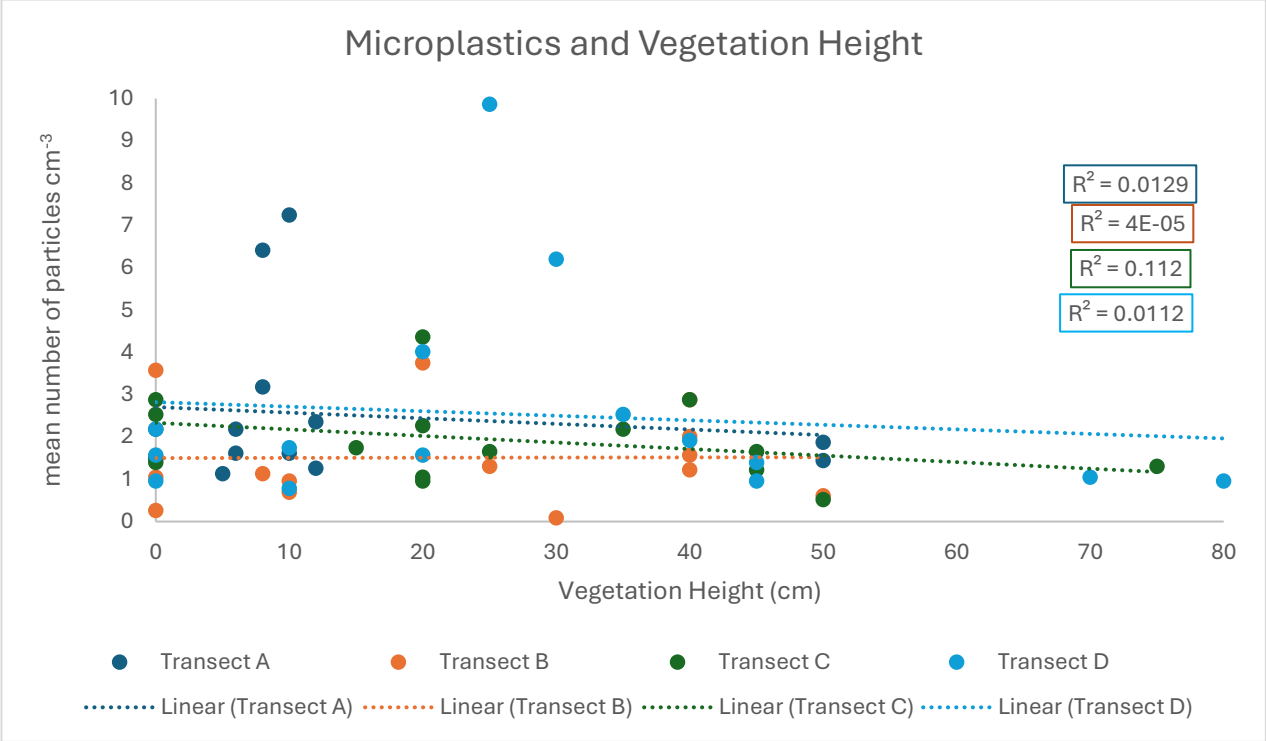


Figure 0.2 - The relationship between Number of Microplastics and Vegetation Height

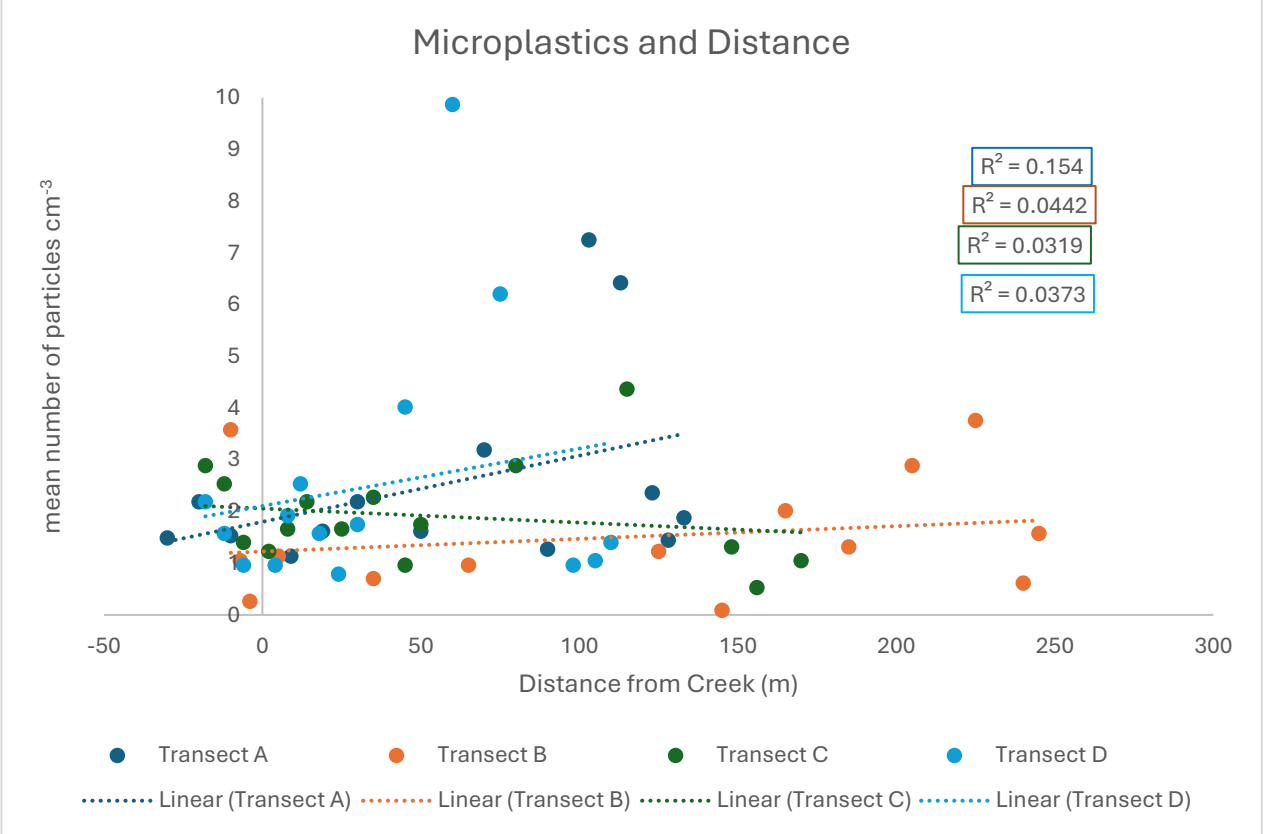


Figure 0.3 - The relationship between Number of Microplastics and Distance from the Salt marsh edge

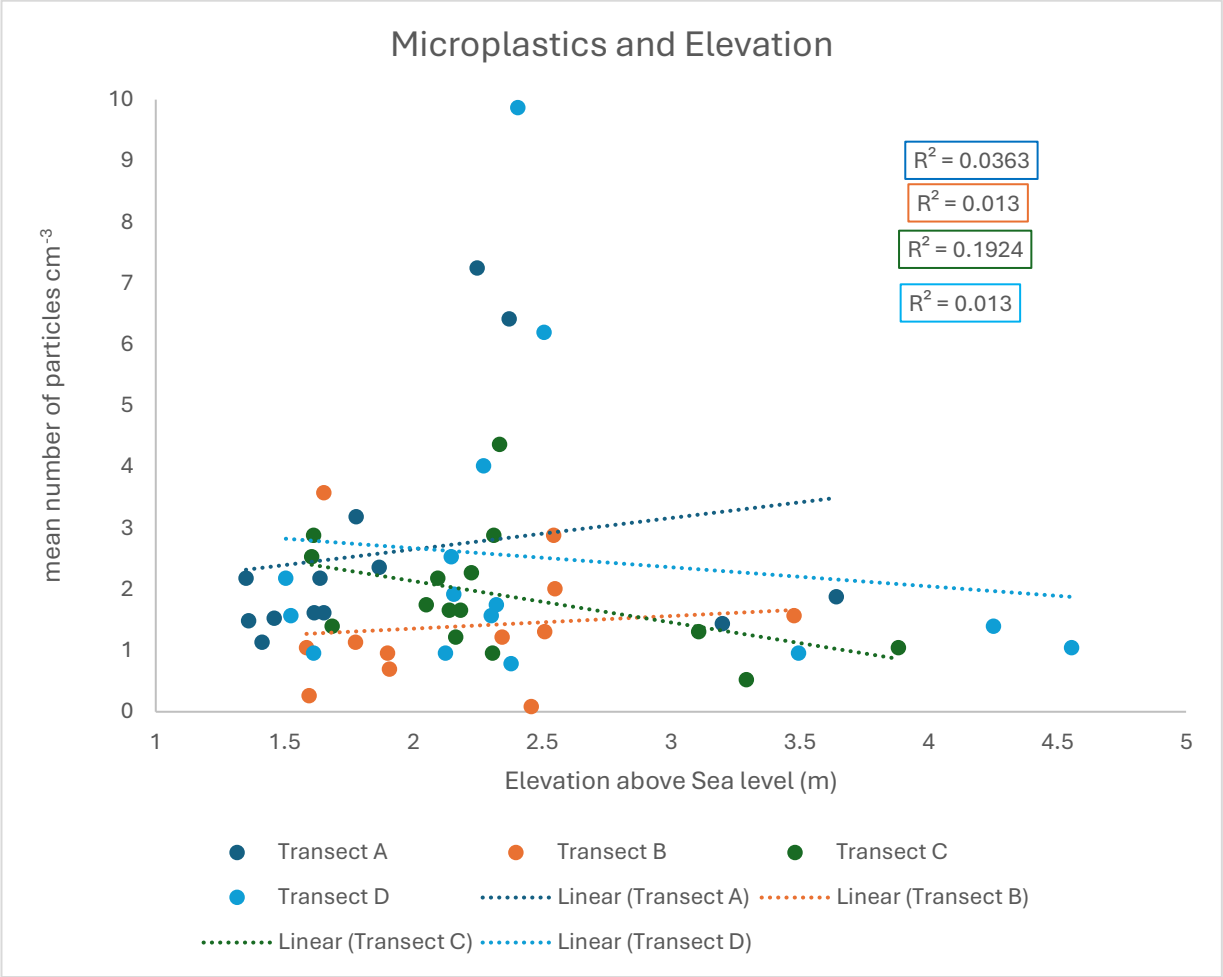


Figure 0.4 - The relationship between Number of Microplastics and Elevation

Appendix 3 – Summary table of papers reviewed in Temporal Study

To draw trends from current research regarding the temporal distribution of microplastics with depth and time, a literature review was carried out. To represent microplastic trends across several decades, temporal studies with cores < 20cm were removed from this review as they on average represent a shorter time scale. The selected studies are summarised in Table 0.6.

Table 0.6 Basic information from studies showing microplastic temporal distribution

Location	Core length and thickness (cm)	Maximum MP abundance (particles/kg)	Time information	Vertical trend of MP abundance
Roter Main River, Germany (Frei *et al.*, 2019)	60; 10	30000 (one core)	\	decreasing for MP size 20-500um

Inde River Floodplain, Germany (Lechthaler *et al.,* 2021)	60-70; 5-10	47.9	0.29-4 cm/year	no depth correlation
Rhine River (Mani *et al.,* 2019)	111	11,070 +-600	\	Decreasing
Qinhuai River, China (Niu *et al.,* 2021)	50; 10	163-563	\	Increasing
Lahn River Floodplain, Germany (Weber and Opp, 2020)	200; 10-50	1.88	0.07 cm/year	Higher on average in top 30cm
Lahn River Floodplain, Germany (Weber *et al.,* 2022)	200; 10-50	2.75	0.5 - 0.91 cm/year	heterogeneous depth distribution
Fuhe River, China (Zhou *et al.,* 2021)	50; 5	5.7 - 570.9	0.49 cm/year	Decreasing
Lake Maharloo, Iran (Abbasi and Turner, 2022)	50; 10	860	1 cm/year	Decreasing
Lake Ontario, Canada (Corcoran *et al.,* 2015b)	30; 2	0.01 - 0.03% weight (each core)	0.1 - 0.4 cm/year	maximum in top 2 cm
Donghu Lake, Wuhan, China (M. Dong *et al.,* 2020)	57; 1.4	741 - 7707	0.95 cm/year	Decreasing
Xinghu Lake, Zhaoqing, China (B. Li *et al.,* 2022)	40; 2-5	523+-140	0.6 cm/year	Maximum near top
Wuliangsuhai Lake, China (Mao *et al.,* 2021)	30; 6	165-724 (surface), 23.3 - 86.7 (bottom)	0.29 cm/year	Decreasing
Hampstead No.1 Pond, UK (Turner *et al.,* 2019)	95; 5	539	0.19 - 1.26 cm/year	maximum at top
Lake in Binhai New Area of Tianjin, China (Yan *et al.,* 2022)	41; 5	11,599	\	Decreasing
Golden Horn Estuary, Turkey (Belivermiş *et al.,* 2021)	104; 2	1545 - 1960	1cm/yr	Variation
Great Bay Estuary, NA (M. L. H. Cheng *et al.,* 2021)	30; 2	116,000 +-21,000	0.12 - 0.6cm/yr	Variation
Louisiana estuaries (Culligan *et al.,* 2022)	72,90; 3	78,502	\	Decreasing

Pearl River Estuary (Fan *et al.,* 2019)	54; 6	140-820	0.97cm/yr	Decreasing
Derwent Estuary, Tasmania, Australia (Willis *et al.,* 2017)	104; 2	2430, 4200	0.43+-0.07cm/yr	Decreasing
Salt marsh, Hangzhou Bay, China (J. Li *et al.,* 2020)	127.5, 162.5; 5	264+- 120	4.89cm/yr	decreasing
Mangrove, Xixi Estuary, Xiaman, China (X. Li *et al.,* 2022)	35; 5-10	261	\	one decreasing, one increasing
Salt marsh, SE New England estuaries, Waquoit Bay estuaries (Lloret *et al.,* 2021)	30; 2	1400	2.82mm/yr	decreasing
Mangrove, Todos os Santos Bay, Brazil (Paes *et al.,* 2022)	30; 3	10,782+-7671	\	no depth correlation
Mangroves Red River Delta and Tien Yen Bay, Vietnam (Viet Dung *et al.,* 2021)	100; 5-15	4941	0.82 - 1.2cm/yr	decreasing
Jiaozhou Bay, Shandong Peninsula, China (Zheng 2020)	45; 8	25 +- 3.1	\	decreasing
Assorted Cores from Japan, Thailand, Malaysia and South Africa (Matsuguma *et al.,* 2017)	50; 11	1845 - 5385	\	decreasing for all cores except one
Marine sediment, East China Sea (Lin *et al.,* 2021)	45, 313; 7	70, 383	\	decreasing
Mangroves, Red Sea (Martin *et al.,* 2020)	170; 6.3	14+-3 per core	\	decreasing
Marine Sediment, Biebu Gulf, China (Xue *et al.,* 2020)	60 ; 10	405±336	\	decreasing
Rockall Trough, Scotland, (Courtene-Jones *et al.,* 2020)	60; 10	19.7 +- 12.9	0.009 – 0.055 cm/year	decreasing
Santa Barbara Basin, California (Brandon *et al.,* 2019)	76; 15	\	0.25 cm/year	decreasing

Kuwait bay, Gulf (Uddin *et al.,* 2021)	59; 7	500	1.15+-0.25 cm/year	decreasing

Appendix 4 – Wadden Sea Data

4.1 – Wadden Sea Microplastic Data

Table 0.7– Microplastic data obtained for the Wadden Sea cores, showing; sample number, number of microplastics, area of lab subsample (cm^2), dry weight of lab subsample (g), calculated number of microplastics per area (m^2) and calculated number of microplastics per dry weight (kg)

Sample	No. of MPs	Area (cm^2)	d.w (g)	No. of MPs/Area (m^2)	No. of MPs /Wgt (kg)
LWC 1	61	14.14	9.15	43140.02829	6666.666667
LWC 2	27	14.14	8.19	19094.76662	3296.703297
LWC 3	49	14.14	10.48	34653.46535	4675.572519
LWC 4	65	14.14	11.59	45968.8826	5608.283003
LWC 5	38	14.14	9.37	26874.11598	4055.496265
LWC 6	105	14.14	10.92	74257.42574	9615.384615
LWC 7	102	14.14	20.65	72135.78501	4939.467312
LWC 8	56	14.14	17.63	39603.9604	3176.403857
LWC 9	66	14.14	14.49	46676.09618	4554.865424
LWC 10	70	14.14	15.42	49504.9505	4539.559014
LWC 11	35	14.14	13.81	24752.47525	2534.395366
LWC 12	12	14.14	11.83	8486.562942	1014.370245
LWC 13	28	14.14	14.56	19801.9802	1923.076923
LWC 14	18	14.14	16.39	12729.84441	1098.230628
LWC 15	35	14.14	13.52	24752.47525	2588.757396
LWC 16	20	14.14	17.23	14144.27157	1160.766106
LWC 17	32	14.14	19.84	22630.83451	1612.903226
LWC 18	12	14.14	25.4	8486.562942	472.4409449
LWC 19	9	14.14	12.89	6364.922207	698.2156711
LWC 20	17	14.14	14.51	12022.63083	1171.605789
SWC 1	7	14.14	10.4	4950.49505	673.0769231
SWC 2	4	14.14	16.03	2828.854314	249.5321273
SWC 3	8	14.14	13.57	5657.708628	589.5357406
SWC 4	4	14.14	22.63	2828.854314	176.7565179
SWC 5	9	14.14	13.89	6364.922207	647.9481641
SWC 6	11	14.14	9.97	7779.349364	1103.30993
SWC 7	5	14.14	19.76	3536.067893	253.0364372
SWC 8	9	14.14	13.62	6364.922207	660.7929515
SWC 9	2	14.14	12.45	1414.427157	160.6425703
SWC 10	29	14.14	17.52	20509.19378	1655.251142
SWC 11	26	14.14	14.16	18387.55304	1836.158192

SWC 12	21	14.14	16.82	14851.48515	1248.513674
SWC 13	14	14.14	15.79	9900.990099	886.6371121
SWC 14	11	14.14	14.85	7779.349364	740.7407407
SWC 15	18	14.14	20.16	12729.84441	892.8571429
SWC 16	9	14.14	23.18	6364.922207	388.2657463
SWC 17	6	14.14	23.43	4243.281471	256.0819462
SWC 18	14	14.14	31.41	9900.990099	445.7179242
SWC 19	12	14.14	24.45	8486.562942	490.797546
SWC 20	6	14.14	20.45	4243.281471	293.398533
SWC 21	21	14.14	15.72	14851.48515	1335.877863
SWC 22	20	14.14	14.36	14144.27157	1392.75766
SWC 23	17	14.14	17.88	12022.63083	950.7829978
SWC 24	8	14.14	20.65	5657.708628	387.409201
SWC 25	6	14.14	15.59	4243.281471	384.8620911

4.2 – Wadden Sea Sediment Data

Table 0.8 – Sediment data obtained from Wadden Sea cores showing: sample, upper and lower depths of subsample slice (cm), Year sediment layer was deposited, and dominant end-member process of that sample

Sample	Upper Depth (cm)	Lower Depth (cm)	Year	Dom End-Mem
LWC 1	0	1	2018.2	Suspension load
LWC 2	2	3	2014.6	Traction load
LWC 3	4	5	2012.8	Suspension load
LWC 4	6	7	2007.5	Suspension load
LWC 5	10	11	2000.3	Suspension load
LWC 6	12	13	1996.7	Suspension load
LWC 7	14	15	1993.2	Dredging/Ditching
LWC 8	16	17	1989.6	Dredging/Ditching
LWC 9	18	19	1987.8	Suspension load
LWC 10	20	21	1983.1	Suspension load
LWC 11	22	23	1980.3	Dredging/Ditching
LWC 12	24	25	1977.4	Dredging/Ditching
LWC 13	26	27	1974.5	Suspension load
LWC 14	28	29	1971.6	Suspension load
LWC 15	30	31	1968.8	Suspension load
LWC 16	32	33	1965.9	Suspension load
LWC 17	34	35	1963.0	Suspension load
LWC 18	36	37	1960.3	Dredging/Ditching
LWC 19	38	39	1957.7	Suspension load
LWC 20	40	41	1955.1	Dredging/Ditching
SWC 1	0	1	2019.1	Suspension load
SWC 2	2	3	2017.9	Traction load
SWC 3	4	5	2017.1	Traction load

SWC 4	6	7	2016.0	Traction load
SWC 5	8	9	2014.4	Traction load
SWC 6	10	11	2011.9	Traction load
SWC 7	12	13	2010.3	Suspension load
SWC 8	14	15	2007.5	Traction load
SWC 9	16	17	2004.1	Traction load
SWC 10	18	19	2002.8	Suspension load
SWC 11	20	21	2001.5	Suspension load
SWC 12	22	23	1999.1	Traction load
SWC 13	24	25	1995.5	Traction load
SWC 14	26	27	1993.0	Traction load
SWC 15	28	29	1991.5	Traction load
SWC 16	30	31	1990.0	Dredging/Ditching
SWC 17	32	33	1988.2	Suspension load
SWC 18	34	35	1986.0	Traction load
SWC 19	36	37	1983.8	Dredging/Ditching
SWC 20	38	39	1981.5	Dredging/Ditching
SWC 21	40	41	1979.2	Traction load
SWC 22	44	45	1973.4	Traction load
SWC 23	48	49	1967.2	Traction load
SWC 24	52	53	1964.0	Traction load
SWC 25	56	57	1961.8	Traction load

For more detailed sediment information on the sediment, age determination model and end-member models, see (Lenz *et al.,* 2023)

4.3 - Control Data for Wadden Sea Samples

Alongside each set of 5 sediment samples, a procedural blank was also run to assess the contamination from the laboratory sources (Table 0.9).

Table 0.9 - Control data for samples from Wadden Sea Salt Marsh (Chapter 4), showing samples extracted in each run (LWC and SWC), and their respective control sample

Samples	Control	Number of Microplastics
LWC 1-5	LWC Lab Control A	5
LWC 6-10	LWC Lab Control B	21
LWC 11-15	LWC Lab Control C	5
LWC 16-20	LWC Lab Control D	6
SWC 1-5	SWC Lab Control A	5
SWC 6-10	SWC Lab Control B	15
SWC 11-15	SWC Lab Control C	7
SWC 16-20	SWC Lab Control D	11
SWC 21-25	SWC Lab Control E	8

An average of 9 ± 1.7 microplastics were found per control. This contamination is to be expected, due to the use of plastic wash bottles and SMI units within the procedure, as well as potential air contamination. This number of particles was subtracted from each sediment sample when calculating the final number of microplastics.

4.4 Regression Analysis

4.4.1 Depth Regression Analysis

Simple linear and non-linear regression was used to investigate whether the depth of deposition significantly affected the abundance of microplastics in core BT02 and BT03 (Table 0.10).

Table 0.10 - Results of regression analysis on microplastic abundance and depth (MPs = microplastics)

Core	Fitted Regression Model	R^2	Significance
BT02 - Linear	No. of MPs = -951.44 (depth) +50855	0.3474	p = 0.006
BT02 - Polynomial	No. of MPs = -47.56 $(depth)^2$ + 1045.5 (depth) + 36875	0.4538	p = 0.006
BT03 - Linear	No. of MPs = 70.26 (depth) + 6588.9	0.0488	p = 0.289
BT03 - Polynomial	No. of MPs = -8.13 $(depth)^2$ + 520.51 (depth) +2395.1	0.2126	p = 0.289

4.4.2 Sediment Composition Regression Analysis

Linear and non-linear regression models were used to analyse the effect sediment composition (% Mud) on the different size fractions of microplastics found (10-100 μm and 101-5000 μm) (Table 0.11 and Table 0.12)

Table 0.11 - Results of regression analysis on 10-100 μm microplastic size fraction (% of total population) and sediment composition (% Mud), (MPs = microplastics)

Core (10-100 μm)	Fitted Regression Model	R^2	Significance
BT02 - Linear	% of total no. of MPs = 53.81 + 35.86 (% Mud)	0.197	0.097
BT02 - Polynomial	% of total no. of MPs = 11.08 - 78.26 (% $Mud)^2$ + 153.44 (% Mud)	0.3168	0.097
BT03 - Linear	% of total no. of MPs = 113.46 - 55.06 (% Mud)	0.2626	0.018

BT03 - Polynomial	% of total no. of MPs = 109.96 - 9.44 (% Mud)^2 - 43.23 (% Mud)	0.2629	0.018

Table 0.12 - Results of regression analysis on 101-5000 μm microplastic size fraction (% of total population) and sediment composition (% Mud), (MPs = microplastics)

Core (101-5000 μm)	Fitted Regression Model	R^2	Significance
BT02 - Linear	% of total no. of MPs = 50.55 - 44.06 (% Mud)	0.2984	0.035
BT02 - Polynomial	% of total no. of MPs = 87.77 + 68.17 (% Mud)^2 - 146.48 (% Mud)	0.3134	0.035
BT03 - Linear	% of total no. of MPs = 10.17 + 25.86 (% Mud)	0.0835	0.018
BT03 - Polynomial	% of total no. of MPs = 26.83 + 40.28 (% Mud)^2 -27.18 (% Mud)	0.0884	0.018

4.4.3 – Linear Regression Analysis of Sediment Deposition Processes

Linear and non-linear regression models were used to determine the relationship between abundance of microplastics, and the three end-member depositional modes: suspension load, flood deposition and dredging/ditching (Table 0.13). No patterns were observed, nor any significance found between the number of microplastics and each factor.

Table 0.13 - Results of regression analysis on microplastic abundance and the 3 depositional processes (MP = microplastics)

Deposition Process	Core	Fitted Regression Model	R^2	Significance
Suspension Load	LWC - Linear	No. of MPs = -7.08 (suspension score) + 30309	1.00E – 08	0.999
	LWC - Polynomial	No. of MPs = 40409 (suspension score)^2 - 46912 (suspension score) + 40083	0.0182	
	SWC - Linear	No. of MPs = 1071.7 (suspension score) + 8180.5	0.0041	0.761
	SWC - Polynomial	No. of MPs = 12769 (suspension score)^2 - 10375 (suspension score) + 8860.5	0.056	
Flood Deposition	LWC - Linear	No. of MPs = -16919 (flood score) + 31237	0.023	0.523
	LWC - Polynomial	No. of MPs = 56817 (flood score)^2 - 61424 (flood score) + 31775	0.0285	
	SWC - Linear	No. of MPs = -1316.2 (flood score) + 9175.6	0.0055	0.724

	SWC - Polynomial	No. of MPs = 1318.5 (flood score)^2 - 2541.9 (flood score) + 9335.6	0.006	
Dredging/Ditching	LWC - Linear	No. of MPs = 6064.4 (ditching score) + 30309	0.0083	0.703
	LWC - Polynomial	No. of MPs = 48662 (ditching score)^2 - 32576 (ditching score) + 31510	0.0367	
	SWC - Linear	No. of MPs = 159.8 (ditching score) + 8368.9	6.00E-05	0.971
	SWC - Polynomial	No. of MPs = -6276.5 (ditching score)^2 + 4958.2 (ditching score) + 8004.7	0.0113	

Appendix 5 - Standard Operating Procedure for Microplastic Extraction from Salt Marsh Samples, and Analysis

Procedure:	The protocols and sequence needed for the successful extraction of microplastics from within salt marsh sediments
Author & Contact Details:	Benjamin Grover b.grover@uea.ac.uk
Date of Creation:	March 2024
Date of Re-assessment and Review:	N/A

This document is to outline the various protocols and sequence used in microplastic extraction for salt marsh samples. This includes the creation of solutions, sample handling, density separation, digestion, staining and imaging steps. Each process has its own SOP, which should be followed in the sequence given in this guide. This will be used by undergraduate students under supervision and postgraduate researchers and staff within the 01.18 CAP CHE lab.

It is recommended that all students and staff that are undertaking work in the CAP building read and familiarise themselves with the data within this document.

Please contact the Author or Scientific Supervisor for further clarification or information.

1. Solutions SOP

1.1 Risk Assessment

PERSONS AT RISK (please X): Employees (X) Contractors ( ) Public ( ) Visitors ( ) Students (X) Others ( )	**Author: Benjamin Grover**	**Location: Sci Faculty**

ACTIVITY/TASK/PROCEDURE:
Creation and use of solutions for;
Density Separation for extraction of Microplastics
Staining and imaging of Microplastics

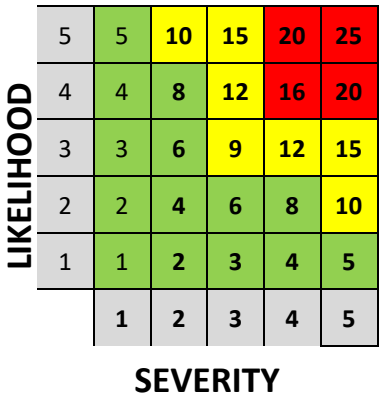
- Use of Acetone for cleaning/degreasing
- General creation and use of solutions:
- Zinc chloride Solution
- Nile Red Dye
- 30% KOH:NaClO
- Working at Height (WAH) to retrieve chemicals
- Use of lab glassware
- Use of electrical equipment
- Vacuum Pumps

Significant Hazard	Potential Consequences of Hazard	INITIAL RISK LEVEL (H/M/L)	Control Measures Required	FINAL RISK LEVEL (H/M/L)
1/Use of Acetone				
Vapours (transfer from bottle and potential spillage)	Eye irritation and breathing problem	M (3 x 3)	Transfer from bottle to wash bottle in fume cupboard only. Transfer into beaker first, then from beaker into wash bottle.	L (1 x 3)
Vapours (when using wash bottle)	Breathing problems	L (2 x 3)	Do not use in confined unventilated space.	L (1 x 3)
Direct exposure	Skin irritation	M (3 x 3)	Wear lab coat, Eye protection (EN166) and gloves suitable for the chemicals being used. Check breakthrough times before use.	L (1 x 3)
Fire	Fatal injuries	M (3 x 5)	Do not use on or close to hot surfaces, source of ignition and flames. Restricted volumes of wash bottle and overall quantities	L (1 x 5)

			of flammable material in laboratory. Store in solvent cabinet.	
2/ Creation and Use of Solutions				
Direct prolonged exposure or inhalation of zinc chloride solution	Mild skin irritation – Hands, Eyes, Internal Organs	L (1 x 3)	Wear gloves when creating large batches to reduce exposure of zinc chloride powder to hands. Wear safety goggles. Wash hands with water and soap shortly after exposure.	L (1 x 3)
Spill of chemicals	Slips resulting in injury	M (3 x 3)	Clean up spills as soon as they are identified. Using absorbent material.	L (1 x 3)
Direct prolonged exposure or inhalation of Nile Red solution	No serious hazards, may cause eye irritation.	M (3x2)	Wear gloves, eye protection. Avoid inhalation and ingestion.	L (1x2)
Direct prolonged exposure or inhalation of 30% KOH solution	Can cause serious irritation and burns to skin and eyes. Harmful if swallowed	M (3x3)	Work within a fume cupboard. Wash hands thoroughly after handling. Wear protective gloves, eye protection and clothing.	L (1x3)
Direct prolonged exposure or inhalation of 30% NaClO solution	May cause irritation to the respiratory tract, (nose and throat). May cause nausea, vomiting if ingested. May cause severe irritation and damage to skin and eyes.	M (3x3)	Wash exposed thoroughly skin after handling. Work within fume cupboard. Wear protective gloves, protective clothing, eye protection.	L (1x3)
3/Working at height to retrieve chemicals				
Fall of person from Height	Personal Injury from fall and potential interaction with something during the fall	L (2x3)	Ensure WAH equipment is fit for use before using. Conduct recorded annual inspection of WAH equipment. Ensure adequate space around you when working. Only work on sound ground. Wear appropriate footwear and clothing. Do not work on WAH equipment if feeling unwell.	L (1x3)

Fall of object from height	Personal Injury from dropped item hitting or bouncing and hitting someone	L (2x3)	Ensure adequate space around you when working. Avoid having item's in hand when mounting the WAH equipment. Ensure adequate space for items in use once on WAH equipment.	L (1x3)
Manual Handing	Musculoskeletal Injury	L (2x3)	All persons to have manual handling training. Only move loads that are suitable for your physique. Plan your items journey before moving.	L (1x3)
4/ Use of lab glassware				
Broken Glass	Personal Injury – Cut	L (2x3)	Broken glass should be cleaned up wearing gloves and minimising contact. Use broken glass bin for disposal	L (1x3)
5/ Use of electrical equipment				
Electrical Shock	Personal Injury	L (1x4)	Do not use electrical equipment with wet hands or material.	L (1x2)

Use the risk matrix below to score your hazard or activity for the probability ('L') likelihood harm will occur and the severity ('S') of the outcome. (6)



RISK
HIGH
MED
LOW

Plot the scores on the matrix to obtain a risk score – then use the colour of that score to determine the Risk Level.

Activities that are **High** must not start (or will need to be suspended), without appropriate controls in place to reduce the risk to an acceptable level.

Activities that are **Medium** should only be tolerated in the short term and then only whilst plans are made to introduce further controls within a defined period.

Activities that are **Low** are largely acceptable, subject to periodic review or after significant changes.

1.2 COSHH

Substance/Chemical Name (No Formula)	H Statements in Full*	Max quantity (with Units)	Exposure Limits (WEL)		SDS Used (company + date)
			Long	Short	
Zinc chloride, anhydrous	H302 (harmful if swallowed), H314 (causes severe skin burns and eye damage), H410 (very toxic to aquatic life with long lasting effects)	10 kg			Alfa Aesar – Thermo Fisher – 29/03/2024
Nile Red	N/A	1 g			Sigma Aldrich – 29/03/2024
Propanol	H225 (highly flammable liquid and vapour), H319 (causes serious eye irritation)	2.5 L	1920 mg/m^3	5760 mg/m^3	Fisher Chemical 29/03/2024
Acetone	H225 (highly flammable liquid and vapour), H319 (causes serious eye irritation), H336 (may cause drowsiness or dizziness)	2.5 L			Fisher Chemical 29/03/2024
Ethanol (Ethyl Alcohol)	H225 (highly flammable liquid and vapour), H319 (causes serious eye irritation)	2.5 L	1920 mg/m^3	5760 mg/m^3	Fisher Chemical 29/03/2024
Potassium Hydroxide	H290 (may be corrosive to metals), H302 (harmful if swallowed), H314 (causes severe skin burns and eye damage), H318 (causes serious eye damage)		2 mg/m^3		

Sodium Hypochlorite	H315 (Causes skin irritation), H318 (causes serious eye damage), H401 (toxic to aquatic life)		1 mg/m^3	2 mg/m^3	

***H334 or H317 may require Health Surveillance. H340, H341, H350, H351, H360, H361 (CMT) and H370, H371, H372, H373 (Long term health) require record keeping.**

1.3 First Aid

Does your process require anything more than the basic first aid listed below? If **yes** please specify in other box.
Eye Contact Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required. **Skin Contact** Wash off immediately with plenty of water for at least 15 minutes. Medical attention maybe required depending on exposer. **Ingestion** Do not induce vomiting. Immediate medical attention is required. **Inhalation** Move to fresh air. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance; induce artificial respiration with a respiratory medical device. Immediate medical attention is required. **Protection of First-aiders** Ensure that medical personnel are aware of the material(s) involved, take precautions to protect themselves and prevent spread of contamination.
Other:

1.4 Disposal/Spillage

Does your process require anything more than the standard SAF waste protocol listed below? If **yes** please specify in other box.
Any substance spilt than cannot be washed down the sink should be collected on the correct absorbent (please specify below), bagged and labelled. All substances going out for disposal must be safely bagged or sealed in an appropriate container . This item must then be clearly labelled using the waste labels provided.

capjobs@uea.ac.uk must then be contacted and will arrange for the removal of the waste ready for disposal by the University contractor.
Other: Zinc chloride waste is to be collected within the zinc chloride waste containers.

1.5 Materials

1.51 Consumables

- Zinc chloride salt (we used Acros Organics 98+% extrapure 2.5 kg as it was the least expensive from our suppliers and you need quite a lot of it. At the high concentrations used you will get significant insoluble visible as a brownish sludge, but do not worry, this will be removed by filtration. Note: it is very hygroscopic, so keep the lid tightly sealed when not in use.)
- Distilled water
- Milli Q water
- N-propanol[1] (we used Analar from Sigma Aldrich)
- Ethanol
- Acetone (for cleaning, we used Fisher)
- Nile Red (we used Acros Organics 99% pure code 415711000. We have also used the equivalent product from Sigma Aldrich, which works fine too but is more expensive from our suppliers).
- Glass Pasteur pipettes (we used Fisherbrand short stem)
- 1 mL pipette tips
- 0.2 µm polycarbonate or regenerated cellulose filters, 47 mm diameter, approximately 1 per 0.5 L solution filtered
- Foil to cover Nile Red solution

[1] Used in place of acetone to address feedback on original method concerning the potentially destructive effect of acetone on polymers.

- Suitable 0.2 µm syringe filter (e.g., 25 mm; PES, Nylon or PTFE, but any brand/type you have should be fine)
- KOH, NaClO

1.52 Non-Consumables

- Buchner flask, one for each solution to be filtered
- All glass filter holder for 47 mm filter discs, filter funnel, and clips
- Glass pipette suitable for 200 µL or 5 mL
- Graduated Cylinders
- Glass beaker, size appropriate for volume of zinc chloride to be made
- Glass bottles or flasks (e.g., conical) with glass stoppers, size appropriate for volume of zinc chloride to be stored
- Red squeezy bottles for storage for zinc chloride
- Glass bottles or flasks (e.g., conical) with glass stoppers for storage Nile Red solution (around 500 mL in size for bulk creation)
- Glass vial in appropriate size/number for volume of Nile Red solution
- Glass flask for storage of filtered digestion solution
- Vacuum pump
- Balance
- Stainless steel spatula
- Stainless steel tweezers
- Glass petri dishes to cover filtration apparatus when not in use
- Waste disposal bottle for zinc chloride waste (should not be disposed of to the drains)

1.6 Solutions

1.61 Zinc Chloride Creation

Made to a density of 1.54 g/cm^3 using the following ratio:

$ZnCl_2$ (g): H_20 (g)
900: 885

Allow ~650 mL of $ZnCl_2$ solution per sample (4 L per 6 samples). This allows plenty of solution for rinsing.

Solutions should be made in a glass beaker and stirred with a magnetic stirrer (if it is too hard for the magnetic stirrer initially, manually stir with a stainless-steel spatula) until all $ZnCl_2$ has dissolved. This process will produce a mild warming of the container.

This $ZnCl_2$ solution must then be filtered through 0.2 µm filters, under vacuum, into a clean Buchner flask and then transferred into clean solvent bottles and the red squeezy bottles.

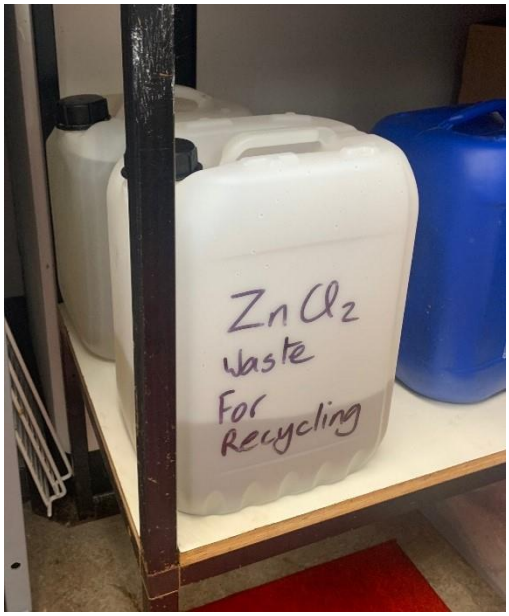
Note: a density of 1.54 g/cm^3 was established as a good medium between floating the plastics and not floating other materials such as crustacean shells often found in sand or aquatic samples. However, this is up to your consideration depending on your sample type. A higher density may be suggested for samples containing denser plastics.

1.62 Zinc Chloride Recycling

Due to the environmental issues, and cost, from using zinc chloride solution for density extraction it is beneficial to recycle the used zinc chloride solution.

If the separators have sat for a while and the solution is settled you can carefully pour this into a storage flask to be filtered, leaving the bottom section with the sample to be disposed of within the $ZnCl_2$ recycling containers (as shown in SOP Figure 1.1). All glassware and storage flasks should be rinsed with filtered zinc chloride solution.

1. Filter some zinc chloride solution, swirl around filter flask, and pour into unfiltered zinc chloride solution, ensuring to rotate the flask 360° to clean the head of the flask completely
2. Continue filtering
3. When you need to fill up the storage flask, pour some of the filtered solution into the flask, swirl and rotate as outlined in step 1. Pour this solution into the unfiltered zinc chloride batch.
4. Proceed with filling up the now clean storage flask with filtered zinc chloride solution
5. Repeat steps as necessary



SOP Figure 1.1 - Zinc Chloride recycling container.

The zinc chloride solution within the containers (SOP Figure 1.1) can be recycled in the same way. Depending on the state or 'dirtiness' of the solution, you may need to change the membrane filter frequently.

Note: we have noticed the density of the solution does not change frequently, it is recommended the density is measured frequently when using a new sample medium and adjusted depending on results.

To measure density

1. Measure weight of clean and dried 100 mL volumetric flask including stopper
2. Fill flask with zinc chloride, using a glass pipette to get to the meniscus to the exact line
3. Measure weight of volumetric flask filled with zinc chloride solution
4. Calculate the density by calculating the weight of the zinc chloride solution
5. Adjust the density of the zinc chloride solution as required. If the density is lower than needed, add zinc chloride powder, and distilled water if the density is too high
6. The solution should then be re-filtered

Note: we recommend having a pre-made solution of zinc chloride that is of higher density that the standard (we used 1.8 g/cm^3) to bring back up the density of recycled solutions.

Table SI 1: Varying Zinc Chloride Densities.

Zinc Chloride (g)	Distilled Water (g)	Density (g/mL)
900	500	1.80
900	885	1.54
420	700	1.38
100	300	1.23
50	350	1.11

SOP Figure 1.2 - Varying Zinc Chloride Densities, and measurements of components

1.63 Nile Red Dye

Dissolve Nile Red in n-propanol at the following ratio:

Nile Red (mg): propanol(mL)
1: 1

Allow 200 µL dissolved Nile Red solution per replicate.

Solution should be made in a glass vial and shaken well to ensure all Nile Red is dissolved. Or if larger quantities are being made, a beaker and magnetic stirrer is preferred.

Solution must be filtered through 0.2 µm syringe filter after dissolution (put filter on after drawing up liquid). When filtering Nile Red solution into small vial, rinse the solution around edges of vial and return to solution beaker to remove dust and MPs.

Once within vial, cover with foil.

Acetone is recommended to clean any glassware after use.

For the staining process, a further step is outlined below. If you wish to only use a small amount, infrequently proceed to Option 1, if you wish to use a large amount of Nile Red solution, for example staining multiple filters frequently proceed to Option 2.

<u>Option 1</u>

Note: these steps should be completed just before use.

1. Measure out 20 mL of Milli Q water.

2. Using a glass Pasteur pipette measure out 200 µL of Nile Red solution and combine with the measured Milli Q water.
3. Ensure all Nile Red is out of the pipette by drawing and releasing the pipette several times.
4. To apply this to your sample, we suggest slowly applying this to your filter, in order to minimise dislodging any potential microplastics.

Option 2

1. Measure out just over 250 mL of ethanol, and filter with clean glassware.
2. With a clean graduated cylinder, measure out 250 mL of the filtered ethanol and add to a 500 mL storage flask
3. Add 250 mL of Milli Q water and swirl flask to mix
4. Add in 5 mL of the Nile Red solution
5. Swirl storage flask in order to mix the solution with the Nile Red, you will notice if it is not completely mixed by a difference or partition in colours
6. Cover storage flask in foil and store within laminar flow cabinet or another appropriate location
7. Refilter before applying to sample

1.64 30% KOH:NaClO

The 1:1 of 30% KOH:NaClO solution is suggested by Enders *et al.* 2016. Note: the exact ratio's used in methodology were changed due to observed particulate matter forming. It is recommended to first test the solution, and then alter the concentrations of KOH of NaClO if necessary.

1. Weigh out 30 g of KOH granules into a large beaker
2. Add roughly 50 mL of MilliQ water and a magnetic stirrer, and allow the solution to mix until clear
3. Add MilliQ water until the solution volume is 100 mL
4. Carefully measure out 50 mL of NaClO solution, and mix with 50 mL of MilliQ water
5. Once the solution has settled, add to the pre-made KOH solution and leave to mix
6. Filter the final digestion solution using a 0.2 µm filter and then leave in a sealed beaker until used

When making up the solution, different volumes may be needed depending on the exact volumes required. It is recommended to have approximately 50 mL of KOH:NaClO solution

per salt marsh sample (so at least 300 mL for a standard sample run), so adjust the volume made accordingly.

Note: it is recommended to make up any digestion solution on the day it is to be used. This prevents potentially hazardous solutions being left in the flow cabinets overnight.

1.7 Filtering of Solutions

All solutions that are involved within microplastic sampling need to be double filtered. This involves:

1. Triple clean all equipment (2x rinse with distilled water, final rinse with Milli Q water) and set up the filtering apparatus (as shown in SOP Figure 1.3). Note: pick an appropriate membrane filter for your purpose. Cellulose nitrate is fine for most solutions, however not appropriate for filtering any digestion solution as the filter will degrade.
2. Filter part of the solution into the buchner flask
3. Turn off the pump and detach the buchner flask
4. Swirl the liquid inside the flask and ensure to rotate 360° to clean the inside of the flask
5. Slowly rotate the flask 360° as you pour the solution into another beaker. Note: the above steps are to ensure the buchner flask is truly and reduce contamination. This process should be repeated for all storage flasks or containers used with the flasks being rinsed with filtered solution from the cleaned buchner flask.
6. Refilter that solution into the now clean buchner flask
7. Use solution appropriately – either immediately or store in storage flask (cleaned in the same way as the Buchner flask as mentioned above

SOP Figure 1.3 - Glass filtering apparatus used to filter all solutions. Buncher flask, filter holder, filter funnel, petri dish to cover funnel and clamp shown.

1.8 Comments on Procedures and Avoiding Contamination

To avoid extraneous microplastics contaminating samples during preparation or processing, a “forensic” approach needs to be taken to remove all sources of such materials insofar as is practically possible. Ideally, a “clean room” (not in a semiconductor manufacturing sense, but one dedicated to these measurements, or where other activities that could introduce microplastics are minimised) should be used, which can be thoroughly cleaned out prior to starting any such work with microplastics determination.

Sensible precautions would be to:

- Thoroughly wash down all benches and surfaces that are accessible with clean water using cotton cloths or paper towels.
- All work should be carried out within the laminar flow cabinets.
- Thoroughly clean the floor with hoover before working. Note: We suggest the best procedure is clean all sides within the lab, and then vacuum. This is due to the vacuum blowing air around which can resuspend dust and microplastics settled on the benches. You should also vacuum on arrival in the lab, and at the end of the day. Particular attention should be taken on Monday morning, or after the lab has been empty for a few days and microplastics in the air may have

settled. It is recommended to empty the vacuum in a bin outside of the microplastics lab.

- Remove all plastic items not actually used for the measurements and keep stocks of such items in cupboards or drawers.
- Wear pure cotton lab coats and avoid wearing any synthetic fibre outer clothing layers while working in the lab.
- Pay attention to any air conditioning or filtration systems and make sure they are not resuspending or introducing particulates into the lab air.
- Work within the laminar flow cabinets for all filtering and processing wherever possible.
- Thoroughly clean all glassware in copious running water. For the density separators and vacuum glassware do a final rinse with Milli Q water. Dry inverted on paper towel.
- Keep stainless steel utensils such as tweezers in a beaker filled with Milli Q water.
- Keep samples covered at all times when they are not actually being used such as store our dried sediments in their glass petri dishes, wrapped in aluminium foil. At the end of the study, a set of samples is archived into glass bottles with screw-top lids. A layer of clean aluminium foil is placed over the bottle before screwing on the cap to isolate the sample from any debris that might shed from the cap or liner. Density separators should be covered at all times with Parafilm. Vacuum glassware covered with foil during the 24 hours between filtering.
- Filter all solutions through membrane filters and store in clean bottles or flasks (finally rinsed with a little of the freshly filtered solution that is being placed in them). Ground glass stoppers are better than screw lids, particularly for solutions that are used frequently (e.g., zinc chloride).
- Cover over filter funnels while samples are being filtered (glass petri dish lids are good for this).

1.9 Procedural Blanks

It is important to run replicate procedural blanks to ensure that the precautions you are taking (e.g. a list similar to the above) are effective in minimising positive staining counts on membranes. If fluorescent particles are found consistently, then the individual solutions and process steps should be checked to try to isolate any source of microplastics.

It is a good idea to use blank Petri dishes in the drying oven and the open bench to collect any particulates arriving from the air. After a period of time (e.g. 24 hours) these can be rinsed and processed (staining and filtration) to test for positive particles. This should give an indication of how generally clean the environment is.

For this procedure, it is essential that the procedural blanks are at or near zero. Processing 5g sediment samples will likely lead to quite low (single figure) counts of positive particles

in many samples, so even low blank counts (e.g. 1 or 2) will significantly increase uncertainty and errors in any subsequent estimates. Following the procedures as described in this SOP have delivered near zero counts in our lab over several months of working, so it is achievable in a general lab with care and attention to detail.

2. Sample Preparation SOP

2.1 Risk Assessment

PERSONS AT RISK (please X): Employees (X) Contractors ( ) Public ( ) Visitors ( ) Students (X) Others ( )	**Author: Benjamin Grover**	**Location: Sci Faculty**

ACTIVITY/TASK/PROCEDURE:
Density Separation for extraction of Microplastics
Staining and imaging of Microplastics

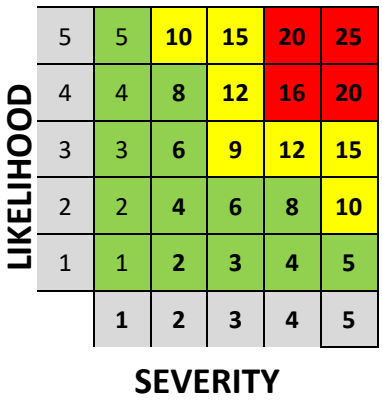
1. Use of Acetone for cleaning/degreasing
2. General use of solutions: (see Solution SOP 1.)
   - Zinc chloride solution
3. Working at Height (WAH) to retrieve chemicals
4. Use of lab glassware
5. Use of electrical equipment
   - Vacuum Pumps

Significant Hazard	Potential Consequences of Hazard	INITIAL RISK LEVEL (H/M/L)	Control Measures Required	FINAL RISK LEVEL (H/M/L)
1/ Use of Acetone				
Vapours (transfer from bottle and potential spillage)	Eye irritation and breathing problem	M (3 x 3)	Transfer from bottle to wash bottle in fume cupboard only. Transfer into beaker first, then from beaker into wash bottle.	L (1 x 3)
Vapours (when using wash bottle)	Breathing problems	L (2 x 3)	Do not use in confined unventilated space.	L (1 x 3)
Direct exposure	Skin irritation	M (3 x 3)	Wear lab coat, Eye protection (EN166) and gloves suitable for	L (1 x 3)

			the chemicals being used. Check breakthrough times before use.	
Fire	Fatal injuries	M (3 x 5)	Do not use on or close to hot surfaces, source of ignition and flames. Restricted volumes of wash bottle and overall quantities of flammable material in laboratory. Store in solvent cupboard.	L (1 x 5)
2/ Creation and Use of Solutions				
Direct prolonged exposure or inhalation of zinc chloride Solution	Mild skin irritation – Hands, Eyes, Internal Organs	L (1 x 3)	Wear gloves when creating large batches to reduce exposure of zinc chloride powder to hands. Wear safety goggles. Wash hands with water and soap shortly after exposure.	L (1 x 3)
Spill of chemicals	Slips resulting in injury	M (3 x 3)	Clean up spills as soon as they are identified. Using absorbent material.	L (1 x 3)
3/Working at height to retrieve chemicals				
Fall of person from Height	Personal Injury from fall and potential interaction with something during the fall	L (2x3)	Ensure WAH equipment is fit for use before using. Conduct recorded annual inspection of WAH equipment. Ensure adequate space around you when working. Only work on sound ground. Wear appropriate footwear and clothing. Do not work on WAH equipment if feeling unwell.	L(1x3)
Fall of object from height	Personal Injury from dropped item hitting or bouncing and hitting someone	L(2x3)	Ensure adequate space around you when working. Avoid having item's in hand when mounting the WAH equipment. Ensure adequate space for items in use once on WAH equipment.	L(1x3)
Manual Handing	Musculoskeletal Injury	L(2x3)	All persons to have manual handling training. Only move loads that are suitable for your physique. Plan your items journey before moving.	L(1x3)
4/ Use of lab glassware				
Broken Glass	Personal Injury – Cut	L(2x3)	Broken glass should be cleaned up wearing gloves and minimising	L(1x3)

			contact. Use broken glass bin for disposal	
5/ Use of electrical equipment				
Electrical Shock	Personal Injury	L(1x4)	Do not use electrical equipment with wet hands or material.	L(1x2)

Use the risk matrix below to score your hazard or activity for the probability ('L') likelihood harm will occur and the severity ('S') of the outcome. (6)



RISK
HIGH
MED
LOW

Plot the scores on the matrix to obtain a risk score – then use the colour of that score to determine the Risk Level.

Activities that are **High** must not start (or will need to be suspended), without appropriate controls in place to reduce the risk to an acceptable level.

Activities that are **Medium** should only be tolerated in the short term and then only whilst plans are made to introduce further controls within a defined period.

Activities that are **Low** are largely acceptable, subject to periodic review or after significant changes.

2.2 COSHH

Substance/Chemical Name (No Formula)	H Statements in Full*	Max quantity (with Units)	Exposure Limits (WEL)		SDS Used (company + date)
			Long	Short	
Zinc chloride, anhydrous	H302 (harmful if swallowed), H314 (causes severe skin burns and eye damage), H410 (very toxic to aquatic life with	10 kg			Alfa Aesar – Thermo Fisher – 25/02/2021

	long lasting effects)				
Acetone	H225 (highly flammable liquid and vapour), H319 (causes serious eye irritation), H336 (may cause drowsiness or dizziness)	2.5 L			Fisher Chemical 23/03/2021

***H334 or H317 may require Health Surveillance. H340, H341, H350, H351, H360, H361 (CMT) and H370, H371, H372, H373 (Long term health) require record keeping.**

2.3 First Aid

Does your process require anything more than the basic first aid listed below? If **yes** please specify in other box.
Eye Contact Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required. **Skin Contact** Wash off immediately with plenty of water for at least 15 minutes. Medical attention maybe required depending on exposer. **Ingestion** Do not induce vomiting. Immediate medical attention is required. **Inhalation** Move to fresh air. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance; induce artificial respiration with a respiratory medical device. Immediate medical attention is required. **Protection of First-aiders** Ensure that medical personnel are aware of the material(s) involved, take precautions to protect themselves and prevent spread of contamination.
Other:

2.4 Disposal/Spillage

Does your process require anything more than the standard SAF waste protocol listed below? If **yes** please specify in other box.
Any substance spilt than cannot be washed down the sink should be collected on the correct absorbent (please specify below), bagged and labelled. All substances going out for disposal must be safely bagged or sealed in an appropriate container. This item must then be clearly labelled using the waste labels provided.

capjobs@uea.ac.uk must then be contacted and will arrange for the removal of the waste ready for disposal by the University contractor.
Other: Zinc chloride waste is to be collected within the zinc chloride waste containers.

2.5. Materials

2.51 Consumables

- Zinc chloride solution. Note: if the solution has been made prior there may be some separation and therefore should be filtered again. Please see solution SOP for more information. Allow ~50 mL of $ZnCl_2$ solution per sample (300 mL per 6 samples). This allows plenty of solution for rinsing.
- Water, for rinsing and cleaning all equipment
- Milli Q water, for final rinse to clean equipment and standard use
- Aluminium foil
- Sediment sample

2.52 Non-Consumables

- Glass petri dishes, size appropriate for volume of sediment sample
- Glass beakers (300 mL)
- Stainless steel spatula
- Stainless steel tweezers
- Buchner flask, for zinc chloride filtration
- Glass bottles or flasks (e.g., conical) with glass stoppers, size appropriate for volume of zinc chloride to be stored
- Wooden pushing block
- Vacuum pump
- Orbital shaker (Rotamax 120, Heidolph)
- Balance
- Vacuum oven

- Recycling waste bottle for zinc chloride waste Note: after use, the zinc chloride should be recycled.

2.6 Methods

2.61 Extracting Sediment

1. Frozen sediment cores are removed from storage (Freezer at -20 ^{o}C) and left in a closed cardboard box within a laminar flow cabinet to thaw for 12 hours.
2. Upon thawing, the aluminium foil wrapping is removed from the cores, and using a fitted wooden block, the mud is pushed up from the bottom and out of the core (SOP Figure 2.1).
3. Using a wooden rule, 1 cm from the top of the sediment core is measured, and then sliced off using a metal blade.
5. The slice is placed into the pre-weighed and pre-cleaned glass petri dish, then placed inside a vacuum oven (40 ^{o}C) and left for 48 hours to dry. If still wet, the sample is further dried in 4-hour periods, until a constant weight is obtained. Upon drying, the sample is reweighed, and the wet and dry weights recorded. Samples should be between 10 – 20 g.
6. This process is then repeated for each sample, with normally 5 – 10 samples being run in a single batch.
7. For each set of 5 samples, a lab blank is run. In this case an empty petri dish is weighed and dried alongside the samples.

SOP Figure 2.1 - Sediment sample being pushed out of core in preparation for slicing

2.62 Deagglomerating Dried Sediment

1. Samples were transferred from the petri dish to a pre-cleaned glass beaker (300 mL). To ensure no sample is lost, the petri dishes were rinsed with zinc chloride, and the rinsate poured into the beaker.
2. Zinc chloride is then added to the beaker until the sediment sample is fully immersed (approximately 20-30 mL), and the beaker covered off using aluminium foil.
3. The beakers are then placed on an orbital shaker (Rotamax 120, 100 rpm), and left to mix for 12 hours (SOP Figure 2.2)
4. After mixing, a spatula is used to gentle break apart any remaining clumps of sediment that remain in the solution.

SOP Figure 2.2 - Samples immersed in zinc chloride solution and agitated on orbital shaker

3. Microplastic Extraction SOP

3.1 Risk Assessment

PERSONS AT RISK (please X): Employees (X) Contractors ( ) Public ( ) Visitors ( ) Students (X) Others ( )	Author: Benjamin Grover	Location: Sci Faculty

ACTIVITY/TASK/PROCEDURE:
Density Separation for extraction of Microplastics
Staining and imaging of Microplastics

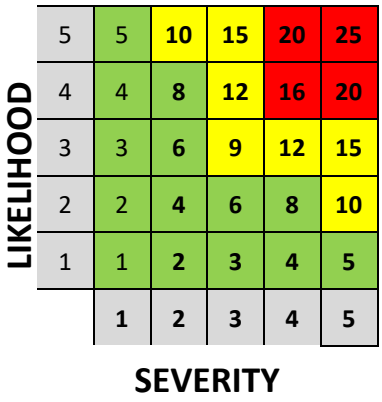
6. Use of Acetone for cleaning/degreasing
7. General use of solutions: (see Solution SOP)
- Zinc chloride solution
8. Working at Height (WAH) to retrieve chemicals
9. Use of lab glassware
10. Use of electrical equipment
- Vacuum Pumps

Significant Hazard	Potential Consequences of Hazard	INITIAL RISK LEVEL (H/M/L)	Control Measures Required	FINAL RISK LEVEL (H/M/L)
1/ Use of Acetone				
Vapours (transfer from bottle and potential spillage)	Eye irritation and breathing problem	M (3 x 3)	Transfer from bottle to wash bottle in fume cupboard only. Transfer into beaker first, then from beaker into wash bottle.	L (1 x 3)
Vapours (when using wash bottle)	Breathing problems	L (2 x 3)	Do not use in confined unventilated space.	L (1 x 3)
Direct exposure	Skin irritation	M (3 x 3)	Wear lab coat, Eye protection (EN166) and gloves suitable for the chemicals being used. Check breakthrough times before use.	L (1 x 3)

Fire	Fatal injuries	M (3 x 5)	Do not use on or close to hot surfaces, source of ignition and flames. Restricted volumes of wash bottle and overall quantities of flammable material in laboratory. Store in solvent cupboard.	L (1 x 5)
2/ Creation and Use of Solutions				
Direct prolonged exposure or inhalation of zinc chloride Solution	Mild skin irritation – Hands, Eyes, Internal Organs	L (1 x 3)	Wear gloves when creating large batches to reduce exposure of zinc chloride powder to hands. Wear safety goggles. Wash hands with water and soap shortly after exposure.	L (1 x 3)
Spill of chemicals	Slips resulting in injury	M (3 x 3)	Clean up spills as soon as they are identified. Using absorbent material.	L (1 x 3)
3/Working at height to retrieve chemicals				
Fall of person from Height	Personal Injury from fall and potential interaction with something during the fall	L (2x3)	Ensure WAH equipment is fit for use before using. Conduct recorded annual inspection of WAH equipment. Ensure adequate space around you when working. Only work on sound ground. Wear appropriate footwear and clothing. Do not work on WAH equipment if feeling unwell.	L(1x3)
Fall of object from height	Personal Injury from dropped item hitting or bouncing and hitting someone	L(2x3)	Ensure adequate space around you when working. Avoid having item's in hand when mounting the WAH equipment. Ensure adequate space for items in use once on WAH equipment.	L(1x3)
Manual Handing	Musculoskeletal Injury	L(2x3)	All persons to have manual handling training. Only move loads that are suitable for your physique. Plan your items journey before moving.	L(1x3)
4/ Use of lab glassware				
Broken Glass	Personal Injury – Cut	L(2x3)	Broken glass should be cleaned up wearing gloves and minimising contact. Use broken glass bin for disposal	L(1x3)
5/ Use of electrical equipment				

Electrical Shock	Personal Injury	L(1x4)	Do not use electrical equipment with wet hands or material.	L(1x2)

Use the risk matrix below to score your hazard or activity for the probability ('L') likelihood harm will occur and the severity ('S') of the outcome. (6)



RISK
HIGH
MED
LOW

Plot the scores on the matrix to obtain a risk score – then use the colour of that score to determine the Risk Level.

Activities that are **High** must not start (or will need to be suspended), without appropriate controls in place to reduce the risk to an acceptable level.

Activities that are **Medium** should only be tolerated in the short term and then only whilst plans are made to introduce further controls within a defined period.

Activities that are **Low** are largely acceptable, subject to periodic review or after significant changes.

3.2 COSHH

Substance/Chemical Name (No Formula)	H Statements in Full*	Max quantity (with Units)	Exposure Limits (WEL)		SDS Used (company + date)
			Long	Short	
Zinc chloride, anhydrous	H302 (harmful if swallowed), H314 (causes severe skin burns and eye damage), H410 (very toxic to aquatic life with long lasting effects)	10 kg			Alfa Aesar – Thermo Fisher – 25/02/2021
Acetone	H225 (highly flammable liquid and vapour), H319 (causes serious eye irritation),	2.5 L			Fisher Chemical 23/03/2021

	H336 (may cause drowsiness or dizziness)				

***H334 or H317 may require Health Surveillance. H340, H341, H350, H351, H360, H361 (CMT) and H370, H371, H372, H373 (Long term health) require record keeping.**

3.3 First Aid

Does your process require anything more than the basic first aid listed below? If **yes** please specify in other box.
Eye Contact Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required. **Skin Contact** Wash off immediately with plenty of water for at least 15 minutes. Medical attention maybe required depending on exposer. **Ingestion** Do not induce vomiting. Immediate medical attention is required. **Inhalation** Move to fresh air. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance; induce artificial respiration with a respiratory medical device. Immediate medical attention is required. **Protection of First-aiders** Ensure that medical personnel are aware of the material(s) involved, take precautions to protect themselves and prevent spread of contamination.
Other:

3.4 Disposal/Spillage

Does your process require anything more than the standard SAF waste protocol listed below? If **yes** please specify in other box.
Any substance spilt than cannot be washed down the sink should be collected on the correct absorbent (please specify below), bagged and labelled. All substances going out for disposal must be safely bagged or sealed in an appropriate container. This item must then be clearly labelled using the waste labels provided. capjobs@uea.ac.uk must then be contacted and will arrange for the removal of the waste ready for disposal by the University contractor.
Other: Zinc chloride waste is to be collected within the zinc chloride waste containers.

3.5. Materials

3.51 Consumables

- Zinc chloride solution (1.54 g/cm^3). Note: if the solution has been made prior there may be some separation and therefore should be filtered again. Please see solution SOP for more information. Allow ~650 mL of $ZnCl_2$ solution per sample (4L per 6 samples). This allows plenty of solution for rinsing.
- Water, for rinsing and cleaning all equipment
- Milli Q water, for final rinse to clean equipment and standard use
- Nile Red solution. Please see solution SOP for more information.
- Glass pasteur pipettes
- 0.2 µm membrane filters, 47 mm diameter, approximately 1 per 0.5 L solution filtered (cellulose nitrate, polycarbonate and regenerated cellulose membranes)
- Aluminium foil
- parafilm

3.52 Non-Consumables

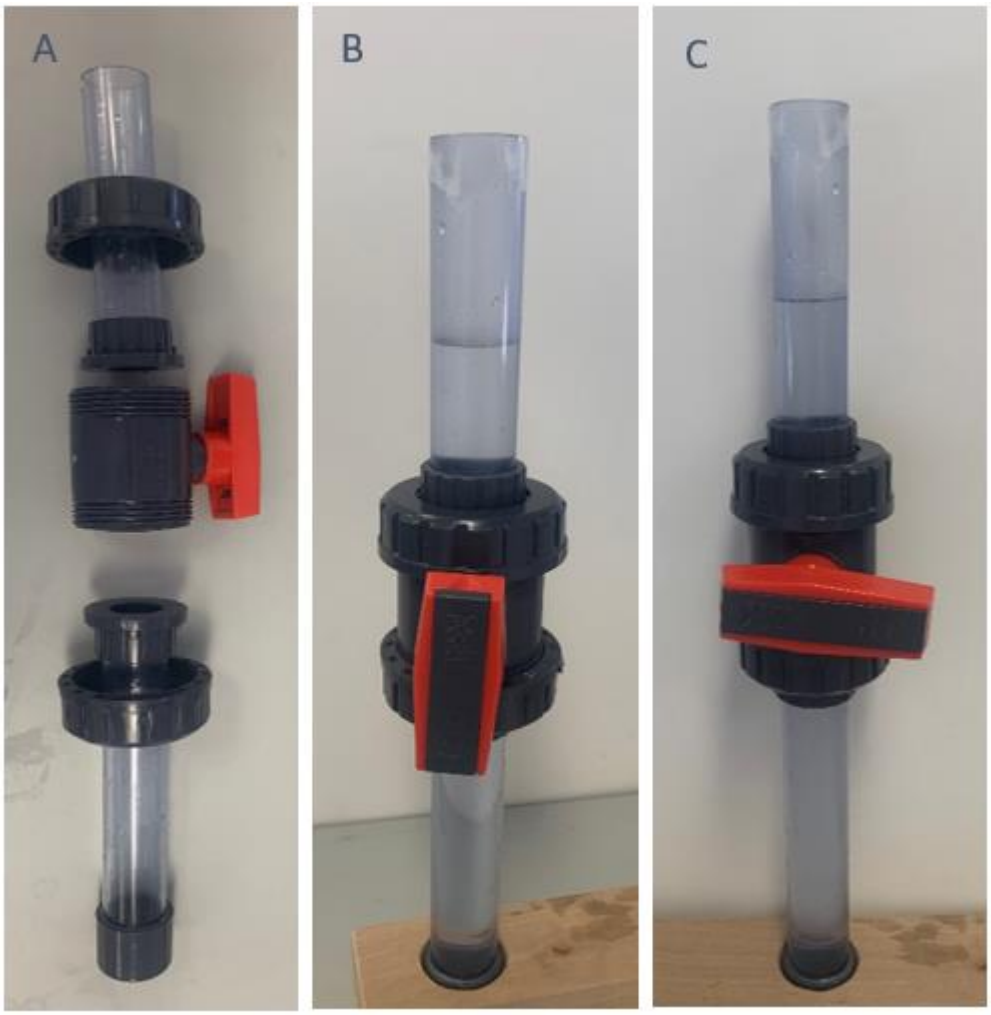
- Sediment Microplastic Isolation (SMI) unit as shown in SOP Figure 3.1 Note: the separators are PET and should be handles accordingly, e.g., do not use acetone or equivalent nearby)
- Wooden standing block for density separators
- 60mm petri dishes, 1 per replicate. Note: for storing the filter after measurement. We use polystyrene ones for this as it is much cheaper and lighter than the glass ones and post-staining, any potential contamination is less of an issue
- Buchner flask, one for zinc chloride filtration and Nile Red solution filtration
- All glass filter holder for 47 mm filter discs, filter funnel and clamp for each sample
- Glass pipette suitable for 200 µL
- Glass beaker, size appropriate for volume of zinc chloride to be made
- Glass bottles or flasks (e.g., conical) with glass stoppers, size appropriate for volume of zinc chloride to be stored
- Vacuum pump
- Stainless steel spatula
- Stainless steel tweezers

- Glass petri dishes to cover filtration apparatus when not in use
- Glass vial, appropriate size/number for volume of Nile Red solution needed
- Recycling waste bottle for zinc chloride waste Note: after use, the zinc chloride should be recycled.

3.6. Method

3.61 Density Separation and Extraction of Microplastics

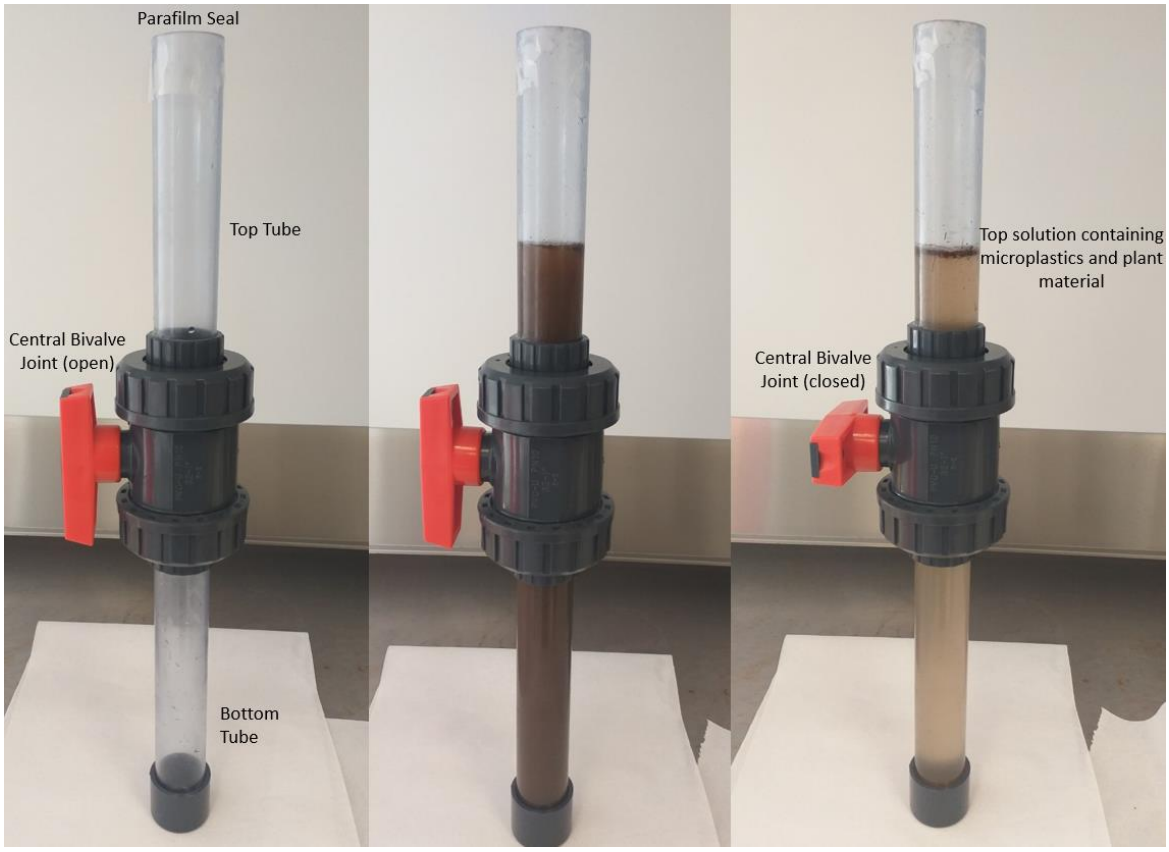
1. Prepare SMI's by rinsing with deionised water (twice) and a final rinse with Milli Q water then assemble. Note: the valves and bottom of the SMI's have been highlighted as an area of potential contamination, see notes in section 3 on how to thoroughly clean the separator parts before rinse stages. Ensure the levers are not stuck and leave open (as shown in SOP Figure 3.1). Place within laminar flow cabinet. Note: for the duration of this experiment, unless covered, the samples, SMI's and equipment should stay within the laminar flow cabinet as much as possible to reduce risk of contamination with airborne microplastics.



SOP Figure3.1 - SMI construction (A), SMI with valve open (B), and valve closed (C).

2. Samples from the sample preparation set (Section 2) were then poured into the SMI, ensuring all sediment is transferred from the beaker by thoroughly rinsing with zinc chloride solution
3. Fill the SMI unit up with further zinc chloride solution, until the level is roughly 5-6cm above the central valve (SOP Figure 3.2)
4. Cover tube with parafilm to create airtight seal
5. Before mixing, carefully open and close the valve several times to remove any air bubbles
6. Carefully invert SMI to dislodge sample from the bottom and continue process until the colour of liquid is uniform, take care with the end covered with parafilm. We

recommend using the palm of your hand to cover end while inverted as shown in SOP Figure 3.3



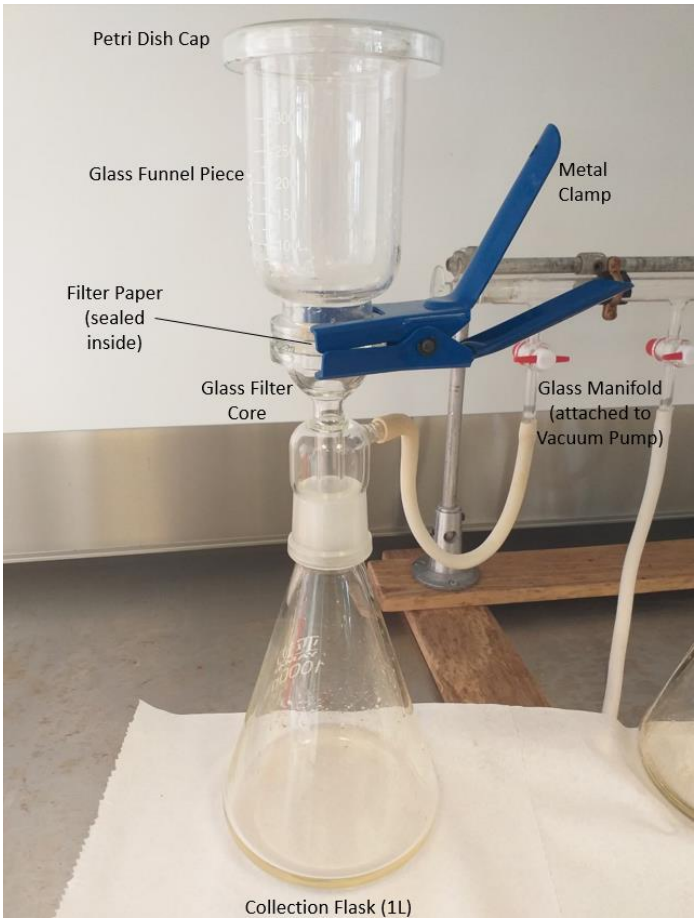
SOP Figure 3.2 - Sediment Microplastic Isolation (SMI) units: empty (left), after mixing, and after separation (right)

SOP Figure 3.2.3 - Use a flat palm to invert the SMI to dislodge the sample and sediment

7. Leave to separate for 24 hours within wooden holding block
8. After 24 hours close the separator valve. We recommend you close all the valves at the same time before starting to filter, this reduces the possibility of forgetting to close the valve as you go along and pouring in all the contents of the separator into the filtration apparatus!
9. Collect the supernatant solution by pouring into a pre-cleaned beaker. Rinse the top half of the SMI thoroughly with zinc chloride solution to collect any material that may be trapped within the tube
10. Seal the beaker with aluminium foil, and then store in laminar flow cabinet until filtration.
11. Opening the central valve, and then repeat steps 3-10. Due this process twice more so that each sample has been separated in the SMI unit 3 times

3.62 Filtering Extracted Microplastics

1. Using pre-cleaned glassware, set up the filtration apparatus (SOP Figure 3.4). Ensure the membrane filter is correct for the solution being filtered (regenerate cellulose or polycarbonate for solutions, cellulose nitrate for samples)



SOP Figure 3.2.4 Filtration apparatus set-up

2. For particularly vegetated samples, place the brass sieve inside the glass funnel piece (SOP Figure 3.5)

SOP Figure 3.2.5 - Custom made brass sieve sitting in filtration apparatus

3. Pour the beaker containing supernatant solution into the apparatus and turn on the vacuum pump to begin filtering Note: If you are filtering multiple samples, it can easily become overcrowded in the laminar flow cabinet. We have found it advantageous to alternate the locations of the clips with some facing towards the back of the cabinet, so the operator has more room to manoeuvre themselves and the SMI.

   Depending on the membrane filter of choice, it can be time consuming separating the filter from the paper separators. If you put the membrane filter onto the glass apparatus, the filter will stick down while the paper separator begins to curl and makes it easy to lift off.
4. Rinse the beaker thoroughly, as well as the brass sieve and any vegetation that has been collected. Allow the rinsate to filter off
5. Remove the brass sieve, and visually inspect the removed vegetation for any larger microplastics/debris that may have been caught in the vegetation. If none dispose of the sieved material
6. Collected the filtered zinc chloride solution for recycling (Section 1). Then, do a final rinse of the apparatus using MilliQ Water to ensure that no zinc chloride remains on the filter.
7. The filters can now be removed and are ready for digestion.

3.7 Comments on Procedures and Contamination Controls

Follow contamination controls as laid out in section 1.7.

For steps that use glass beakers, use the beakers from earlier stages in the protocol (sample preparation). Clean/rinse as necessary, however reducing the amount of glassware and maintaining the same beaker for each sample will prevent any cross contamination between samples.

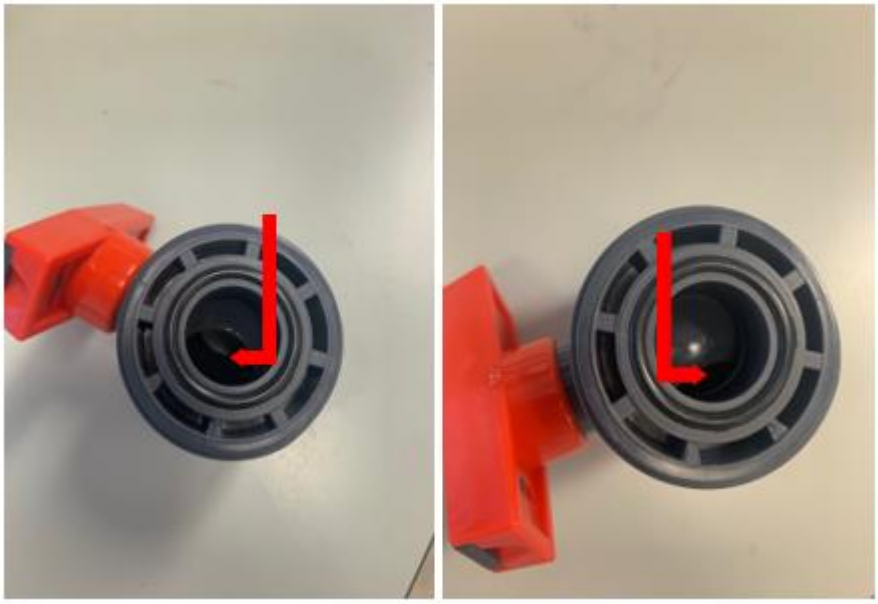
The valve section of the density separating equipment has been highlighted as an area of concern for contamination.

Due to the mechanics of the valve compartment, there are areas within the valve that are only accessible as the valve is turned. There is the possibility that sample material creeps

into these voids as the valve is rotated. These areas can easily be missed in routine cleaning of the equipment. This has potential to leave contamination from past samples within the valve, which could be carried over and contaminate working areas or future samples.

To counteract this, before and after each use of the density separators you should

1. Submerge the valve compartment (SOP Figure 3.6) in clean hot water and open and close the valve several times. Make sure to open and close the valve holding it vertically in both directions to ensure no water is trapped inside the valve.
2. With the valve open, move the valve compartment horizontally back and forth whilst submerged in the water to flush the water through.
3. Using a bristle brush, clean the inside of the valve. Pay particular attention to the white seal rings on the inside of the valve. Note: a plastic free bristle brush would be optimal, however if that is unavailable, please ensure to thoroughly rinse through the compartment after this step.
4. Repeat steps 1-3 with the valve quarter closed and then half closed. Make sure to use a bristle brush to clean the compartment that is exposed when the valve is partially closed (shown below). For thorough cleaning, it is recommended the bristle brush is gently pushed inside the valve, twisted around and then gently removed. This should be repeated three times from either end.
5. Inspect the valve and repeat any steps you feel necessary.



SOP Figure 3.2.6 Central valve showing valve compartment

4. Digestion Steps SOP

4.1 Risk Assessment

PERSONS AT RISK (please X): Employees (X) Contractors ( ) Public ( ) Visitors ( ) Students (X) Others ( )	**Author: Benjamin Grover**	**Location: Sci Faculty**

ACTIVITY/TASK/PROCEDURE:

Digestion step used for extraction of microplastics from samples

- Use of lab glassware
- Use of electrical equipment
- Vacuum Pumps
- Vacuum Oven
- Shaker Incubator
- Balance
- General creation and use of solutions (see **all** risks on Solution: SOP)
- 30% KOH:NaClO
- Working at Height (WAH) to retrieve chemicals

Significant Hazard	Potential Consequences of Hazard	INITIAL RISK LEVEL (H/M/L)	Control Measures Required	FINAL RISK LEVEL (H/M/L)
1/ Use of lab glassware				
Broken Glass	Personal Injury – Cut	L(2x3)	Broken glass should be cleaned up wearing gloves and minimising contact. Use broken glass bin for disposal	L(1x3)
2/ Use of electrical equipment				
Electrical Shock	Personal Injury	L(1x4)	Do not use electrical equipment with wet hands or material.	L(1x2)
Burn from Oven or Shaker Incubator	Personal Injury	L(2x1)	Do not place hands into hot oven, wear protective gloves if needed.	L(1x1)
Entrapment from Shaker Incubator	Personal Injury	L(1x3)	Do not place hands near moving parts of the shaker incubator.	L(1x2)
3/ General creation and use of solutions				
Spill of chemicals	Slips resulting in injury	M(3x3)	Clean up spills as soon as they are identified. Using absorbent material.	L (1 x 3)
Direct prolonged exposure or	Can cause serious irritation and burns to skin and eyes.	M(3x3)	Work within a fume cupboard. Wash hands thoroughly after handling. Wear protective gloves,	L(1x3)

inhalation of 30% KOH: NaClO solution	Harmful if swallowed		eye protection and clothing. Wash exposed thoroughly skin after handling.	
Direct prolonged exposure or inhalation of 30% H_2O_2 or Fentons reagent	Irritation of nose, throat and airway. Causes irritation of the eyes. May cause burns and skin irritation. Nausea, vomiting. Irritation of the mouth, throat, oesophagus and gastrointestinal tract if ingested	M(3x3)	Wear protective gloves, suitable clothing and eye protection. Work within a fume cupboard. Wash hands, or contaminated areas thoroughly after use.	L(1x3)
4/Working at height to retrieve chemicals				
Fall of object from height	Personal Injury from dropped item hitting or bouncing and hitting someone	L(2x3)	Ensure adequate space around you when working. Avoid having item's in hand when mounting the WAH equipment. Ensure adequate space for items in use once on WAH equipment.	L(1x3)
Fall of person from Height	Personal Injury from fall and potential interaction with something during the fall	L (2x3)	Ensure WAH equipment is fit for use before using. Conduct recorded annual inspection of WAH equipment. Ensure adequate space around you when working. Only work on sound ground. Wear appropriate footwear and clothing. Do not work on WAH equipment if feeling unwell.	L(1x3)
Manual Handing	Musculoskeletal Injury	L(2x3)	All persons to have manual handling training. Only move loads that are suitable for your physique. Plan your items journey before moving.	L(1x3)

Use the risk matrix below to score your hazard or activity for the probability ('L') likelihood harm will occur and the severity ('S') of the outcome. (6)

LIKELIHOOD						
5	5	10	15	20	25
4	4	8	12	16	20
3	3	6	9	12	15
2	2	4	6	8	10
1	1	2	3	4	5
	1	2	3	4	5
	SEVERITY				

RISK
HIGH
MED
LOW

Plot the scores on the matrix to obtain a risk score – then use the colour of that score to determine the Risk Level.

Activities that are **High** must not start (or will need to be suspended), without appropriate controls in place to reduce the risk to an acceptable level.

Activities that are **Medium** should only be tolerated in the short term and then only whilst plans are made to introduce further controls within a defined period.

Activities that are **Low** are largely acceptable, subject to periodic review or after significant changes.

4.2 COSHH

Substance/Chemical Name (No Formula)	H Statements in Full*	Max quantity (with Units)	Exposure Limits (WEL)		SDS Used (company + date)
			Long	Short	
Potassium Hydroxide	H290 (may be corrosive to metals), H302 (harmful if swallowed), H314 (causes severe skin burns and eye damage), H318 (causes serious eye damage)		2 mg/m^3		VWR/VWR
Sodium Hypochlorite	H315 (Causes skin irritation), H318 (causes serious eye damage),		1 mg/m^3	2 mg/m^3	VWR/VWR

	H401 (toxic to aquatic life)				

***H334 or H317 may require Health Surveillance. H340, H341, H350, H351, H360, H361 (CMT) and H370, H371, H372, H373 (Long term health) require record keeping.**

4.3 First Aid

Does your process require anything more than the basic first aid listed below? If **yes** please specify in other box.
Eye Contact Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required. **Skin Contact** Wash off immediately with plenty of water for at least 15 minutes. Medical attention maybe required depending on exposer. **Ingestion** Do not induce vomiting. Immediate medical attention is required. **Inhalation** Move to fresh air. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance; induce artificial respiration with a respiratory medical device. Immediate medical attention is required. **Protection of First-aiders** Ensure that medical personnel are aware of the material(s) involved, take precautions to protect themselves and prevent spread of contamination.
Other:

4.4 Disposal/Spillage

Does your process require anything more than the standard SAF waste protocol listed below? If **yes** please specify in other box.
Any substance spilt than cannot be washed down the sink should be collected on the correct absorbent (please specify below), bagged and labelled. All substances going out for disposal must be safely bagged or sealed in an appropriate container. This item must then be clearly labelled using the waste labels provided. capjobs@uea.ac.uk must then be contacted and will arrange for the removal of the waste ready for disposal by the University contractor.
Other: KOH:NaClO waste can be poured down the sink, however should only be done so in small volumes (<1 L) in rinsed with lots of water

4.5. Materials

4.51 Consumables

- Water, for rinsing and cleaning all equipment
- Milli Q water
- Cleaning Utensils
- KOH pellets
- NaClO solution (14% active chlorine)
- 47 mm membrane filter of your choice, 1 per replica. 0.45 µm pore sizes is suggested (polycarbonate or glass fibre for samples, regenerated cellulose for filtering solutions)

4.52 Non-Consumables

- Glass petri dishes, size appropriate for volume of sample to cover beakers
- Glass beakers large enough for sample and digestion solution
- Graduated measuring cylinder
- Vacuum pump
- Shaker Incubator (ES-80, Grant Instrument)
- Buncher flask and all glass apparatus for filtering
- Balance
- Stainless steel spatula
- Stainless steel tweezers
- Glass petri dishes to cover filtration apparatus when not in use
- Magnetic hot place
- Magnetic stirrer

4.6. Method

4.61 Preparing Digestion Solution

1. Weigh out 30 g of anhydrous KOH pellets out into a pre-cleaned beaker

2. Dissolve the KOH in approximately 80 mL of MilliQ water, using the magnetic stirrer if necessary. Once fully dissolved, use MilliQ water to make the volume 100 mL. This makes the 30% KOH solution.
3. Carefully measure out 50 mL of NaClO solution (inside a fume cupboard) into a separate beaker.
4. Mix with 50 mL of MilliQ water and leave for 10 minutes.
5. Finally, combine the KOH and NaClO solutions, and leave to mix for 5 minutes.
6. Repeat steps if more digestion solution is required (approximately 300 mL used in a standard sample run), or recalculate the weights and volumes required before starting the procedure.

4.62 Digesting Microplastic Filters

1. Using the glass filtration apparatus (Section 3), filter the KOH:NaClO solution. Note: ensure not to use a regenerated cellulose or cellulose nitrate filter, as they will be partially dissolved by the solution
2. Take the filter membrane containing microplastics and vegetation from the extraction protocol (Section 3) and place in a clean beaker.
3. Rinse the glass funnel piece by placing it so it sits on the beaker (SOP Figure 4.1) and pour through approximately 50 mL of KOH: NaClO solution.
4. Using a small amount of MilliQ Water, rinse down any material that got stuck to the side of the beaker, ensuring it is all within the digestion solution.
5. Seal the beaker using two layers of aluminium foil.
6. Place the beakers within a shaker incubator (50 ^{o}C, 120 rpm), then leave to digest for 72 hours (SOP Figure 4.2).
7. Set up the filtration apparatus once more (use either polycarbonate or glass fibre filter membranes).
8. Upon completed digestion, filter off KOH: NaClO solution (rinse through with MilliQ water to remove all solution) before preparing to stain the new filters.

SOP Figure 4.1 - Glass funnel piece placed so that it sits within the beaker

SOP Figure 4.2 - Shaker incubator containing beakers with samples in digestion solution

4.7 Comments on Procedures and Contamination Controls

Follow contamination controls as laid out in section 1.7.

For steps that use glass beakers, use the beakers from earlier stages in the protocol (sample preparation and microplastic extraction). Clean/rinse as necessary, however reducing the amount of glassware and maintaining the same beaker for each sample will prevent any cross contamination between samples.

5. Staining SOP

5.1 Risk Assessment

PERSONS AT RISK (please X): Employees (X) Contractors ( ) Public ( ) Visitors ( ) Students (X) Others ( )	Author: Benjamin Grover	Location: Sci Faculty

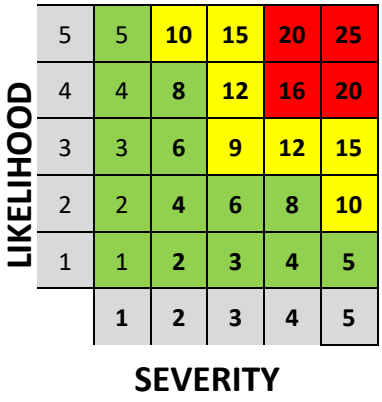
ACTIVITY/TASK/PROCEDURE:
Density Separation for extraction of Microplastics
Staining and imaging of Microplastics

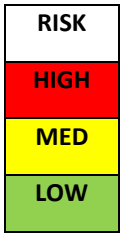
- Use of Acetone for cleaning/degreasing
- General use of solutions: (see Solution SOP document)
- Nile Red Dye
- Use of lab glassware
- Use of electrical equipment
- Vacuum Pumps

Significant Hazard	Potential Consequences of Hazard	INITIAL RISK LEVEL (H/M/L)	Control Measures Required	FINAL RISK LEVEL (H/M/L)
1/Use of Acetone				
Vapours (transfer from bottle and potential spillage)	Eye irritation and breathing problem	M (3 x 3)	Transfer from bottle to wash bottle in fume cupboard only. Transfer into beaker first, then from beaker into wash bottle.	L (1 x 3)
Vapours (when using wash bottle)	Breathing problems	L (2 x 3)	Do not use in confined unventilated space.	L (1 x 3)

Direct exposure	Skin irritation	M (3 x 3)	Wear lab coat, Eye protection (EN166) and gloves suitable for the chemicals being used. Check breakthrough times before use.	L (1 x 3)
Fire	Fatal injuries	M (3 x 5)	Do not use on or close to hot surfaces, source of ignition and flames. Restricted volumes of wash bottle and overall quantities of flammable material in laboratory. Store in ---	L (1 x 5)
2/ Creation and Use of Solutions				
Spill of chemicals	Slips resulting in injury	M (3 x 3)	Clean up spills as soon as they are identified. Using absorbent material.	L (1 x 3)
3/ Use of lab glassware				
Broken Glass	Personal Injury – Cut	L(2x3)	Broken glass should be cleaned up wearing gloves and minimising contact. Use broken glass bin for disposal	L(1x3)
4/ Use of electrical equipment				
Electrical Shock	Personal Injury	L(1x4)	Do not use electrical equipment with wet hands or material.	L(1x2)

Use the risk matrix below to score your hazard or activity for the probability ('L') likelihood harm will occur and the severity ('S') of the outcome. (6)





Plot the scores on the matrix to obtain a risk score – then use the colour of that score to determine the Risk Level.

Activities that are **High** must not start (or will need to be suspended), without appropriate controls in place to reduce the risk to an acceptable level.

Activities that are **Medium** should only be tolerated in the short term and then only whilst plans are made to introduce further controls within a defined period.

Activities that are **Low** are largely acceptable, subject to periodic review or after significant changes.

5.2 COSHH

Substance/Chemical Name (No Formula)	H Statements in Full*	Max quantity (with Units)	Exposure Limits (WEL)		SDS Used (company + date)
			Long	Short	
Nile Red		1 g			Sigma Aldrich – 25/02/2021
Propanol	H225 (highly flammable liquid and vapour), H319 (causes serious eye irritation)	2.5 L	1920 mg/m^3	5760 mg/m^3	Fisher Chemical 25/02/2021
Acetone	H225 (highly flammable liquid and vapour), H319 (causes serious eye irritation), H336 (may cause drowsiness or dizziness)	2.5 L			Fisher Chemical 23/03/2021
Ethanol (Ethyl Alcohol)	H225 (highly flammable liquid and vapour), H319 (causes serious eye irritation)	2.5 L	1920 mg/m^3	5760 mg/m^3	Fisher Chemical 25/02/2021

***H334 or H317 may require Health Surveillance. H340, H341, H350, H351, H360, H361 (CMT) and H370, H371, H372, H373 (Long term health) require record keeping.**

5.3 First Aid

Does your process require anything more than the basic first aid listed below? If **yes** please specify in other box.
Eye Contact Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required. **Skin Contact** Wash off immediately with plenty of water for at least 15 minutes. Medical attention maybe required depending on exposer. **Ingestion**

Do not induce vomiting. Immediate medical attention is required. **Inhalation** Move to fresh air. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance; induce artificial respiration with a respiratory medical device. Immediate medical attention is required. **Protection of First-aiders** Ensure that medical personnel are aware of the material(s) involved, take precautions to protect themselves and prevent spread of contamination.
Other:

5.4 Disposal/Spillage

Does your process require anything more than the standard SAF waste protocol listed below? If **yes** please specify in other box.
Any substance spilt than cannot be washed down the sink should be collected on the correct absorbent (please specify below), bagged and labelled. All substances going out for disposal must be safely bagged or sealed in an appropriate container. This item must then be clearly labelled using the waste labels provided. capjobs@uea.ac.uk must then be contacted and will arrange for the removal of the waste ready for disposal by the University contractor.
Other: Zinc chloride waste is to be collected within the zinc chloride waste containers.

5.5 Materials

5.51 Consumables

- Water, to clean utensils and equipment
- Milli Q water
- Nile Red solution. Please see solution SOP for more information. You will need ~2mL per sample.
- 0.45 µm membranes, 47 mm diameter. You will need 1 for the Nile Red solution, and 1 per sample. We recommend the polycarbonate membrane filters for staining processes but act according to your research objectives.
- Acetone (this can be used for cleaning Nile Red solution out of the filter head if needed)

5.52 Non-Consumables

- 60mm petri dishes, 1 per replicate (for storing the filter after measurement. We use polystyrene ones for samples that are being stored for a long time as it is much cheaper and lighter than the glass ones and post-staining, any potential contamination is less of an issue. If you are not keeping the filter after imaging etc a glass petri dish is sufficient to use)
- Glass filtering apparatus: Buchner flask, glass vacuum funnel, all glass filter holder, clamp. You will need one for filtering of Nile Red solution and appropriate number for the samples themselves
- Vacuum pump
- Stainless steel tweezers
- Glass beaker, to fill with Milli Q water to store stainless steel tweezers in
- Glass petri dishes to cover filtration apparatus when not in use
- Solution SOP document

5.6 Method

5.61 Staining Processes

1. Make up Nile Red following solutions SOP (section 1) or use pre-made Nile Red solution.
2. Wash and set up the glass filtration apparatus, you will need one for filtering the Nile Red solution, and then one each for each sample you wish to stain. If following from the digestion step, you can leave the filters in the apparatus after rinsing with MilliQ Water and leaving to dry.
3. Filter Nile Red solution.
4. Carefully measure out 5 mL of Nile Red solution and apply to the filter. Note: it is beneficial to not dislodge the microplastic particles from the filter as it could mean they stick to the side and not end up on the filter. A suggestion is to pipette the Nile Red solution onto the filter.
5. Leave solution on the filter for 30 minutes.

6. Filter solution under vacuum until filter is superficially “dry” (typically about 5 minutes)
7. Carefully transfer filter membrane to a clean glass petri dish with clean stainless-steel tweezers, ensuring filter is kept horizontal to keep all potential microplastics on the filter.
8. Move onto to appropriate imaging techniques (see Imaging SOP, section 6) or ATR processes.

5.7. Comments on Procedures and Contamination Controls

Follow contamination controls as laid out in section 1.7.

If after staining you plan to directly move onto Imaging or Chemical Analysis, filters can be transferred directly to the instrument in a glass petri dish. If the stamps are to be stored, they can be put in the freezer (for future analysis) or transferred to polystyrene petri dishes in analysis has been completed.

6. Imaging SOP

6.1 Risk Assessment

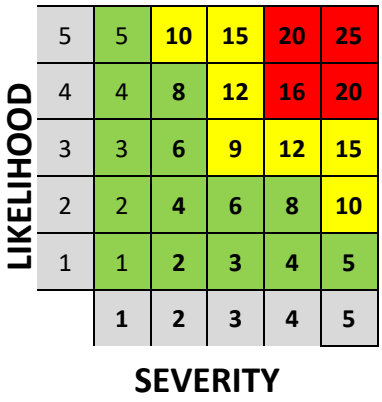
PERSONS AT RISK (please X): Employees (X) Contractors ( ) Public ( ) Visitors ( ) Students (X) Others ( )	**Author: Benjamin Grover**	**Location: Sci Faculty**

ACTIVITY/TASK/PROCEDURE:
Density Separation for extraction of Microplastics
Staining and imaging of Microplastics

- Use of lab glassware
- Use of electrical equipment
- Use of UV Crimelights

Significant Hazard	Potential Consequences of Hazard	INITIAL RISK LEVEL (H/M/L)	Control Measures Required	FINAL RISK LEVEL (H/M/L)
1/ Use of lab glassware				
Broken Glass	Personal Injury – Cut	L(2x3)	Broken glass should be cleaned up wearing gloves and minimising contact. Use broken glass bin for disposal	L(1x3)
2/ Use of electrical equipment				
Electrical Shock	Personal Injury	L(1x4)	Do not use electrical equipment with wet hands or material.	L(1x2)
3/ Use of UV Crimelights				
UV exposure	Eye Damage	L (2x3)	Wear Orange Filter Goggles	L (1x3)

Use the risk matrix below to score your hazard or activity for the probability ('L') likelihood harm will occur and the severity ('S') of the outcome. (6)



RISK
HIGH
MED
LOW

Plot the scores on the matrix to obtain a risk score – then use the colour of that score to determine the Risk Level.

Activities that are **High** must not start (or will need to be suspended), without appropriate controls in place to reduce the risk to an acceptable level.

Activities that are **Medium** should only be tolerated in the short term and then only whilst plans are made to introduce further controls within a defined period.

Activities that are **Low** are largely acceptable, subject to periodic review or after significant changes.

6.2 COSHH

Substance/Chemical Name (No Formula)	H Statements in Full*	Max quantity (with Units)	Exposure Limits (WEL)		SDS Used (company + date)
			Long	Short	
N/A					

***H334 or H317 may require Health Surveillance. H340, H341, H350, H351, H360, H361 (CMT) and H370, H371, H372, H373 (Long term health) require record keeping.**

6.3 First Aid

Does your process require anything more than the basic first aid listed below? If **yes** please specify in other box.
Eye Contact Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required. **Skin Contact** Wash off immediately with plenty of water for at least 15 minutes. Medical attention maybe required depending on exposer.

Ingestion Do not induce vomiting. Immediate medical attention is required. **Inhalation** Move to fresh air. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance; induce artificial respiration with a respiratory medical device. Immediate medical attention is required. **Protection of First-aiders** Ensure that medical personnel are aware of the material(s) involved, take precautions to protect themselves and prevent spread of contamination.
Other:

6.4 Disposal/Spillage

Does your process require anything more than the standard SAF waste protocol listed below? If **yes** please specify in other box.
Any substance spilt than cannot be washed down the sink should be collected on the correct absorbent (please specify below), bagged and labelled. All substances going out for disposal must be safely bagged or sealed in an appropriate container . This item must then be clearly labelled using the waste labels provided. capjobs@uea.ac.uk must then be contacted and will arrange for the removal of the waste ready for disposal by the University contractor.
Other:

6.5 Materials

6.51 Consumables

- Water, to clean utensils and equipment
- Milli Q water
- Paper towels

6.52 Non-Consumables

- Stainless steel tweezers
- Glass beaker, to fill with Milli Q water to store stainless steel tweezers in
- UV Crime-Lite (420-470 nm Blue)
- Imaging Rig (consisting of programmable, movable electronics rig, and Canon EOS 6000 with Hoya 55 mm Orange lens

- Computer with EOS Utility Software (connected to camera)
- Computer with Mach 3 CNC software (connected to rig)
- Computer with ImageJ software

6.6 Methods

6.61 Imaging the Filter

1. Using MilliQ water, rinse the filter platform on the rig, as well as the detachable weight.
2. Turn on the Camera and start up the rig control program (Mach 3 CNC), and the camera program (EOS Utility).
3. Load the imaging script into Mach 3 CNC.
4. In EOS Utility, check the camera settings (Shutter Speed 1/20, Aperture F5.6, Iso:800) and pre-load a file to save the images to. Note it is recommended to have separate subfiles for each filter that is being images, so create a new folder each time.
5. Place the filter membrane in the holder on the rigs frame.
6. Turn off the lights and turn on the Crime-Light.
7. In EOS Utility open the Camera feed, and using the controls in Mach 3 CNC, move the camera until it is positioned over a fluorescent particle. Then, by altering the z axis of the rig, adjust the camera height until the particle is focus.
8. In Mach 3 CNC, selected “Rewind to start” and then Click Run.
9. The Rig will then follow the pre-programmed route, taking 24 stops in a 4x6 block.
10. Using the controls in EOS utility, manually take a picture as the rig stops at each point. Note: it is best to wait one second after the rig stops moving so that there is no motion blur in the image. The rig will remain stopped for a couple of seconds before moving onto the next point.
11. Once the rig has completed the route, open the images in the premade folder which they saved too. Enlarge the images until they form the 4x6 grid and ensure that the entire filter has been imaged.
12. Once the run is complete, remove the filter membrane, and repeat steps 1-11 for the next sample.

6.62 Stitching and Analysing the Images

1. Transfer the images from the Camera to a USB/separate folder.
2. Ensure images are saved on the computer which you will use for stitching.
3. Open up the software ImageJ.
4. Select “File – Import – Image Sequence”, then select the folder in which you saved the images you wish to stitch. Ensure all images are selected, then click import.
5. Once all the images have been loaded, select “Image – Stacks – Make Montage” and then in the pop-up box, ensure the montage is in a 4x6 grid. Then select run.
6. When finished this has created a stitched montage of all 24 images into one, showing the filter as a single image. Save this image alongside the 24 individual images.
7. To count any fluorescent particles on the filter, first select “Image – Type – 8 Bit” which should convert the image to greyscale.
8. Once the image is greyscale, select “Image – Adjust – Threshold”. The image should now be mostly red with a threshold of 0. As you increase the threshold, the darker background disappears leaving only brighter particles (shown in red). Once a suitable threshold is reached, select apply. Note: when determining the correct threshold, it is good to have the stitched filter image pulled up for comparison. Increase the threshold until the red dots only match that of the fluorescent particles on the filter. A good indicator can be brighter patches of the edge of the filter, the threshold must be high enough to remove these patches. Whilst the brightness of each filter will vary and thus have their own unique thresholds, a threshold in the range of 80 – 120 is normally effective for particles stained and imaged by following this SOP.
9. Finally, select “Analyse – Analyse particles”. In the pop-up window, ensure that the minimum particle size is set to 9, to remove any chance of counting random bright spots from the lens. Then select run. The resulting output should be a notepad with the number of particles, as well as information on their dimensions, area etc. Save this alongside the images, and then repeat steps 1 – 9 for the next set of images. Note: During the pop-up window it is possible to highlight/outline the particles, as well as change the information and counting process. These are not necessary but could be useful depending on the information you are trying to extract from the filter.

6.7. Comments on Procedures and Contamination Controls

Follow contamination controls as laid out in section 1.7.

To ensure minimum contamination whilst the filter is exposed during imaging, it is recommended to ensure all the programs are set up beforehand so that once the filter is placed onto the rig, imaging can begin immediately. Furthermore, if imaging is planned for that day, it is recommended to use the mini-hoover in order to clean the workspace on and around the imaging rig.

7. Chemical Confirmation of Microplastics – Infrared Spectrometry

7.2 Risk Assessment

PERSONS AT RISK (please X): Employees (X) Contractors ( ) Public ( ) Visitors ( ) Students (X) Others ( )	**Author: Benjamin Grover**	**Location: Sci Faculty**

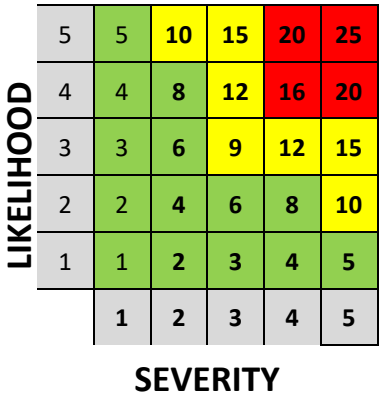
ACTIVITY/TASK/PROCEDURE:
Density Separation for extraction of Microplastics
Staining and imaging of Microplastics

- Use of lab glassware
- Use of electrical equipment
- Use of UV light
- Handling Liquid Nitrogen

Significant Hazard	Potential Consequences of Hazard	INITIAL RISK LEVEL (H/M/L)	Control Measures Required	FINAL RISK LEVEL (H/M/L)
1/ Use of lab glassware				
Broken Glass	Personal Injury – Cut	L(2x3)	Broken glass should be cleaned up wearing gloves and minimising contact. Use broken glass bin for disposal	L(1x3)
2/ Use of electrical equipment				
Electrical Shock	Personal Injury	L(1x4)	Do not use electrical equipment with wet hands or material.	L(1x2)
3/ Use of UV Crimelights				
UV exposure	Eye Damage	L (2x3)	Wear Orange Filter Goggles	L (1x3)

4/Handling Liquid Nitrogen				
Skin contact /Inhalation	Frostbite	L (2x4)	Ensure user is properly trained in handling Liquid Nitrogen. Use of goggles and heavy gloves recommended.	L (1x4)

Use the risk matrix below to score your hazard or activity for the probability ('L') likelihood harm will occur and the severity ('S') of the outcome. (6)



RISK
HIGH
MED
LOW

Plot the scores on the matrix to obtain a risk score – then use the colour of that score to determine the Risk Level.

Activities that are **High** must not start (or will need to be suspended), without appropriate controls in place to reduce the risk to an acceptable level.

Activities that are **Medium** should only be tolerated in the short term and then only whilst plans are made to introduce further controls within a defined period.

Activities that are **Low** are largely acceptable, subject to periodic review or after significant changes.

7.2 COSHH

Substance/Chemical Name (No Formula)	H Statements in Full*	Max quantity (with Units)	Exposure Limits (WEL)		SDS Used (company + date)
			Long	Short	
N_2	H281(contains refrigerated gas; may cause cryogenic burns or injury)	1 L	N/A	N/A	Fisher Chemical 26/07/2024
Ethanol (Ethyl Alcohol)	H225 (highly flammable liquid and vapour), H319 (causes	2.5 L	1920 mg/m^3	5760 mg/m^3	Fisher Chemical 25/02/2021

	serious eye irritation)				

***H334 or H317 may require Health Surveillance. H340, H341, H350, H351, H360, H361 (CMT) and H370, H371, H372, H373 (Long term health) require record keeping.**

7.3 First Aid

Does your process require anything more than the basic first aid listed below? If **yes** please specify in other box.
Eye Contact Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Immediate medical attention is required. **Skin Contact** Wash off immediately with plenty of water for at least 15 minutes. Medical attention maybe required depending on exposer. **Ingestion** Do not induce vomiting. Immediate medical attention is required. **Inhalation** Move to fresh air. If breathing is difficult, give oxygen. Do not use mouth-to-mouth resuscitation if victim ingested or inhaled the substance; induce artificial respiration with a respiratory medical device. Immediate medical attention is required. **Protection of First-aiders** Ensure that medical personnel are aware of the material(s) involved, take precautions to protect themselves and prevent spread of contamination.
Other:

7.4 Disposal/Spillage

Does your process require anything more than the standard SAF waste protocol listed below? If **yes** please specify in other box.
Any substance spilt than cannot be washed down the sink should be collected on the correct absorbent (please specify below), bagged and labelled. All substances going out for disposal must be safely bagged or sealed in an appropriate container. This item must then be clearly labelled using the waste labels provided. capjobs@uea.ac.uk must then be contacted and will arrange for the removal of the waste ready for disposal by the University contractor.
Other:

7.5 Materials

7.51 Consumables

- Ethanol, to clean utensils and equipment
- Milli Q water
- Paper towels
- Liquid Nitrogen
- Cotton buds

7.52 Non-Consumables

- Stainless steel tweezers
- Glass beaker, to fill with Milli Q water to store stainless steel tweezers in
- Infrared Spectrometer (Hyperion 2000 with micro-ATR crystal, Bruker)
- Mercury Lamp
- Computer with OPUS software

7.6 Methods

7.61 Preparing the Instrument

1. Begin by switching on the Mercury Lamp and pressing the ignite switch so that the light comes on (SOP Figure 7.1A). Allow 5 minutes for the lamp to warm up.
2. Check the cooling light on the bottom of the spectrometer (SOP Figure 7.1B). If turned on liquid nitrogen is required.
3. Insert the funnel into the top of the spectrometer (SOP Figure 7.1C), and then carefully pour in liquid nitrogen. Continue to pour in small increments until the light on the spectrometer turns off. Note: spillage is very common during this step, however there is no need to clean up as the liquid nitrogen evaporates almost instantly.
4. Once the spectrometer is cooled, open OPUS 7.8 on the computer and selected the correct apparatus in the pop-up box.

SOP Figure 7.1 - Showing; (left) **A** - Mercury lamp (turned off), (mid) **B** – Spectrometer cooling light (on so liquid nitrogen required), (right) **C** – Liquid Nitrogen funnel inserted into the top of the spectrometer

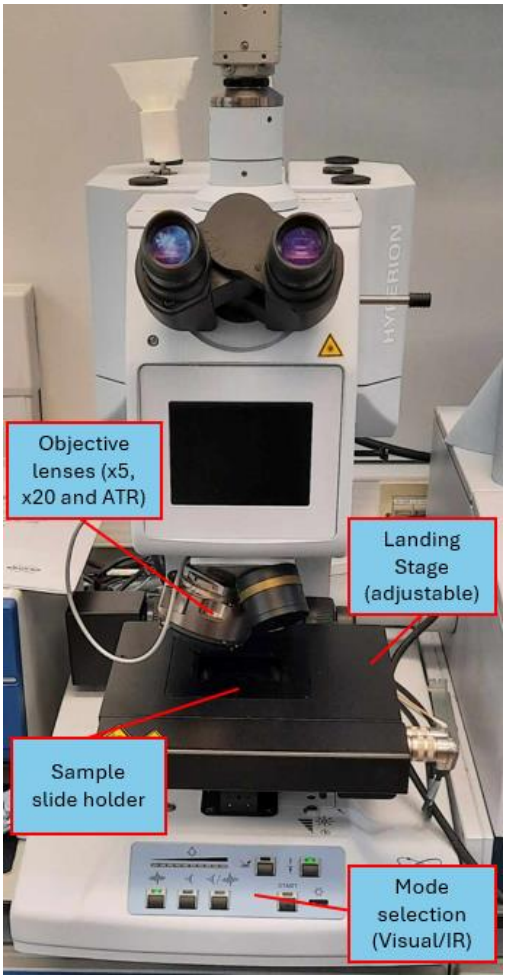
7.62 Analysing a Sample

1. Apply some ethanol to a cotton bud, and then gently clean the tip of the ATR objective lens.
2. In OPUS, under the headings, select “Measure – Set up measurement parameters” and then in the new window load the file “Pike_Miracle”.
3. In this window you can also adjust where to save the measurements, expected number of scans (this can also be done later) and how you would like the spectra to appear.
4. On the instrument, take your sample and place it on the provided sample plate. This then slots into the space on the Landing stage (SOP Figure 7.2). Note: there are two sampling plate, one plain and one with internal reference squares. If using a filter, it is recommended to tape down the edge of the filter so that it doesn’t move during analysis.
5. Ensure that the microscopes objective lens is set to Visual (5x), and that the landing moves in response to the joystick controller.
6. In OPUS, select “Measure – Start Video Wizard” and in the pop-up box “Select Device” select the Hyperion 2000 ATR.
7. In the next window, collect an image of the sample.
8. To find a particle, use the joystick to move the landing stage around, whilst observing the image of the sample on the computer screen. Once a suitable particle has been

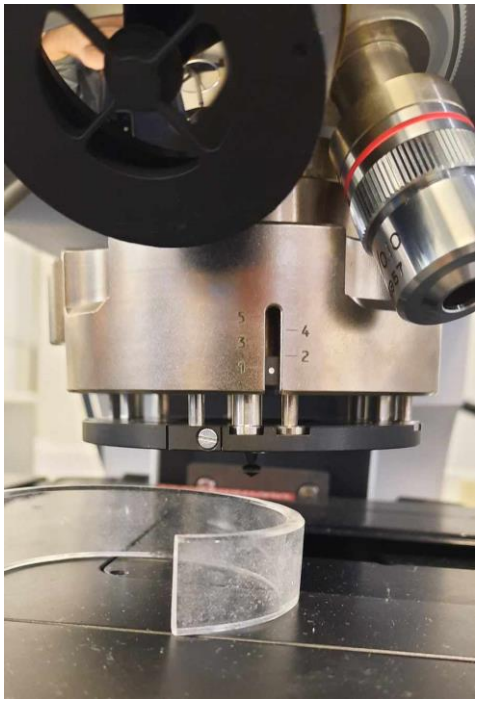
found, on the instrument swap to the 20x lens, and then refocus using the manual landing stage adjuster on the side of the instrument. Once in focus, the landing stage may need to be moved horizontally to find the particle again. Repeat this process but swapping to the ATR lens. Ensure on the computer the lens is also set to ATR, and then if the particle is satisfactory, select “Single Image”. Note: it can often be easier to find particles using fluorescence. First ensure all lights in the laboratory are turned off. Then, pull the lever (marked fluorescence) on box at the back of the spectrometer to select blue light for the sample. In OPUS, select “Direct Command Entry” and then enter the command “mot56=14” to turn on the UV filter. Fluorescent particles should now appear as bright orange against the black background of the filter, making them much easier to find at each stage of the process. Once a particle has been found, the lever ca be pushed back in, and the direct command repeated to return to normal visual light.

9. At this stage you can save the image of the picture and alter other settings according to your preference. Once the image contained the particle you wish to test, select Next.
10. The next step to measure a background spectrum. If measuring a single particle, in the new window select “Measure background once”. If measuring multiple particles, or several times select “Measure background after each sample measurement”.
11. To run a background spectrum, the background position must be set. In the next window, if using the reference plate select “Internal reference”. Otherwise select “User defined background position”, and on the previously taken image of your sample, select a point of the background to run. Ensure that the mode on the spectrometer is set to ATR, and that the distance ring has been placed around your sample (SOP Figure 7.3). Then select “Measure background spectrum”. Note: where you select for the background on your image doesn’t really matter, as the distance ring will prevent the ATR from touching your sample regardless. Furthermore, when inserting the distance ring, it is fine if the stage needs to be manually wound down, as it will automatically raise itself during the spectral acquisition.
12. Follow the instructions of the pop-up box and lower the outer cylinder on the ATR lens. To do so, push the lever on the side and then drop the cylinder down until it clicks into the “1” position. Then select Run.
13. The spectrometer will then automatically run the background, taking about 1-2 minutes.

14. Once the background has been collected, in the final window you can set the parameters for running the spectra of the particle.
15. First, on the previously taken image, select a point/points in which the spectra will be taken from (Note: it is recommended to be near the centre of the particle if possible). From this window you can also select the resolution of the spectra, as well as the number of scans you want to be run. Finally, ensure that the spectra destination is saved in the folder of your choosing (Note: this must be done every sample, it will not automatically save in the previous location). Ensure that the distance ring has been removed from the landing stage, and then select “Run Spectra”.
16. From the pop-up box, ensure that the ATR lens is once again locked in the 1 position, then select Okay. The instrument will then obtain the spectra by pressing the ATR tip into the particle. This process will take 2-3 minutes, or longer if multiple points were selected for spectra (an estimate is given in OPUS).
17. Once completed, OPUS will automatically open the spectral output in another window. From here it can be corrected using various tools, as well as peaks outlined or compared to a library. Ensure that the spectrum is saved before closing this window.
18. If running other particles, repeat steps 1 – 17 to the next filter/particle.
19. Once analysis has been completed, first wipe the ATR tip with ethanol and a cotton bud once more. Then, ensure the mercury lamp is turned off, and close the OPUS software.



SOP Figure 7.2 - Hyperion 2000 ATR spectrometer with components labelled



SOP Figure 7.3 - Landing Stage, showing Distancing Ring applied and ATR lens in the 1 position

7.63 Analysing Spectra

Once a spectrum has been obtained, it is compared with Bruker's built in library in OPUS, as well as an online library, OpenSpecy.

1. In OPUS, load the spectra you wish to analyse.
2. From the icons in the top bar, select "Spectrum Search".
3. In the new window, fill in the wavenumber range you wish to search (normally 4000 – 300), the select search library.
4. Several spectra will then load alongside your original, with information such as material name and hit quality given in a box below. These spectra can be sorted, and by comparing with your original spectra, help to identify your particles. Note: The hit quality uses various factors in its calculation, including peak wavenumber, peak height and the area under the peaks. This can often be very different between your sample and the reference library spectra's, therefore whilst hit quality can be an effective guideline, we recommend focussing more on the key peaks of functional groups to determine whether your sample matches a reference or not.
5. To analyse your spectra using OpenSpecy, first save your spectra in OPUS as a .csv file (if using an earlier version of OPUS, you may need to save normally and then use additional software to convert the file to .csv).
6. Load a webpage and search for Open Specy.
7. When in Open Specy, click the Analyse Spectra tab on the left-hand side of the webpage.
8. Upload your spectra by either by selecting "browse" and then selecting your file or dragging and dropping your file into the box on the webpage.
9. Select the Identification bar at the top of the window and wait for the spectra to be analysed.
10. After about 30 seconds, the webpage will load a list of possible matches in a table beneath your spectra, showing data such as the material, hit quality and where the spectrum came from. Selecting a material from the box loads the spectra beneath your loaded one for comparison. Images can also be saved of your spectra, as well with

comparison to the libraries references. Note: OpenSpecy has the same issue when calculating hit quality, however it has a much larger library of references. You may therefore see several different versions of the same material, each with slightly different hit qualities due to how the peak areas are measured. This makes comparing your sample to various spectra of the same material much more efficient, allowing for more confident identification.

11. To analyse another spectra, simply load another .csv file as done in step 8, and repeat the process.