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**The spectroscopic detection and bacterial  
colonisation of synthetic microplastics in  
coastal marine sediments**

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## Abstract

Synthetic microplastics ( $\leq 5$ -mm fragments) are anthropogenic contaminants that are rapidly accumulating in coastal environments worldwide. The distribution, abundance, ecological impacts and fate of these pollutants are poorly understood. In this study, a novel reflectance micro-FT-IR spectroscopy method was developed to detect microplastics in sediments from 17 sites (UK), using polyethylene (PE) as the model polymer. Additionally, a 14-day laboratory microcosm experiment was used to characterise bacterial succession on low-density polyethylene (LDPE) fragments over time and across three sediment types (ranging from sand to silt) from the Humber Estuary, UK. In contrast with ATR-FT-IR measurements, micro-FT-IR measurements in reflectance mode were susceptible to refractive error when analysing irregularly shaped PE fragments. However, molecular mapping by reflectance micro-FT-IR spectroscopy successfully detected PE within polymer-spiked sediments and in a non-spiked sediment retentate. Moreover, reflectance micro-FT-IR spectra of PE were consistent across all 17 sampling sites. Bacteria were found to rapidly colonise LDPE fragments, with bacterial community structure and diversity differing significantly from those in bulk sediments, as demonstrated by scanning electron microscopy, T-RFLP analysis and 16S rRNA gene sequencing. The composition of LDPE-colonising assemblages within different sediment types increasingly converged over time, with 16S rRNA gene sequencing analysis identifying site-specific populations of the genera *Arcobacter* (Epsilonproteobacteria) and *Colwellia* (Gammaproteobacteria) as dominant members (up to 93% of sequences) of the plastic-associated communities after 14 days of exposure. Log-fold increases in the relative abundance of LDPE-associated bacteria occurred within 7 days of exposure with bacterial abundance differing significantly across sediment types, as shown by Q-PCR amplification of 16S rRNA genes.

Attachment of bacterial cells and specifically of *Colwellia* spp. onto LDPE surfaces was demonstrated by CARD-FISH analysis. These results provide a foundation to both developing improved spectroscopy methods to detect microplastics, and characterising ecological interactions between microorganisms and microplastic debris within marine sediments.

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It is a pleasure to dedicate this work to my family and all those who are closest to me. I am fortunate to have been surrounded by exceptional friends during my time in Sheffield and would like to extend my gratitude to each of them.

## List of Abbreviations

°C	degrees Celsius
μ	micro (10 <sup>-6</sup> )
%	per cent
ANOSIM	analysis of similarity
ANOVA	analysis of variance
ATR	attenuated total reflectance
ASW	artificial seawater
au	arbitrary units
bp	base pair
CARD-FISH	catalysed reporter deposition <i>in situ</i> fluorescence hybridisation
c (as prefix)	centi (10 <sup>-2</sup> )
C <sub>t</sub>	cycle threshold value
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
<i>E</i>	amplification efficiency
EPS	extracellular polymeric substances
<i>et al.</i>	“and others”
FPA	focal plane array
FT-IR	Fourier-transform infrared
g	gramme
<i>g</i> (in italics)	gravitational acceleration
<i>H'</i>	Shannon's index of diversity
Hz	hertz
k (as prefix)	kilo (10 <sup>3</sup> )
l	litre
LB	Luria-Bertani
LDPE	low-density polyethylene
LOI	loss on ignition

m	metre
m (as prefix)	milli ( $10^{-3}$ )
M	molar
n (as prefix)	nano ( $10^{-9}$ )
<i>n</i> (in italics)	number of replicates
NA	numerical aperture
nMDS	non-metric multidimensional scaling
nt	nucleotides
OC	organic carbon
OTU	operational taxonomic unit
<i>p</i>	<i>p</i> -value (statistical significance)
PCR / Q-PCR	polymerase chain reaction / quantitative polymerase chain reaction
pH	measure of the acidity or basicity of a solution
ppm	parts per million
PSD	particle size distribution
$r^2$	coefficient of determination
rRNA	ribosomal ribonucleic acid
SD	standard deviation
SE	standard error
SIP	stable isotope probing
T-RFLP	terminal-restriction fragment length polymorphism
U	unit
UHMW PE	ultra-high molecular weight polyethylene
UK	United Kingdom
V	volt
v / v	volume / volume
w / v	weight / volume
X-Gal	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside
$\nu$	stretching
$\delta$	bending
$\delta_s$	scissoring

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# Chapter 1

## Introduction

Aspects of this introduction have been published as:

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Interactions between microorganisms and marine microplastics:

a call for research. *Marine Technology Society Journal* **45**: 12-20.



## 1.1. Background

We live in the Plastic Age, with industrialised nations now reliant on synthetic polymers in most aspects of our lives. The worldwide demand for plastics is estimated to have increased annually by 10% since the 1950s, with their total mass of production reaching 245 million tonnes in 2006 (PlasticsEurope, 2008; Andrady and Neal, 2009). As a consequence of this 600-fold increase in predicted consumption during the past 60 years, synthetic thermoplastics (*e.g.* polyethylene) now comprise the most abundant and rapidly growing component of anthropogenic debris entering the Earth's oceans (Derraik, 2002; Moore, 2008; Barnes *et al.*, 2009; Law *et al.*, 2010). The increasing significance of this debris as a descriptor of the ecological integrity of marine ecosystems is recognised in environmental treaties across the globe, including the multilateral European Marine Strategy Framework Directive (Cheshire *et al.*, 2009; Galgani *et al.*, 2010; GESAMP, 2010a, 2010b).

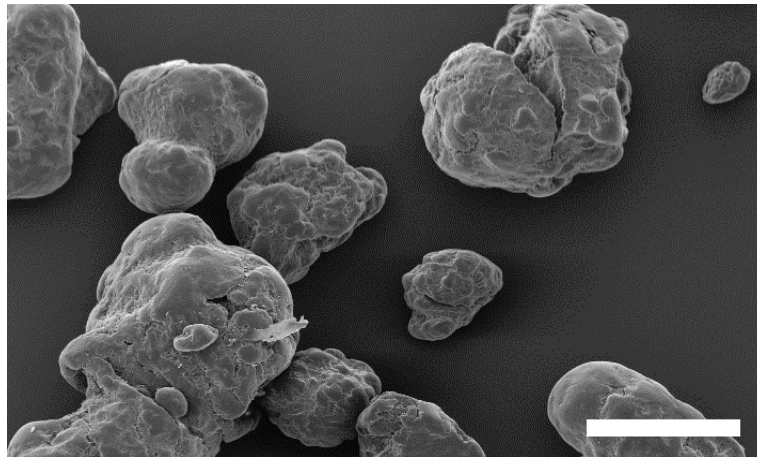
Plastic waste is globally distributed across both surface waters and sediments within the marine environment, reflecting the widespread use of polymer products and their ability to resist physical and biological degradation for centuries (Galgani *et al.*, 2000; Moore *et al.*, 2001; Katsanevakis and Katsarou, 2004; Sudhakar *et al.*, 2007b; Andrady and Neal, 2009). The environmental fate of this waste is controlled by both human activities and hydrogeological factors (*e.g.* littering, accidental disposal and oceanic circulation), with an excess of 200,000 plastic fragments having been discovered within a square kilometre of water in the North Atlantic Subtropical Gyre (Galgani *et al.*, 2000; Katsanevakis and Katsarou, 2004; Morishige *et al.*, 2007; Law *et al.*, 2010; Browne *et al.*, 2011). Since the majority of synthetic polymers sink in seawater, sediments function as sinks for the accumulation of plastic debris (Thompson *et al.*, 2004; Reddy *et al.*, 2006; Moore, 2008; Barnes *et al.*, 2009; Browne *et al.*, 2011;

Claessens *et al.*, 2011; Cole *et al.*, 2011; Hidalgo-Ruz *et al.*, 2012). For example, up to 47.4 kilogrammes per square kilometre of anthropogenic debris have been discovered in the Eastern Mediterranean seabed, over half of which was comprised of plastic (Koutsodendris *et al.*, 2008). In comparison, 5.1 kilogrammes per square kilometre of floating plastics have been described in the North Pacific Central Gyre (Moore *et al.*, 2001; Ryan *et al.*, 2009).

The ubiquity and persistence of synthetic polymers are promoting global public concern about the impacts of plastic pollution on marine wildlife. These impacts are most apparent when considering the risks of entanglement in and ingestion of readily visible (> 5-mm) fragments of plastic by higher organisms, such as birds and fish (Laist, 1987; Derraik, 2002; Moore, 2008; Gregory, 2009). Other impacts of plastic waste on marine animals include the transport of invasive species and alterations in the structure of microbial communities in the seabed (Barnes, 2002; Katsanevakis *et al.*, 2007; Gregory, 2009). For example, both the numerical abundance and species diversity of benthic epifauna have been shown to increase in the presence of marine litter, with potential long-term impacts upon intra- and interspecific interactions between different organisms residing on the seafloor (Katsanevakis *et al.*, 2007).

Within the last decade, increasing attention has been directed towards the proliferation and potential environmental impacts of microplastics (defined as  $\leq 5$ -mm fragments) in marine ecosystems (Figure 1.1) (Thompson *et al.*, 2004; Barnes *et al.*, 2009; NOAA, 2009; Andrady, 2011; Claessens *et al.*, 2011; Hidalgo-Ruz *et al.*, 2012). However, we only possess an elementary understanding of the distribution, abundance and fate of microplastics in the marine environment, partially due to the difficulty of detecting and quantifying these pollutants (NOAA, 2009; GESAMP, 2010b; Cole *et al.*,

2011; Hidalgo-Ruz *et al.*, 2012). The ecological interactions of microplastic debris with marine organisms are also poorly understood (Barnes *et al.*, 2009). For example, although microorganisms (bacteria, archaea and microbial eukaryotes) mediate functions that sustain life in our oceans and could facilitate the breakdown of microplastic-associated chemical compounds or even of the debris itself, little research has been directed towards assessing the interactions between microplastics and microbial communities in marine ecosystems.



**Figure 1.1.** Scanning electron micrograph of polyethylene microplastics. The scale bar is 1 mm.

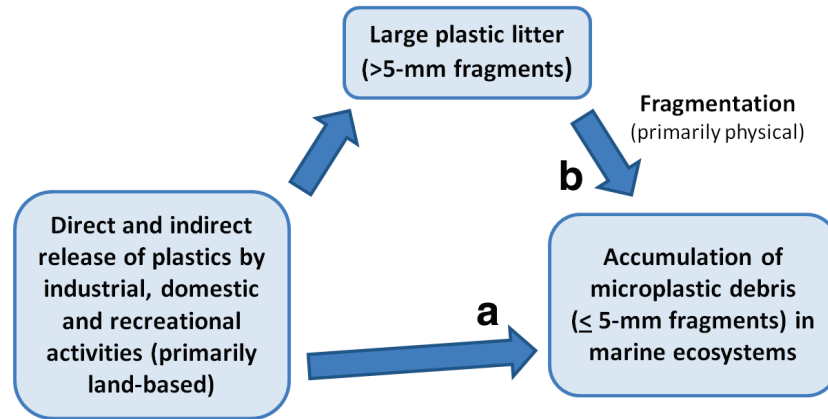
Herein, our knowledge concerning the distribution and detection of microplastics in marine ecosystems, and the potential interactions of these pollutants with marine organisms are discussed in order to identify avenues for novel research. Our understanding concerning methods for the detection, identification and enumeration of microplastics is evaluated. Subsequently, an appraisal of existing research into the ecological impacts of microplastics on higher organisms is provided. Following a brief overview of the role of microorganisms as drivers of the functioning of marine ecosystems, research into microbial-plastic interactions and the fate of marine plastic litter is explored with the aim of identifying key opportunities for future investigation.

Given the role of the sediment environment as a sink for plastic pollution (Thompson *et al.*, 2004; Reddy *et al.*, 2006; Moore, 2008; Barnes *et al.*, 2009; Browne *et al.*, 2011; Claessens *et al.*, 2011), emphasis is given to methods for the detection of microplastics and to investigate microbial-plastic interactions within benthic marine habitats.

## **1.2. Microplastic Pollution in Marine Ecosystems**

### **1.2.1. Sources, distribution and abundance of microplastics**

Although the accumulation of small fragments of plastics in the marine environment has been recognized since the 1970s, research into their sources, distribution and abundance has only gained momentum during the last decade, following the identification of microplastics as a distinct category of anthropogenic debris (Carpenter *et al.*, 1972; Carpenter and Smith, 1972; Colton *et al.*, 1974; Morris and Hamilton, 1974; Thompson *et al.*, 2004; NOAA, 2009; Cole *et al.*, 2011). Since then, it has become evident that microplastics are entering marine habitats at a global scale and particularly in the Northern Hemisphere, either as components of industrial and domestic waste or as a result of the photo-oxidative, hydrolytic and mechanical breakdown of larger plastics (Figure 1.2) (Thompson *et al.*, 2004; Koutny *et al.*, 2006; Ng and Obbard, 2006; Browne *et al.*, 2007; Moore, 2008; Barnes *et al.*, 2009; Corcoran *et al.*, 2009; Fendall and Sewell, 2009; Andrady, 2011; Browne *et al.*, 2011; Cole *et al.*, 2011). For example, sewage contaminated with synthetic polymer fibres created as a by-product of washing clothes and use of exfoliating cleansers has been identified as a major source of microplastics in marine habitats (Fendall and Sewell, 2009; Browne *et al.*, 2011; Cole *et al.*, 2011). Wind-blown plastics from landfills may also become transported into the marine environment (Barnes *et al.*, 2009). Other sources of marine microplastics include the manufacture of plastic pellets for industry, commercial and recreational fishing, aquaculture and coastal tourism, among other activities (Cole *et al.*, 2011).



**Figure 1.2.** Schematic diagram of the sources and transport of synthetic microplastics into the marine environment. Microplastics may be transported into marine habitats either a) directly (termed primary microplastics) or b) indirectly due to fragmentation of larger plastic fragments (secondary microplastics) (Cole *et al.*, 2011). For a detailed discussion concerning the sources, transport, distribution and abundance of microplastics in marine ecosystems, see Ryan *et al.* (2009), Cole *et al.* (2011) and Hidalgo-Ruz *et al.* (2012).

Microplastics are likely to constitute the numerically most abundant type of plastic debris in marine ecosystems, particularly in coastal environments where these pollutants may represent as much as 80% of plastic litter (Browne *et al.*, 2007; Barnes *et al.*, 2009; Corcoran *et al.*, 2009; Browne *et al.*, 2010; Browne *et al.*, 2011; Claessens *et al.*, 2011). For example, at least two billion microscopic fragments of plastic have been estimated to have entered Californian coastal waters over three days, merely via two rivers (Moore *et al.*, 2005). Near a polyethylene production site in Sweden, an excess of 100,000 microplastic fragments has been recorded within a cubic metre of seawater (OSPAR, 2009). Moreover, at an intertidal site near a ship-wrecking yard in India, microplastics have been discovered in the sediment at a concentration of 81 parts per million by mass (Reddy *et al.*, 2006). Although sediments and seawater from other study sites across the globe have typically been found to contain comparatively lower concentrations of microplastics (see Claessens *et al.*, 2011), the abundance of these

pollutants in the marine environment is expected to increase in the future as a result of increased consumption of plastic products and population growth (Thompson *et al.*, 2004; Barnes *et al.*, 2009; Browne *et al.*, 2011; Claessens *et al.*, 2011). Indeed, significant increases in the number of microplastic fragments over the last few decades have already been demonstrated both within marine sediments and seawater (Thompson *et al.*, 2004; Barnes *et al.*, 2009; Claessens *et al.*, 2011). Additionally, whilst the highest abundances of microplastics have frequently been reported within densely populated areas and in proximity to land-based human activities (Barnes *et al.*, 2009; Browne *et al.*, 2011; Cole *et al.*, 2011), it is probable that future research will detect increasing concentrations of microplastics in geographically remote locations, such as Arctic and Antarctic environments (Derraik, 2002; Moore, 2008; Barnes *et al.*, 2010; Zarfl and Matthies, 2010).

### **1.2.2. Methods for the detection of microplastics**

Currently, there are no standardised protocols for surveying, quantifying and monitoring microplastics in natural ecosystems (NOAA, 2009; Ryan *et al.*, 2009; GESAMP, 2010b). Previously described methods for enumerating microplastics in marine waters and sediments have typically involved separation of putative polymer fragments from other materials by flotation in saline solution, followed by filtration and/or sieving of the fragments, and their visual separation and examination by light microscopy (reviewed by Hidalgo-Ruz *et al.*, 2012; see Thompson *et al.*, 2004; Ng and Obbard, 2006; Reddy *et al.*, 2006; Corcoran *et al.*, 2009; Andrady, 2011; Claessens *et al.*, 2011). Due to difficulties associated with the efficient separation of microplastics from seawater and sediments in particular, only limited confidence can be placed on present estimates of the extent of microplastic pollution in the marine environment (Ryan *et al.*, 2009; GESAMP, 2010b; Claessens *et al.*, 2011; Hidalgo-Ruz *et al.*, 2012). Additionally,

microplastics frequently resemble sediment particles and other non-polymeric materials in appearance, and their characterisation by visual means is both labour-intensive and prone to error (GESAMP, 2010b). As such, spectroscopic techniques and specifically micro-Fourier-transform infrared (micro-FT-IR) spectroscopy (Skoog *et al.*, 2007) have been used to detect microplastics in marine habitats by distinguishing the molecular structure of different plastic types from other materials (Hidalgo-Ruz *et al.*, 2012).

Infrared spectroscopy is a well-established technique to identify chemical functional groups in a molecule based on their vibration modes at different infrared frequencies (Skoog *et al.*, 2007). The functional groups of synthetic plastics have been elucidated in detail and there are extensive databases for the identification of unknown polymers. Therefore, the identity of putative plastics in environmental samples may be determined by comparing the absorbance or transmission spectra of the analysed materials with those in a standard spectral library (Ng and Obbard, 2006; Browne *et al.*, 2010; Browne *et al.*, 2011). The two main FT-IR operating modes are transmittance and reflectance (Skoog *et al.*, 2007). Transmittance analyses are likely to be of limited suitability for identifying microplastics because the sample must be either transparent or sufficiently thin to transmit the infrared beam. Reflectance measurements are particularly useful for characterising solid samples that cannot be analysed in transmission mode (*e.g.* Ojeda *et al.*, 2009). A separate reflective method is known as attenuated total reflectance FT-IR (ATR-FT-IR) spectroscopy (Skoog *et al.*, 2007). In contrast with other FT-IR methods, in ATR-FT-IR spectroscopy the infrared beam is directed onto a highly refractive crystal in contact with the sample surface, enabling measurements with high signal-to-noise ratios and minimal spectral distortion even in the presence of irregularly shaped sample surfaces (Zhongsheng *et al.*, 2007; Davis *et al.*, 2010). All of the above techniques are compatible with microscopy (termed micro-

FT-IR spectroscopy). Additionally, micro-FT-IR analyses may be used to visualise the spatial distribution of different functional groups within a sample, either by molecular mapping approaches or focal plane array (FPA)-based imaging (described in Levin and Bhargava, 2005). Briefly, mapping involves the spectral visualisation of a given area based on sequential measurements collected at spatially separated, user-defined point intervals. In contrast, FPA-based imaging enables rapid visualisation of an entire field of view based on simultaneous collection of spectra from the sample surface (Levin and Bhargava, 2005).

Whilst the need for improved methods for separating microplastics from seawater and sediments is widely recognised (NOAA, 2009; GESAMP, 2010b), the development of improved spectroscopy techniques for identifying these pollutants has received little attention. For example, although micro-FT-IR spectroscopy is regarded as the most reliable method for detecting microplastics in marine ecosystems (Barnes *et al.*, 2009; Hidalgo-Ruz *et al.*, 2012), the suitability of different FT-IR techniques to identify these pollutants has not been assessed. Moreover, there has been no research into the spectral visualisation of microplastics. To date, conventional micro-FT-IR analyses in both transmittance and reflectance mode (Skoog *et al.*, 2007) have been used to identify microplastics within coastal sediments (Ng and Obbard, 2006; Browne *et al.*, 2010). Attenuated total reflectance FT-IR (ATR-FT-IR) spectroscopy and near-infrared spectroscopy (Skoog *et al.*, 2007) have also been employed to analyse microplastics within beaches and open-water environments (Corcoran *et al.*, 2009; Hirai *et al.*, 2011). Additionally, a single study has used Raman microspectroscopy to identify microplastics within crustacean gut samples (Murray and Cowie, 2011). Even so, all presently known methods for the spectroscopic analysis of microplastics are subject to a



broad range of uncertainties introduced by both sampling error and the prior visual selection of fragments for characterisation.

### **1.3. Ecological Impacts of Microplastics on Higher Organisms**

Whilst microplastics may represent a physical hazard to marine animals, as in the case of larger plastic fragments (*e.g.* via ingestion and entanglement), their prevalence, high bioavailability and surface area-to-volume ratio have promoted significant additional concern over the ability of this debris to function as a substrate for the accumulation on and transport of plastic additives (*e.g.* organotins and phthalates) and of persistent organic pollutants (*e.g.* polynuclear aromatic hydrocarbons and polychlorinated biphenyls) (Mato *et al.*, 2001; Masó *et al.*, 2003; Rios *et al.*, 2007; Teuten *et al.*, 2007; Karapanagioti and Klontza, 2008; Teuten *et al.*, 2009; Colabuono *et al.*, 2010). The selective ingestion and subsequent bioaccumulation of microplastics has been demonstrated for suspension- and deposit-feeding invertebrates at the base of marine food webs, and there is emerging evidence for the transport of these fragments to higher trophic levels (Eriksson and Burton, 2003; Thompson *et al.*, 2004; Teuten *et al.*, 2007; Browne *et al.*, 2008; Graham and Thompson, 2009; Teuten *et al.*, 2009; Cole *et al.*, 2011; Murray and Cowie, 2011). For example, a recent survey of plastic ingestion by the sediment-dwelling crustacean *Nephrops norvegicus* reported the presence of microplastics in 83% of gut content samples obtained from the Clyde Sea (Murray and Cowie, 2011). Moreover, it has been estimated that the presence of a single microgramme of phenanthrene-contaminated polyethylene in a gramme of sediment significantly increased the body burden of this priority contaminant for the lugworm *Arenicola marina* (Teuten *et al.*, 2007). Desorption of plastic-associated contaminants in the gut has also been demonstrated for the streaked shearwater (*Calonectris leucomelas*) (Teuten *et al.*, 2009). As such, microplastics may constitute a threat to the

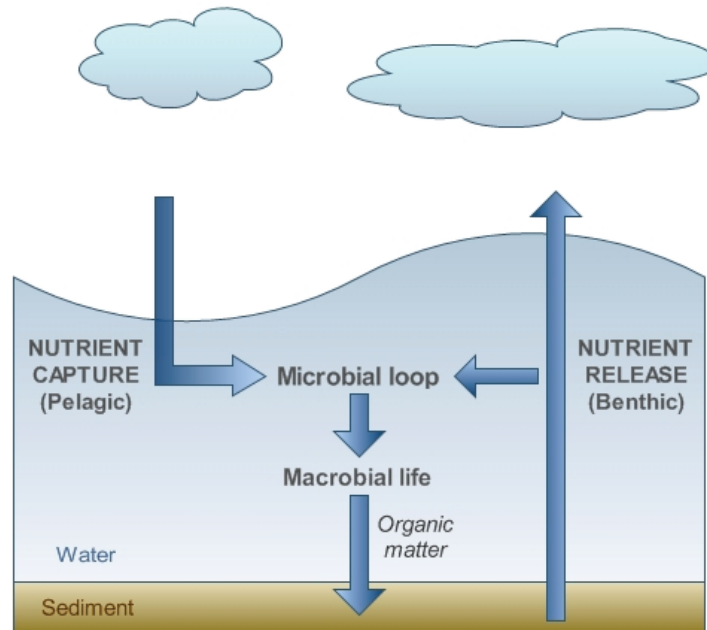
functioning of marine ecosystems which parallels that of engineered nanomaterials (Zhu *et al.*, 2006; Blickey and McClellan-Green, 2008; Koehler *et al.*, 2008; Andrady, 2011).

#### **1.4. Interactions between Microorganisms and Microplastics**

In the following sections, the significance of microorganisms in maintaining the ecological integrity of the marine environment and in potential interactions with synthetic microplastics is illustrated. For a comprehensive discussion of how microorganisms drive the biogeochemistry of marine ecosystems, the reader is referred to reviews by Arrigo (2005), Azam and Malfatti (2007), Karl (2007), Falkowski *et al.* (2008) and Strom (2008).

##### **1.4.1. Microorganisms and the marine biosphere**

Microorganisms are incredibly abundant in marine ecosystems and may reach up to hundreds of millions of bacterial cells in a gramme of wet marine sediment (Amann *et al.*, 1995; Sievert *et al.*, 1999). The total number of prokaryotic cells in marine subsurface habitats has been estimated as  $355 \times 10^{28}$ , with a total of  $170 \times 10^{26}$  cells occurring within the top 10 cm of open-ocean sediments (Whitman *et al.*, 1998). The metabolically active fraction of these cells underpins the functioning of marine food webs by catalysing redox reactions that control primary productivity and the cycling of nutrients (including nitrogen, phosphorus and sulphur) in the oceans (Figure 1.3) (López-Urrutia *et al.*, 2006; Hamasaki *et al.*, 2007; Falkowski *et al.*, 2008; Gasol *et al.*, 2008). Moreover, phototrophic microorganisms including algae and cyanobacteria (*e.g.* the genera *Prochlorococcus* and *Synechococcus*) are responsible for carbon fixation within pelagic marine environments (Falkowski *et al.*, 1998; DeLong and Karl, 2005). As such, the entire marine carbon cycle is tightly coupled to elemental transformations that are exclusively mediated by microorganisms (Arrigo, 2005; Madsen, 2008).



**Figure 1.3.** Schematic diagram providing a simplified overview of nutrient assimilation and remineralisation by microorganisms in marine ecosystems. The conversion of inorganic nutrients to microbial biomass underpins the functioning of macrobial food webs in the marine environment, which in turn represents a major source of sediment organic matter. Organic matter deposited within sediments is subsequently remineralised by benthic microorganisms. For information concerning oceanic primary production, the microbial loop and pelagic elemental cycling, see Falkowski *et al.* (1998), DeLong and Karl (2005), Azam and Malfatti (2007) and Pomeroy *et al.* (2007).

Marine sediments provide habitats for microbial growth that are fundamentally different from those in the water column (Falk *et al.*, 2007). Horizontal and vertical gradients of physical and geochemical parameters (*e.g.* pH, oxygen and redox conditions, organic carbon, nutrients and light) structure the local composition and activities of microbial communities in the seafloor at the scale of millimetres (Edlund *et al.*, 2008; Köster *et al.*, 2008, Wu *et al.*, 2008). The responsiveness of these communities to variation in habitat physicochemistry and the availability of growth substrata render the composition and metabolic activities of microbial assemblages within the seafloor potentially vulnerable to perturbation. Moreover, most of the biogeochemical transformations mediated by marine microorganisms occur in coastal

sediments which are at greater risk of exposure to human activities (including the disposal of synthetic plastics) than offshore sites (Walsh, 1991; Moore, 2008; Wu *et al.*, 2008).

Given the high metabolic potential of microbial communities in the seafloor and the role of coastal benthic habitats as sinks for the deposition of plastic litter (Section 1.2.1), investigation of the interactions between microorganisms and plastics within coastal sediments is of particular interest to understanding of the ecological impacts and fate of marine microplastic debris. In order to guide research into the responses of microorganisms to microplastics in marine habitats, it is pertinent to consider existing research into microbial-plastic interactions.

#### **1.4.2. Investigation of microbial-plastic interactions**

Microorganisms and particularly bacteria are known to function as primary colonists of several types of artificial materials present in marine ecosystems, including synthetic plastics (Dang and Lovell, 2000; Dang *et al.*, 2008; Lee *et al.*, 2008). Therefore, it is possible that plastic-colonising microbial assemblages participate in activities contributing to the absorption and/or desorption of plastic-associated compounds, and potentially even polymer biodegradation. Within marine habitats, microplastic particles may also function as sites for colonisation by microbial taxa that possess the capacity to influence the ecology and resident microflora of higher organisms following their ingestion (Deines *et al.*, 2007; Graham and Thompson, 2009). A summary of the potential, yet primarily uncharacterised interactions between microplastics, plastic-associated additives, contaminants, microbial assemblages and higher organisms is provided in Figure 1.4.



no studies have characterised fundamental processes of microbial plastic colonisation in marine sediments that function as sinks for the accumulation of plastic debris (Thompson *et al.*, 2004; Reddy *et al.*, 2006; Barnes *et al.*, 2009; Browne *et al.*, 2011; Claessens *et al.*, 2011; Cole *et al.*, 2011).

Despite detailed accounts of the likely prerequisites and mechanisms underlying the microbial biodegradation of plastics (Chiellini *et al.*, 2003; Gu, 2003; Kawai *et al.*, 2004; Koutny *et al.*, 2006; Lucas *et al.*, 2008; Shah *et al.*, 2008; Eubeler *et al.*, 2010), little research has been conducted into the biodegradation of plastic litter in marine habitats. The biodegradability of synthetic polymers is thought to depend upon the type and chemical properties of the plastic, the environment (*e.g.* seasonality and the availability of oxygen) and metabolic interactions within plastic-associated biofilms (Bonhomme *et al.*, 2003; Gilan *et al.*, 2004; Artham *et al.*, 2009). Although research into the biotransformation of plastics has focused on microorganisms from terrestrial habitats (*e.g.* Chiellini *et al.*, 2003; Arkatkar *et al.*, 2009), a limited number of experiments have characterised the capacity of microbial assemblages in the water column to utilise synthetic polymers as a resource for growth (Table 1.1). For example, low-density polyethylene has been estimated to degrade more rapidly than high-density polyethylene, polycarbonate and polypropylene immersed in seawater (Sudhakar *et al.*, 2007b; Sudhakar *et al.*, 2008; Artham *et al.*, 2009). A positive relationship between the degree of polymer oxidation and rates of *in vitro* plastic degradation has also been demonstrated for polyethylene films in freshwater (Chiellini *et al.*, 2007). However, only two studies have examined the potential for sediment microorganisms in marine ecosystems to biodegrade plastic debris (Kumar *et al.*, 2007; Balasubramanian *et al.*, 2010).

Overall, the rates of degradation of plastics in marine systems are likely to be significantly lower than in their terrestrial counterparts, due to the low availability of nutrients, oxygen and light (Barnes *et al.*, 2009). Additionally, whilst several methods (*e.g.* microscopy, mass loss, FT-IR spectroscopy, calorimetry and respirometry) have been used to measure biodegradation of plastic litter in the environment, there is a lack of direct experimental evidence demonstrating the utilisation of synthetic plastics as a source of carbon for microbial growth (Lucas *et al.*, 2008). Therefore, unequivocal evidence for the biodegradation of plastics is yet to emerge, because it is unclear whether microbial activities degrade the plastic itself, exploit plastic-associated chemicals or both (Koutny *et al.*, 2006; Lucas *et al.*, 2008; Eubeler *et al.*, 2010).

**Table 1.1.** Experimental investigations of the biodegradation of synthetic polymers by aquatic microorganisms.

<b>Microbial taxa</b>	<b>Plastic type</b>	<b>Environment</b>	<b>Exposure</b>	<b>Comments</b>	<b>References</b>
<i>Pseudomonas</i> sp. B2	Polycarbonate film containing bisphenol A (BPA)	Seawater	<i>In situ</i> and <i>in vitro</i>	9% loss of mass over 12 months, leaching of BPA	Artham and Doble (2009)
Unidentified consortium	Polycarbonate, polyethylene and polypropylene coupons	Seawater	<i>In situ</i>	Degradation dependent on plastic type and season	Artham <i>et al.</i> (2009)
Unidentified consortium	Polyethylene film containing pro-oxidant additives	Freshwater	<i>In situ</i>	Degradation dependent on degree of polymer oxidation	Chiellini <i>et al.</i> (2007)
Unidentified consortium	Polyethylene films with and without starch additive	Seawater	<i>In situ</i> and <i>in vitro</i>	Little or no evidence for degradation over 20 months	Rutkowska <i>et al.</i> (2002a)
Unidentified consortium	Polyurethane sheets	Seawater	<i>In situ</i> and <i>in vitro</i>	Degradation dependent on polymeric cross-linking	Rutkowska <i>et al.</i> (2002b)
<i>Bacillus cereus</i> , <i>B. sphericus</i> , <i>Vibrio furnisii</i> , <i>Brevundimonas vesicularis</i>	Nylon pellets	Seawater	<i>In vitro</i>	Degradation varied across microbial taxa (highest for <i>B. cereus</i> )	Sudhakar <i>et al.</i> (2007a)
<i>Pseudomonas</i> spp., <i>Clostridium</i> spp., unidentified anaerobic, hetetrophic and iron-reducing bacteria, fungi	Polyethyelene and polypropylene sheets	Seawater	<i>In situ</i>	Degradation dependent on study site, plastic type and season	Sudhakar <i>et al.</i> (2007b)
<i>B. cereus</i> subgroup A, <i>B. sphericus</i> GC subgroup IV	Polyethylene sheets	Seawater	<i>In vitro</i>	Degradation dependent on type of polyethylene	Sudhakar <i>et al.</i> (2008)



## **1.5. Opportunities for Future Research**

Whilst several aspects concerning the extent and environmental impacts of marine microplastic pollution are poorly understood, this introduction has highlighted three key areas for novel research concerning both the detection of synthetic microplastics and characterising microbial-plastic interactions in marine ecosystems:

### *1. Research into the development of improved spectroscopic methods for detecting and visualising microplastics within the marine environment*

Our ability to understand the transport, distribution, abundance and ecological impacts of microplastics in marine ecosystems depends upon the development of technically robust and standardised methods for the quantification of these pollutants in environmental samples (NOAA, 2009; Ryan *et al.*, 2009; GESAMP, 2010b; Hidalgo-Ruz *et al.*, 2012). Due to the inherent limitations of previously described techniques for the sampling, detection and quantification of microplastics, only limited confidence can be placed on present estimates of the extent of microplastic pollution in the marine environment. Yet, whilst the need for improved approaches to separate microplastics from marine habitats has already been acknowledged internationally (NOAA, 2009; GESAMP, 2010b), little attention has been directed towards optimising, assessing and comparing the applicability of different spectroscopy methods for the analysis of microplastics. Since micro-FT-IR spectroscopy represents the most commonly used method for identifying microplastics (Hidalgo-Ruz *et al.*, 2012), there is a particular need to experimentally evaluate the performance of this technique across different marine habitats and sample types, and between research groups. Moreover, since all presently known methods for detecting microplastics are labour-intensive and prone to

error due to their dependence on the visual selection of putative polymers for analysis, there is a need to develop novel spectroscopy techniques that circumvent this step.

*2. Fundamental research into determining the potential for microplastics to function as sites for bacterial attachment over time and within different types of coastal sediments*

In order to advance our understanding of the ecological impacts of microplastic waste, research efforts must be focused on determining how the deposition of this debris into sediments affects those organisms that are most likely to be exposed to microplastics and which mediate ecosystem services that sustain marine life (NOAA, 2009; Galgani *et al.*, 2010). Whilst we are beginning to address these questions with reference to invertebrates and other higher organisms, microorganisms underpin the functioning of marine ecosystems by driving primary productivity and elemental cycling (Madsen, 2005; Falkowski *et al.*, 2008). Microbial activities also facilitate the mineralisation of anthropogenic pollutants. Nevertheless, there is a lack of ecological information concerning the potential for microplastics in marine ecosystems to function as sites for the attachment of microbial assemblages originating from the wider environment. This is particularly true for sediments, where processes involved in the microbial colonisation of plastics remain essentially unknown (Section 1.4.2). Since previous studies of microbial-plastic interactions within seawater have identified bacteria as primary colonists of synthetic polymer surfaces (Dang and Lovell, 2000; Dang *et al.*, 2008; Lee *et al.*, 2008), it is necessary to characterise processes involved in the colonisation of microplastics by these organisms within (coastal) marine sediments. In order to enable microbial research into the ecological impacts and fate of microplastics in the benthic marine environment, there is a particular need to understand fundamental

patterns of bacterial colonisation on microplastic surfaces over time and within different sediment types.

### *3. Culture-independent investigation of the ecological interactions between microorganisms and microplastics in the marine environment*

Our understanding concerning the ecological interactions between microorganisms and synthetic polymers in marine ecosystems is constrained by the fact that most prior research into this topic has been based on the utilisation of culture-based methodologies or unidentified microbial assemblages within seawater (Section 1.4). Even so, only one per cent or fewer of all naturally occurring bacterial taxa are typically cultivable under laboratory conditions (Amann *et al.*, 1995). Therefore, research employing culture-independent molecular methods is required to elucidate the structure, diversity and taxonomic identities of plastic-colonising microorganisms in the marine environment and particularly within sediments. Additionally, it is likely that most plastic-colonising marine microbial taxa possessing the capacity to influence the fate and/or potential long-term biodegradation of microplastics and plastic-associated compounds remain undiscovered and could be characterised by culture-independent analyses. In this respect, the applications of molecular microbial ecology including microscopy techniques and PCR-based methods such as community fingerprinting and 16S rRNA gene sequencing (Liu *et al.*, 1997; Suzuki *et al.*, 2000; Pernthaler *et al.*, 2002; Daims *et al.*, 2005; Röling and Head, 2005) are bound to offer significant opportunities to advance our knowledge about the ecological impacts of microplastics within marine ecosystems.

## 1.6. Aims and Objectives

Synthetic microplastics have now been recognised for nearly a decade as rapidly emerging and persistent anthropogenic pollutants within marine ecosystems (Thompson *et al.*, 2004). Even so, very little is known about both the abundance and biological impacts of this waste. Therefore, this study aims to advance research into understanding the distribution and abundance, ecological impacts, biotic interactions and fate of microplastic pollutants in marine ecosystems by addressing two important yet understudied topics. Firstly, an optimised method for detecting microplastics in sediments by reflectance micro-FT-IR spectroscopy was developed and compared with ATR-FT-IR analyses (Chapter 2). The infrared spectra of microplastics were assessed across plastic-spiked sediments from a total of seventeen UK coastal and offshore sampling sites. Additionally, a novel approach to the molecular mapping of microplastics without the need for visual selection of fragments for analysis was explored, with the objective of informing investigation into the development of high-throughput techniques for imaging these pollutants within environmental samples.

Secondly, a laboratory microcosm experiment was used to evaluate successional patterns of bacterial colonisation on microplastics deposited into both sandy and silty coastal sediments collected from the Humber Estuary, UK (Chapter 3). This research aimed to investigate variation in the structure, diversity and taxonomic identities of plastic-associated bacterial communities using culture-independent analyses. Terminal-restriction fragment polymorphism (T-RFLP) analysis of PCR-amplified bacterial 16S rRNA genes (Liu *et al.*, 1997; Osborn *et al.*, 2000) was used to examine patterns of primary succession within the plastic-sediment interface. T-RFLP fingerprinting is a well-established and reproducible method for comparing the relative structure, diversity and temporal dynamics of microbial communities in natural ecosystems (Osborn *et al.*,

2000). Additionally, the predominant bacterial genera attaching to plastic surfaces in sediments were identified using sequencing analyses of bacterial 16S rRNA gene clone libraries (see Røling and Head, 2005).

Whilst T-RFLP analysis and 16S rRNA gene sequencing analysis provide valuable information concerning the structure, diversity and taxonomic composition of microbial communities in the environment, these methods do not provide fully quantitative data concerning the abundance of microorganisms in different samples (von Wintzingerode *et al.*, 1997). Hence, an additional objective of this study was to quantify the extent of bacterial attachment to microplastic surfaces over time and across both sandy and silty sediment types (Chapter 4), using the laboratory microcosm experiment described above. 16S rRNA gene numbers of plastic-associated bacteria were quantified as a proxy of relative bacterial abundance, using a quantitative real-time PCR (Q-PCR) assay (Becker *et al.*, 2000; Suzuki *et al.*, 2000; Smith *et al.*, 2006; Smith and Osborn, 2009). Q-PCR analysis is a widely used method for quantitatively measuring the abundance of selected target genes amplified from environmental DNA (Smith and Osborn, 2009). Moreover, this study aimed to visually investigate attachment by bacterial cells and specifically, key members of the plastic-colonising bacterial genera identified by 16S rRNA gene sequencing analyses. Bacterial attachment onto microplastic surfaces was examined using catalysed reporter deposition fluorescence *in situ* hybridisation (CARD-FISH) analysis, a microscopy-based technique that is independent of PCR amplification and is frequently used to demonstrate the presence of specific microbial taxa within environmental samples (Pernthaler *et al.*, 2002; Sekar *et al.*, 2003; Wendeberg *et al.*, 2010).

All aims and objectives of this present study were addressed using low-density polyethylene (LDPE) as the model polymer due to both its commercial importance and prevalence as a component of marine plastic debris (Andrady and Neal, 2009; Barnes *et al.*, 2009; Browne *et al.*, 2011; Hidalgo-Ruz *et al.*, 2012). Due to the absence of previous research into the topics examined within this study, the overarching focus of the present investigation was to generate primary empirical data to inform further research (Section 5.2) into the development of reliable methods for quantifying microplastics in marine habitats, and to characterise microbial-plastic interactions within a broad range of sediment environments. The microbiological aspect of this study was based on the following *a priori* predictions:

1. Naturally occurring bacterial assemblages are able to colonise microplastics deposited into coastal marine sediments.
2. The structure and diversity of microplastic-colonising bacterial assemblages vary over time and across different sediment types (ranging from sand to silt).
3. The taxonomic composition of microplastic-associated bacterial assemblages varies across different sediment types (ranging from sand to silt).
4. The abundance of plastic-associated bacteria varies with time and across different sediment types (ranging from sand to silt).

## **Chapter 2**

### **The Applicability of Reflectance Micro-Fourier-transform Infrared Spectroscopy for the Detection of Synthetic Microplastics in Marine Sediments**

This research has been published as:

Harrison, J.P., Ojeda, J.J. and Romero-González, M.E. (2012) The applicability of reflectance micro-Fourier-transform infrared spectroscopy for the detection of synthetic microplastics in marine sediments. *Science of the Total Environment* **416**: 455-463.

## 2.1. Abstract

Synthetic microplastics ( $\leq 5$ -mm fragments) are globally distributed contaminants within coastal sediments that may transport organic pollutants and additives into food webs. Although micro-Fourier-transform infrared (micro-FT-IR) spectroscopy represents an ideal method for detecting microplastics in sediments, this technique lacks a standardised operating protocol. Herein, an optimised method for the micro-FT-IR analysis of microplastics in vacuum-filtered sediment retentates was developed. Reflectance micro-FT-IR analyses of polyethylene (PE) were compared with attenuated total reflectance FT-IR (ATR-FT-IR) measurements. Molecular mapping as a precursor to the imaging of microplastics was explored in the presence and absence of 150- $\mu\text{m}$  PE fragments, added to sediment at concentrations of 10, 100, 500 and 1000 ppm. Subsequently, polymer spectra were assessed across plastic-spiked sediments from fifteen offshore sites. Whilst all spectra consisted of evenly shaped plastics were typical to PE, reflectance micro-FT-IR measurements of irregularly shaped materials must account for refractive error. Additionally, the present research provides the first evidence that mapping successfully detects microplastics without their visual selection for characterisation, despite this technique relying on spectra from small and spatially separated locations. Flotation of microplastics from sediments only enabled a fragment recovery rate of 61 ( $\pm 31$  SD)%. However, mapping 3- $\text{mm}^2$  areas (within 47-mm filters) detected PE at spiking concentrations of 100 ppm and above, displaying 69 ( $\pm 12$  SD)% of the fragments in these locations. Additionally, mapping detected a potential PE fragment in a non-spiked retentate. These data have important implications for research into the imaging of microplastics. Specifically, the sensitivity and spatial resolution of the present protocol may be improved by visualising the entire filter with high-throughput detection techniques (*e.g.* focal plane array-based imaging). Additionally, since micro-FT-IR analyses depend on methods of sample collection, these results



emphasise the urgency of developing efficient and reproducible techniques to separate microplastics from sediments.

## 2.2. Introduction

Synthetic microplastics ( $\leq 5$ -mm fragments) are globally distributed pollutants in marine ecosystems that have been discovered particularly in coastal sediments (Barnes *et al.*, 2009; Browne *et al.*, 2010; Claessens *et al.*, 2011; Hidalgo-Ruz *et al.*, 2012). Despite their ability to transport organic pollutants and additives into food webs, our understanding concerning the abundance and fate of microplastics is insufficient, owing to the difficulty of quantifying these contaminants (NOAA, 2009; GESAMP, 2010b). Reflectance micro-Fourier-transform infrared (micro-FT-IR) spectroscopy represents an ideal method for the detection of microplastics, due to its non-destructive nature, minimal need for sample preparation and ability to produce IR absorption spectra for thick and opaque materials (Ojeda *et al.*, 2009). To date, most attempts to employ FT-IR analyses for the detection of microplastics in sediments have been preceded by the flotation and filtration of buoyant materials in a saline solution, followed by the light microscopy-based separation of putative polymers for spectroscopy (Thompson *et al.*, 2004; Ng and Obbard, 2006; Reddy *et al.*, 2006; Corcoran *et al.*, 2009; Claessens *et al.*, 2011; Hidalgo-Ruz *et al.*, 2012). However, this approach to the FT-IR analysis of microplastics is both labour-intensive and prone to error due to its reliance on the visual selection of particles for characterisation (Corcoran *et al.*, 2009; Ng and Obbard, 2006; Reddy *et al.*, 2006; Thompson *et al.*, 2004). Additionally, spectroscopic approaches for the characterisation of microplastics have not been evaluated between different research groups and across different types of marine habitats. Due to these challenges, limited confidence can be placed on estimates concerning the extent of microplastic pollution in the environment. Hence, the development of reliable methods for the collection,

detection and quantification of microplastics is pivotal to the advancement of research into the distribution, fate and impacts of plastic debris (NOAA, 2009; Ryan *et al.*, 2009; GESAMP, 2010b).

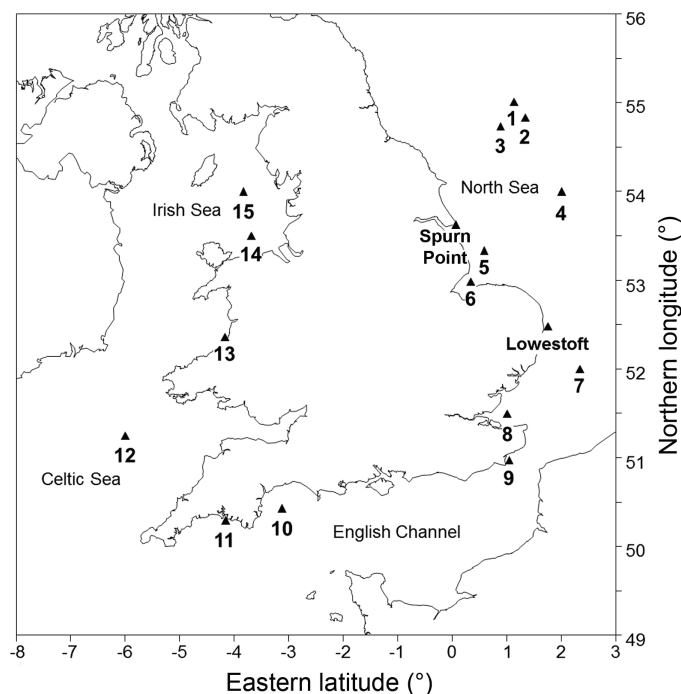
In order to form a basis for research into developing improved techniques for the FT-IR-based detection microplastics in the environment, this research assessed the applicability of reflectance micro-FT-IR analyses for the identification of synthetic microplastics in vacuum-filtered marine sediments. Following an optimisation of instrumental scanning parameters, as described by Mastalerz and Bustin (1995) and Ojeda *et al.* (2009) for the analysis of opaque samples, the reliability of reflectance micro-FT-IR spectroscopy was compared with attenuated total reflectance FT-IR (ATR-FT-IR) analyses and with reference to evenly and unevenly shaped microplastics. Furthermore, the potential for the molecular mapping of microplastics was explored as a step towards the spectral imaging of these pollutants without the need for visual selection of fragments for analysis (Levin and Bhargava, 2005). Finally, the variability of microplastic spectra across fifteen offshore sampling sites was investigated. Throughout the study, polyethylene (PE) was used as a model polymer due to its role as a dominant component of marine plastic debris (Barnes *et al.*, 2009).

## **2.3. Materials and Methods**

### **2.3.1. Sediment sampling and analysis**

Sediments were collected between April and July 2009 from Lowestoft, Spurn Point (within the Humber Estuary) and fifteen offshore sites (UK), with distances of 50 – 500 km between stations (Figure 2.1). Samples from Lowestoft and Spurn Point were collected from the surficial centimetre of the seabed at low tide, using a stainless steel spatula. Offshore samples were obtained using bulk sediments collected by a 0.1 m<sup>2</sup>

Day Grab. All sediments were stored at 4 °C until use. Offshore sediments were analysed for organic carbon content as described by Sapp *et al.* (2010).



**Figure 2.1.** The locations (▲) of field sites used for sediment sampling (UK). Offshore sites are identified by the numbers 1 – 15.

### 2.3.2. Spiking of sediments with microplastics

For all analyses with the exception of molecular mapping, 10 g of wet homogenised sediment from each site were mixed with a single, evenly shaped 1 × 5 × 5-mm LDPE square (Goodfellow Cambridge Ltd., Huntingdon, UK) and 50 ± 5 mg of unevenly shaped 1-mm LDPE granules (Goodfellow). For molecular mapping, sediment from a single sampling site (Lowestoft, UK) was spiked with 10, 100, 500 or 1000 ppm of unevenly shaped 150-µm ultra-high molecular weight (UHMW) PE granules (Goodfellow). Non-spiked sediment retentates were also examined, using sediment collected from Lowestoft and Spurn Point, Humber Estuary (UK). All samples were prepared in triplicate.

### **2.3.3. Vacuum filtrations of plastic-spiked sediments**

Microplastic-spiked sediments were stored at 4 °C for 48 hours and covered up to 50 ml with hypersaturated artificial seawater prepared using 1.2 kg l<sup>-1</sup> of a standard salt mixture (ZM Systems, Hampshire, UK) (Thompson *et al.*, 2004; Ng and Obbard, 2006; Browne *et al.*, 2010). The samples were mixed by inversion, vortexed for 30 seconds at 40 Hz and kept overnight at 4 °C. The resulting supernatants (30 ml) were vacuum-filtered through 47-mm Isopore<sup>TM</sup> polycarbonate membrane filters with a pore size of 0.2 µm (Millipore, USA). The filters were air-dried and stored at room temperature until analysis.

### **2.3.4. Recovery of microplastics**

Recovery (%) of artificially added microplastics was calculated using sediments spiked with 150-µm UHMW PE fragments ( $n = 3$ ). The mean mass of non-plastic residues on the filter (determined using retentates of sediment and water only;  $n = 3$ ) was subtracted from the total mass of materials retained on the filter surfaces following vacuum filtration.

### **2.3.5. FT-IR spectroscopy**

Fourier-transform infrared measurements were performed using reflectance micro-FT-IR spectroscopy and attenuated total reflectance FT-IR (ATR-FT-IR) spectroscopy. Spectra were collected of artificially added microplastics within sediment retentates and of the membrane filter surface following vacuum filtration (Sections 2.3.2 and 2.3.3), using a Perkin-Elmer Spotlight imaging system and a Perkin-Elmer Spectrum One FT-IR spectroscope (Ojeda *et al.*, 2009). For ATR-FT-IR measurements, a Specac Silver Gate ATR accessory was used, consisting of a germanium crystal at an incidence angle of 45 degrees. Scans were performed using a spectral resolution of 4 cm<sup>-1</sup>. Reflectance

spectra were Kramers-Kronig-transformed to correct for specular reflection (Mastalerz and Bustin, 1995, 1996; Hacura *et al.*, 2003). The micro-FT-IR analyses were performed individually or by molecular mapping. Individual measurements were obtained for randomly selected positions on the sample surface. Molecular micrographs were obtained for randomly selected areas of 3 mm<sup>2</sup>, with spectra collected at intervals of 150 µm.

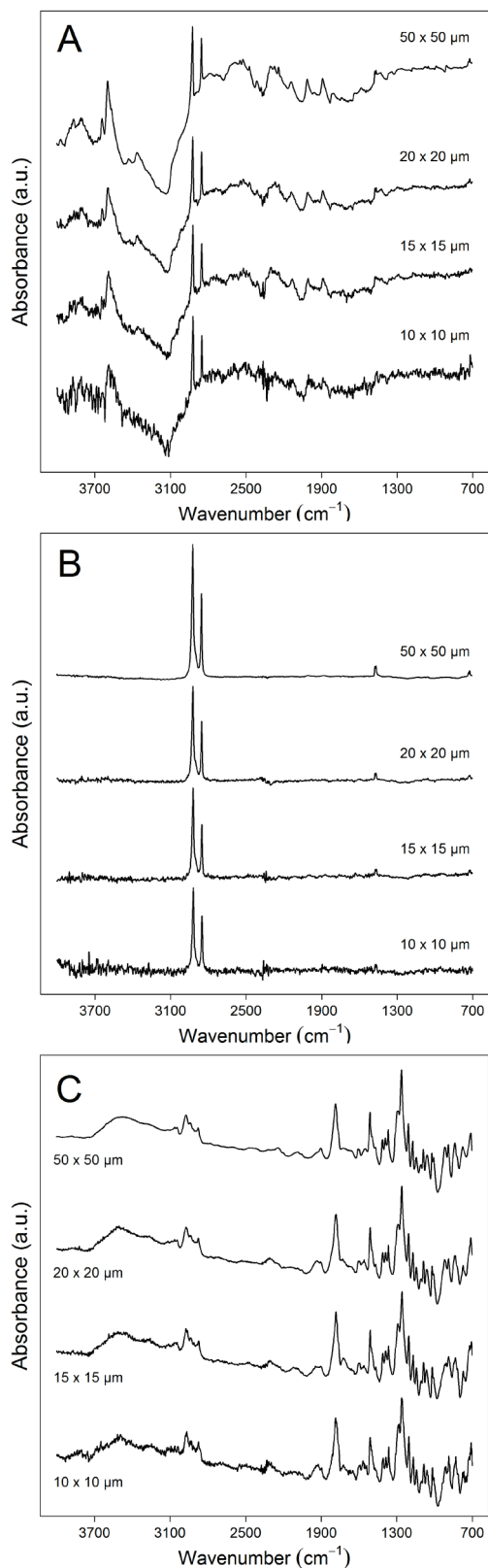
## **2.4. Results and Discussion**

Although micro-FT-IR spectroscopy represents an ideal method for the detection of microplastics in environmental samples (Thompson *et al.*, 2004; Ng and Obbard, 2006; Ojeda *et al.*, 2009), this method has not been experimentally evaluated. This study assessed the applicability of reflectance micro-FT-IR analyses for the detection of synthetic microplastics in retentates of vacuum-filtered marine sediments, using polyethylene as a model plastic. Following an examination of polymer spectra as a function of aperture size and the frequency of scans, reflectance micro-FT-IR analyses were compared with ATR-FT-IR measurements. Molecular mapping analyses were explored as a step towards the spectral imaging of microplastics. Finally, the spectra of plastics were compared across fifteen offshore sampling sites.

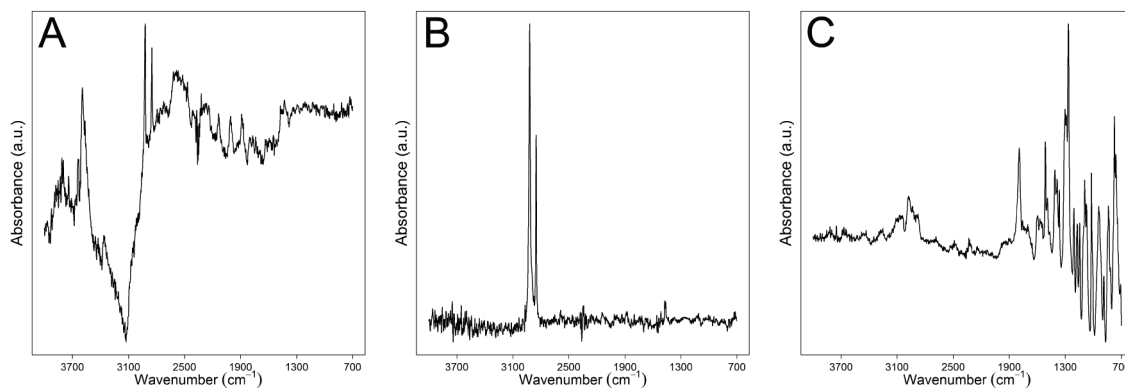
### **2.4.1. Signal-to-noise ratio as a function of aperture size and the frequency of scans**

Despite previous attempts to detect synthetic microplastics in marine sediments (Thompson *et al.*, 2004; Ng and Obbard, 2006; Reddy *et al.*, 2006; Corcoran *et al.*, 2009), the instrumental parameters for the reflectance micro-FT-IR analysis of microplastics have not been optimised. Therefore, spectra were collected of granule- and square-shaped LDPE fragments within a vacuum-filtered retentate of plastic-spiked sediment from Lowestoft (UK) and of the polycarbonate membrane following filtration,

using 100 co-added scans at aperture sizes of 10, 15, 20 and 50  $\mu\text{m}^2$  (Fig. 2.2). The filtration step did not affect the positions of absorbance bands, as shown by a comparison with spectra collected prior to filtration (Figure 2.3). The positions of absorbance bands and the ratios between peaks were consistent for all samples when examined as a function of aperture size. No interference was detected from the background signal originating from the polycarbonate membrane when collecting spectra for LDPE (Figure 2.2). Although the signal-to-noise ratios of the spectra improved (*i.e.* became increasingly well-resolved) with increasing aperture size, large apertures may limit the detection of small plastic particles in heterogeneous matrices. Small aperture sizes provide high spatial resolution and are likely to facilitate the molecular mapping of microplastics, favouring the use of small apertures during spectral collection. It was observed that an aperture size of 15  $\times$  15  $\mu\text{m}$  provided well-resolved spectra and included all absorbance peaks that were found with larger apertures. An aperture of 10  $\times$  10  $\mu\text{m}$  was also tested. However, the spectra obtained showed low signal-to-noise ratios (Figure 2.2), reducing the ability to reliably discriminate between the absorbance bands. For this reason, an aperture size of 15  $\times$  15  $\mu\text{m}$  was selected for subsequent measurements.



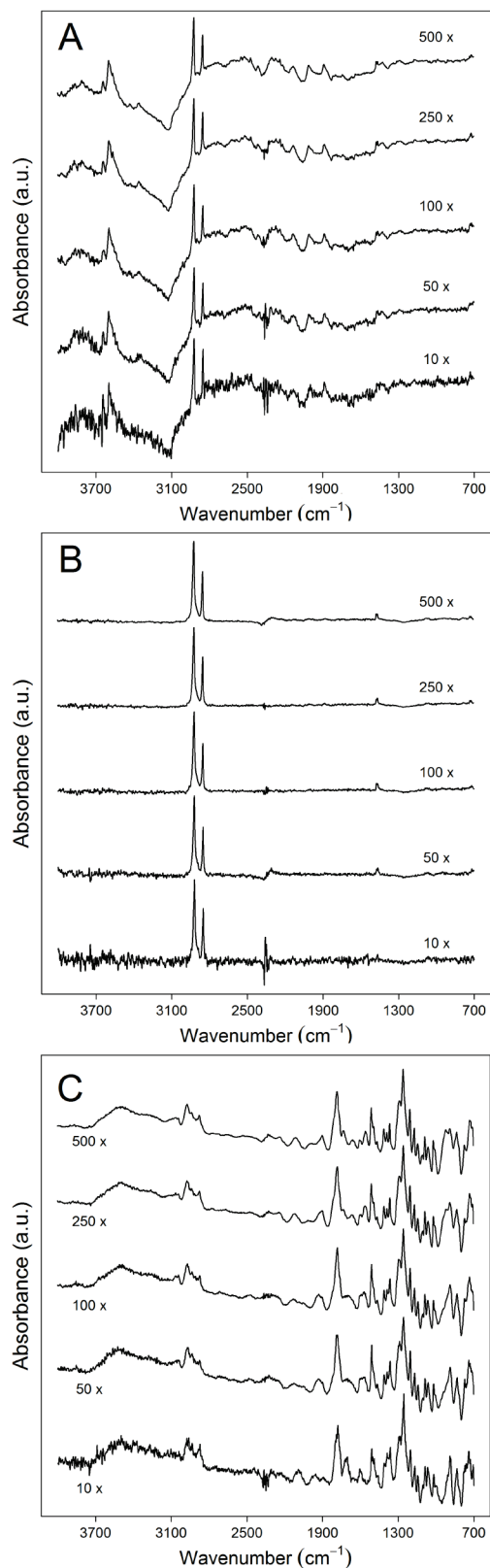
**Figure 2.2.** Reflectance micro-FT-IR spectra of A) granule-shaped LDPE fragments (1 mm) and B) square-shaped LDPE fragments ( $1 \times 5 \times 5$  mm) within a vacuum-filtered retentate of plastic-spiked sandy sediment (Lowestoft, UK), and C) the surface of the polycarbonate membrane following filtration. Spectra are presented as a function of aperture size ( $\mu\text{m}$ ) while using 100 co-added scans per measurement, with absorbance recorded as arbitrary units (a.u.).



**Figure 2.3.** Reflectance micro-FT-IR spectra of A) granule-shaped LDPE fragments, B) square-shaped LDPE fragments and C) the polycarbonate membrane prior to the filtration of microplastics, with absorbance recorded as arbitrary units (a.u.).

The optimal number of scans was determined by collecting spectra of the filtered LDPE fragments on the polycarbonate membrane using 10, 50, 100, 250 and 500 co-added scans (Figure 2.4). There was an increase in the quality of the signal-to-noise ratio between 10 and 50 scans at a spectral resolution of  $4\text{ cm}^{-1}$ . However, after 100 scans the improvement in spectral quality was less evident. Five minutes were required to obtain 50 co-added scans, whereas 0.5, 10, 25 and 50 minutes were needed for 10, 100, 250 and 500 scans, respectively. No shifts occurred in the positions of the absorbance bands or the ratios between peaks as a function of the number of scans, similarly to results obtained for the calibration of aperture size. Based on these findings, one hundred co-added scans were selected for the further collection of spectra. However, in order to reduce the data burden and measurement time required for molecular mapping, the lowest reliable frequency of scans was used (Ojeda *et al.*, 2009). Therefore, during mapping, ten co-added scans were obtained for individual spectra.

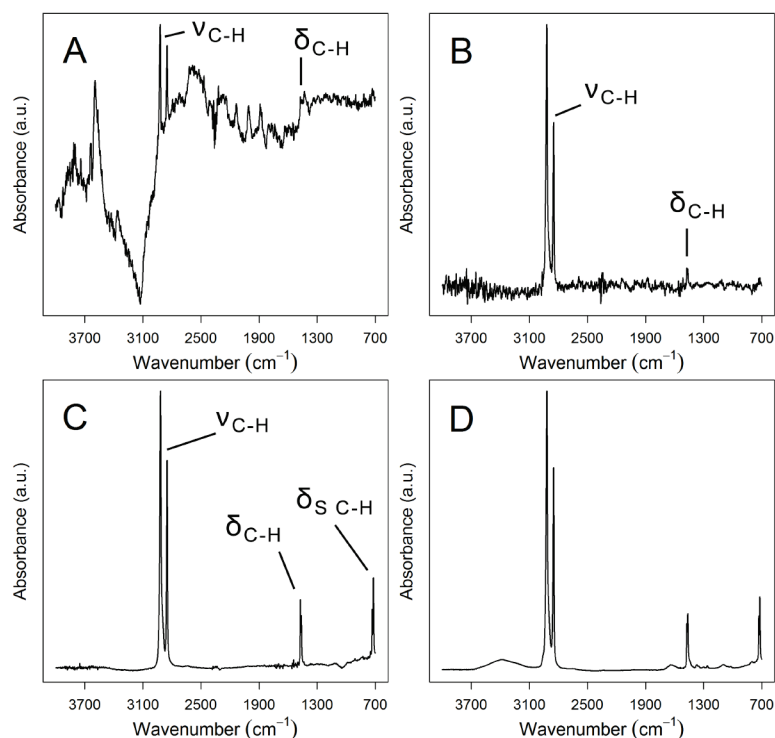




**Figure 2.4.** Reflectance micro-FT-IR spectra of A) granule-shaped LDPE fragments (1 mm), B) square-shaped LDPE fragments (1 × 5 × 5 mm) within a vacuum-filtered retentate of plastic-spiked sandy sediment (Lowestoft, UK), and C) the surface of the polycarbonate membrane following filtration. Spectra are presented as a function of the number of scans while using an aperture of 15 × 15 μm, with absorbance recorded as arbitrary units (a.u.).

### 2.4.2. Impact of the mode of analysis on sample spectra

In this study, it was found that the reflectance micro-FT-IR profiles of square-shaped LDPE fragments in retentates of vacuum-filtered sediments were similar to the standard spectrum of this polymer (Cooper and Corcoran, 2010), when examined across a broad range of aperture sizes and frequencies of co-added scans. Nonetheless, they differed from the corresponding measurements obtained for granule-shaped LDPE fragments (Figure 2.2). In order to determine whether micro-FT-IR analyses in reflectance mode resulted in distortions of the spectra due to differences in polymer shape, these profiles were compared with ATR-FT-IR measurements. Regions of absorbance intrinsic to the stretching of C-H ( $\nu_{C-H}$ ) (3000 – 2770  $\text{cm}^{-1}$ ) and the bending of C-H ( $\delta_{C-H}$ ) (1500 - 1450  $\text{cm}^{-1}$ ) bonds were present for all fragments, irrespective of the mode of analysis (Figure 2.5).



**Figure 2.5.** Comparison between absorbance spectra of granule- and square-shaped LDPE fragments obtained by reflectance micro-FT-IR spectroscopy (A and B, respectively) and attenuated total reflectance FT-IR (ATR-FT-IR) spectroscopy (C and D, respectively). Regions of absorbance corresponding to the stretching, bending and scissoring ( $\nu$ ,  $\delta$  and  $\delta_s$ , respectively) of C-H bonds are indicated with reference to the spectra.

However, comparing the micro-FT-IR and ATR-FT-IR spectra demonstrated the potential for spectral distortion when analysing irregularly shaped plastics in reflectance mode. Although this may arise due to scattering of the infrared beam (Budevskas, 2000; Földes *et al.*, 2003; Bassan *et al.*, 2009; Davis *et al.*, 2010), no previous research has been conducted into the impacts of the morphology of microplastics upon their micro-FT-IR spectra. Yet, alterations in the shapes of spectra due to variation in sample morphology may complicate the identification of microplastics.

Given the susceptibility of reflectance micro-FT-IR spectroscopy to refractive error, this research emphasises the need to base the identification of microplastics on using a combination of polymer-specific regions of absorbance that are insensitive to variation in sample morphology (Cooper and Corcoran, 2010). Analyses by ATR-FT-IR spectroscopy could also facilitate identification of microplastics by detecting absorbance bands that cannot be discerned by reflectance micro-FT-IR spectroscopy alone. As seen from the present results, bands corresponding to the bending of C-H bonds ( $1500 - 1450 \text{ cm}^{-1}$ ) were more efficiently distinguished by ATR-FT-IR measurements than by reflectance micro-FT-IR spectroscopy (Figure 2.5). Additionally, ATR-FT-IR measurements enabled detection of bands attributed to the scissoring of C-H bonds ( $750 - 700 \text{ cm}^{-1}$ ) that were indiscernible by reflectance micro-FT-IR analyses.

### **2.4.3. Molecular mapping of microplastics in sediment retentates**

#### **2.4.3.1. Rationale for mapping analyses**

Previous attempts to identify microplastics in filtered marine sediments have frequently relied on the visual selection of fragments for analysis (Thompson *et al.*, 2004; Ng and Obbard, 2006; Reddy *et al.*, 2006; Browne *et al.*, 2010; Hidalgo-Ruz *et al.*, 2012). Given the low precision of this approach, developing improved protocols for the

detection of microplastics is pivotal (NOAA, 2009). Molecular imaging by FT-IR microspectroscopy has been used to obtain high-resolution data concerning the distributions of several molecular groups in heterogeneous media, including biofilms (Ojeda *et al.*, 2009), tissues and cancer cells (Yu *et al.*, 2003; Faibish *et al.*, 2005; Levin and Bhargava, 2005; Petibois and Déléris, 2006; Bhargava, 2007; Petibois *et al.*, 2007). Interestingly, the technique has also been employed to visualise silicone and poly(ethylene terephthalate) in human tissues (Kidder *et al.*, 1997).

If successfully applied, FT-IR imaging would enable identification of microplastics without the visual separation of fragments for analysis (Bhargava *et al.*, 2000; Levin and Bhargava, 2005). Here, the applicability of this technique was assessed by utilising a point measurement-based approach for molecular mapping. Mapping performed on conventional FT-IR microscopes is slow and inefficient in contrast with modern focal plane array (FPA)-based imaging (Bhargava *et al.*, 2000; Levin and Bhargava, 2005). However, it is a useful precursor to the further imaging of microplastics, due to its affordability and broad comparability with FPA-based analyses.

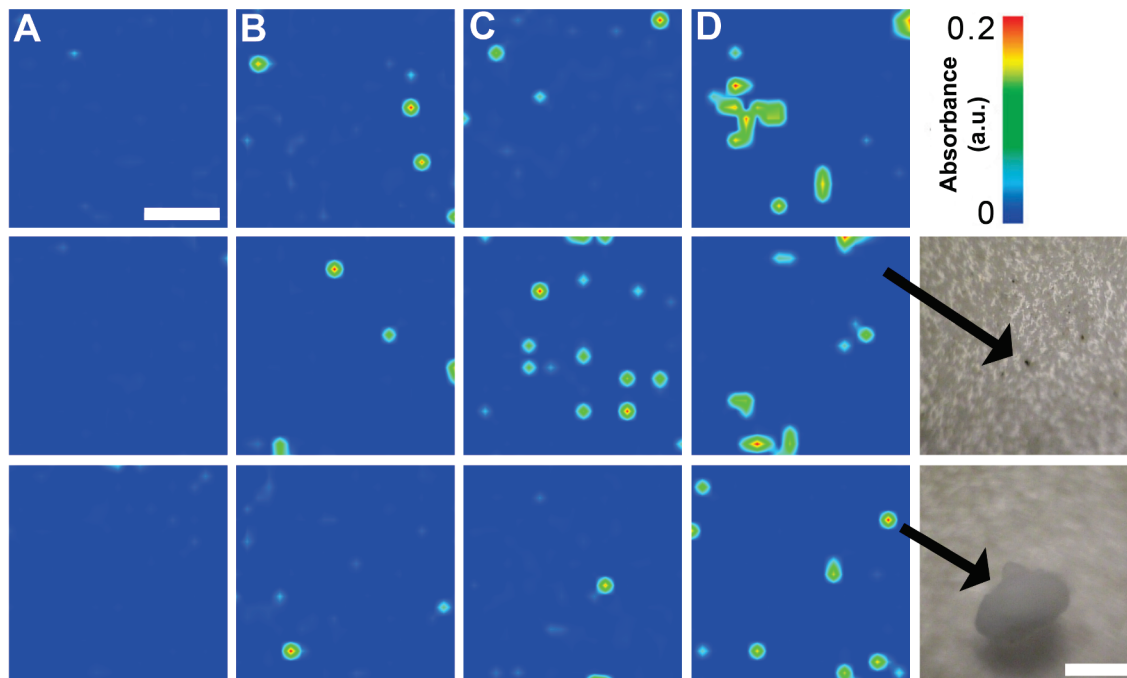
#### **2.4.3.2. Development of mapping protocol**

Prior results described herein have demonstrated that the main absorbance bands of both evenly and irregularly shaped LDPE fragments observed by reflectance micro-FT-IR spectroscopy were intrinsic to the stretching and bending of C-H bonds at 3000 - 2770  $\text{cm}^{-1}$  and 1500 – 1450  $\text{cm}^{-1}$ , respectively (Section 2.4.2). The need to base the identification of microplastics on polymer-specific regions of absorbance that are insensitive to variation in sample shape has also been discussed. Therefore, based on these regions of absorbance, molecular micrographs were obtained for randomly selected surface areas of 3  $\text{mm}^2$ , using retentates of vacuum-filtered sandy sediment

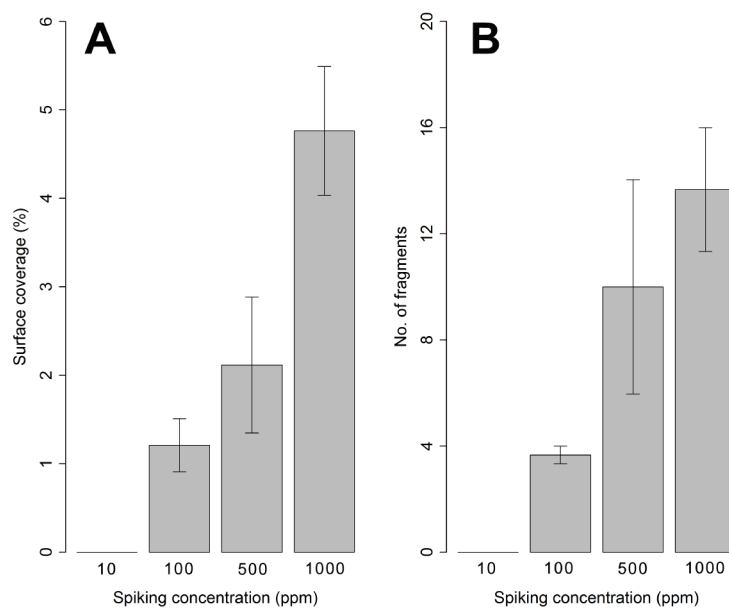
(Lowestoft, UK) on 47-mm polycarbonate membranes, and in the presence and absence of artificially added microplastics. Micrographs were similarly obtained for retentates of non-spiked silty sediment collected from Spurn Point (Humber Estuary, UK) (Figure 2.1). Ultra-high molecular weight (UHMW) PE granules (150- $\mu\text{m}$  diameter) were used for mapping in order to improve the ability of the micro-FT-IR instrument to focus on the plastic fragments.

A comparison of false-colour micrographs across several spiking concentrations of UHMW PE microplastics within retentates of vacuum-filtered sandy sediment (Lowestoft, UK) is presented in Figure 2.6. At the lowest concentration of spiking (10 ppm; Figure 2.6A), no microplastics were detected within the selected areas of 3 mm<sup>2</sup>. This may have been due to the employment of small surface areas for molecular mapping and methodological bias caused by the low and highly variable rates of recovery (%) estimated for UHMW PE fragments retrieved from sediment by flotation ( $\bar{x} = 61 \pm 31$  SD) ( $n = 3$ ). Interestingly, Claessens *et al.* (2011) reported particle recovery rates within a similar range of efficiency. Moreover, mapping was only found to detect 69 ( $\pm 12$  SD) % of the total frequencies of fragments within the visualised areas, most likely due to the reliance of this technique on collecting spectra from small and spatially separated locations. Despite these limitations, mapping successfully detected PE fragments within all examined sediment retentates when the nominal concentration of spiking was 100 ppm or higher. Regions of comparatively high absorbance within the micrographs (Figure 2.6B-D) corresponded to the presence of microplastics, as confirmed by optical images. No evidence was found for interference from the background signal originating from the polycarbonate membrane (Figure 2.6). Moreover, the surface coverage (%) of spectra corresponding to PE in the micrographs increased in proportion with the visually calculated frequencies of microplastics

detected by mapping, as based on light microscopy (Figure 2.7). The calculated errors were also comparable (Figure 2.7).



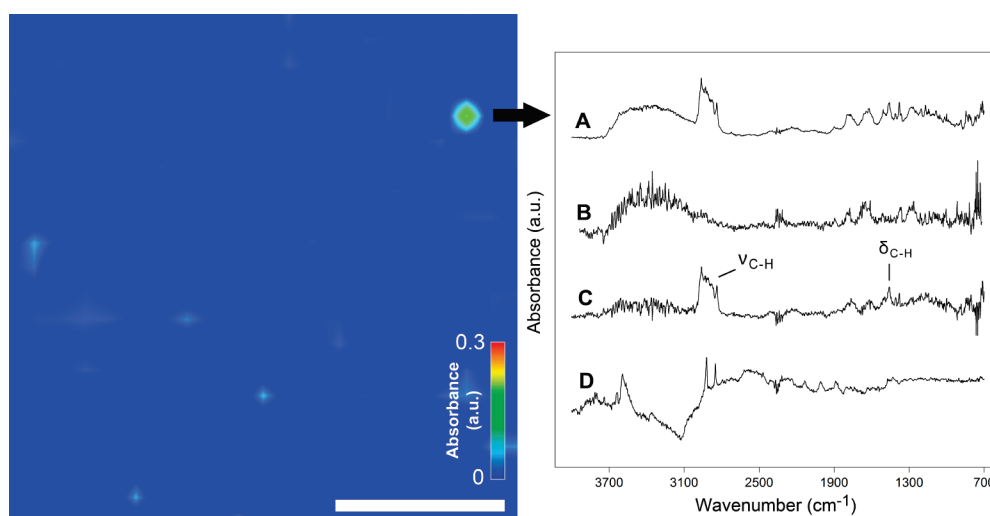
**Figure 2.6.** Molecular micrographs showing artificially added 150- $\mu\text{m}$  UHMW PE fragments in retentates of vacuum-filtered sandy marine sediment (Lowestoft, UK). False-colour images were obtained for sediments spiked with A) 10, B) 100, C) 500 and D) 1000 ppm of microplastics. The micrographs are based on measurements obtained by the integration of absorbance peaks corresponding to both the stretching and bending of C-H bonds (3000 – 2770 and 1500 – 1450  $\text{cm}^{-1}$ , respectively). Analyses were performed in triplicate and represent randomly selected areas of 3  $\text{mm}^2$ . The arrows and optical images show the surface of the polycarbonat membrane filter (above) and an UHMW PE fragment (below). The scale bars are 1 mm for the micrographs and 100  $\mu\text{m}$  for the optical images. Absorbance is recorded as arbitrary units (a.u.).



**Figure 2.7.** Comparison between the A) mean surface coverage (%) of micro-FT-IR spectra corresponding to PE in false-colour micrographs and B) visually calculated frequencies of 150-µm UHMW PE granules detected by molecular mapping. Micrographs were obtained for randomly selected 3-mm<sup>2</sup> surfaces in filtrates of microplastic-spiked sandy sediment (Lowestoft, UK). Error bars represent one standard error ( $n = 3$ ).

Corresponding mapping analyses using non-spiked silty sediment from Spurn Point (Humber Estuary, UK) detected an irregularly shaped polymer fragment with a surface area of approximately 15 µm<sup>2</sup> (Figure 2.8). Whilst the spectrum of this fragment differed from the absorbance profiles typical to the PE particles employed in the present study, this may have resulted from signal interference originating from the surrounding polycarbonate membrane. Interference may have occurred due to the area of the fragment being approximately equal to the scanning aperture (Section 2.4.1). Indeed, subtracting the IR signal of the polycarbonate membrane from that of the detected fragment enabled identification of clear absorbance bands intrinsic to the stretching and bending of C-H bonds (3000 - 2770 cm<sup>-1</sup> and 1500 – 1450 cm<sup>-1</sup>, respectively) within the resulting spectrum (Figure 2.8C). Whilst these bands are characteristic of the IR spectrum of PE (Cooper and Corcoran, 2010), it must be noted that the resulting

absorbance profile was still different from that of the reflectance micro-FT-IR spectrum of the PE granules used in this study (Figure 2.8D). Therefore, a fully conclusive polymer type assignment could not be performed, highlighting the challenges of using reflectance micro-FT-IR spectroscopy for identifying micrometre-scale fragments in environmental samples that are likely to contain a mixture of several different polymer types possessing a broad range of surface morphologies (Browne *et al.*, 2011).



**Figure 2.8.** A molecular micrograph showing an approximately  $15 \times 15 \mu\text{m}$  microplastic fragment in a retentate of vacuum-filtered silty marine sediment (Spurn Point, Humber Estuary, UK). The micrograph is based on measurements obtained by the integration of absorbance peaks corresponding to the stretching and bending of C-H bonds ( $3000 - 2770$  and  $1500 - 1450 \text{ cm}^{-1}$ , respectively). The scale bar is 1 mm. Absorbance spectra are given next to the micrograph, corresponding to A) the detected fragment, B) the surface of the polycarbonate membrane following filtration, C) the difference between the fragment and the polycarbonate membrane, and D) a granule-shaped LDPE fragment (reference). Absorbance is recorded as arbitrary units (a.u.).

Despite the inherent limitations of the present analytical protocol, these results provide the first proof of principle for the feasibility of molecular mapping by reflectance micro-FT-IR spectroscopy for the detection of microplastics in sediment retentates. Further protocol development is now particularly necessary in order to



optimise spectral visualisation methods for the analysis of multiple polymer types in complex environmental matrices and across several sampling sites.

#### **2.4.3.3. Considerations for further research into the imaging of microplastics**

This research has provided the first evidence that reflectance micro-FT-IR spectroscopy and molecular mapping analyses based on utilising a suitable combination of polymer-specific regions of absorbance may be successfully applied to visualise microplastics in both plastic-spiked and untreated sediment retentates. As such, this study provides a foundation for the further development of methodologies for the spectral visualisation of these pollutants in environmental samples.

In order to guide further research into the spectroscopic visualisation of microplastics, it must be recognised that the concentrations of microplastic particles in marine ecosystems are likely to be significantly lower than those employed in these experiments (Barnes *et al.*, 2009), with a maximal concentration of 81 ppm by mass having been reported for coastal sediments (Reddy *et al.*, 2006). Even so, the present protocol successfully detected PE in a non-spiked sediment retentate (see Figure 2.8) and further investigation is now required for the development of molecular mapping and imaging techniques for detecting microplastics at environmentally relevant concentrations. Whilst this approach towards mapping aimed to provide a proof of principle for the spectral visualisation of microplastics, it would be necessary to scan the entire surface of the membrane filter to detect all plastic fragments dispersed within the sediment retentate. Although mapping methods are unfeasible for visualising large surface areas, FPA-based imaging is likely to improve our ability to detect plastics within sediment retentates, because this technique rapidly captures spectra from the entire sample surface instead of small and spatially separated locations (Levin and

Bhargava, 2005). Where very high spatial resolution is required, Raman microspectroscopy may also provide an alternative to FT-IR analyses of microplastics (GESAMP, 2010b; Andrady, 2011; Murray and Cowie, 2011).

Whilst polyethylene was employed as a model polymer in the present study due to its ubiquity in the marine environment (Barnes *et al.*, 2009), FT-IR spectroscopy has also been shown to be capable of identifying several types of plastics in marine sediments across the globe, including acrylic, nylon, polyamide, polyester, polystyrene, polyvinylchloride and propylene (Thompson *et al.*, 2004; Ng and Obbard, 2006; Browne *et al.*, 2011; Claessens *et al.*, 2011). As such, it is now possible to develop working protocols for the spectral visualisation of multiple types of polymers. Since measurements used for the generation of molecular micrographs consist of full-range IR spectra ( $4000 - 700 \text{ cm}^{-1}$ ), several types of plastic may be imaged within a single micrograph by selecting different combinations of absorbance bands characteristic to a given type of plastic, following mapping and/or imaging.

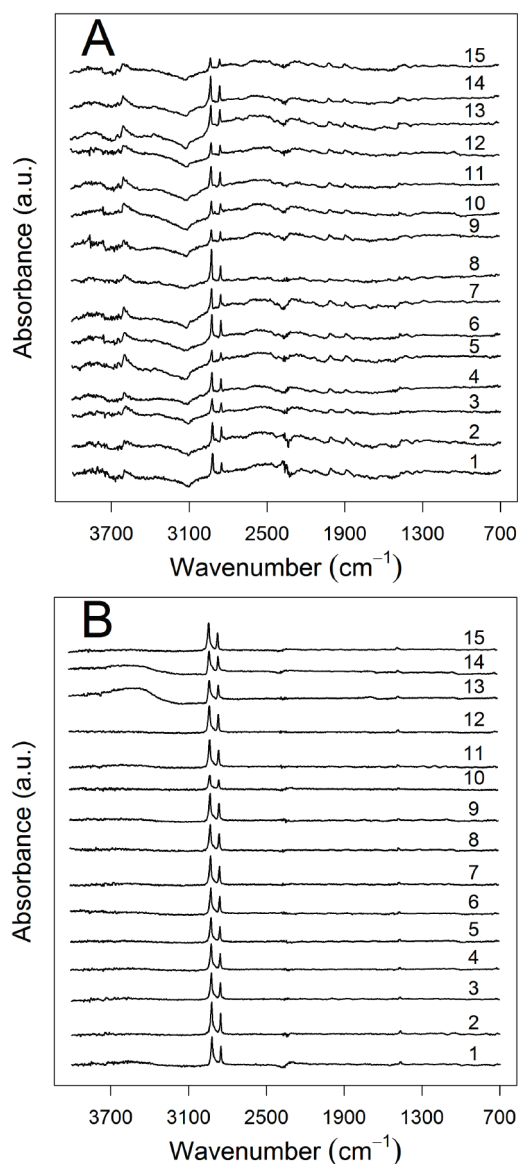
Importantly, due to the low and highly variable rates of recovery obtained by separating microplastics from sediments by flotation (see Sections 2.3.3 and 2.4.3.2), these results highlight the need for more efficient and reproducible methods to separate microplastics from sediments (NOAA, 2009). A coarse filtration step for the removal of meso- and macrolitter has been suggested previously (Andrady, 2011). Additionally, elutriation methods have been proposed as an alternative to the suspension and filtration of microplastics (GESAMP, 2010b). The pre-concentration or repeated extraction of plastics using a given sediment sample prior to filtration may also improve the ability of spectral visualisation techniques to detect these pollutants (Claessens *et al.*, 2011).

#### 2.4.4. Impacts of sampling site on FT-IR spectra

Research by Ng and Obbard (2006) has invoked naturally occurring particulate matter as a source of uncertainty when evaluating the micro-FT-IR spectra of microplastics in sediments. The spectra could also be influenced by plastic-associated pollutants (*e.g.* polynuclear aromatic hydrocarbons) and/or naturally occurring organic materials in marine ecosystems (Mato *et al.*, 2001; Endo *et al.*, 2005; Rios *et al.*, 2007; Teuten *et al.*, 2007; Ogata *et al.*, 2009; Teuten *et al.*, 2009). Therefore it can be speculated that the absorbance spectra of microplastics vary across sampling sites, reflecting differences in the organic composition of the sediment, among several other factors. This study has provided a baseline for further research into this topic by characterising variation in the spectra of artificially added granule- and square-shaped LDPE microplastics across sediments obtained from fifteen offshore sampling sites across the UK coast (Figure 2.1).

Typical LDPE spectra were observed across all sites, irrespective of fragment shape (Figure 2.9). Whilst these data support the potential for reflectance micro-FT-IR spectroscopy to identify artificially added microplastics in a wide range of sediments, it must be recognised that further research is required to confirm their applicability to true (*i.e.* non-spiked) environmental samples, despite the ability of the present molecular mapping protocol to detect a microplastic fragment in a non-spiked sediment retentate (see Figure 2.8). Even so, the polymer-spiked offshore sediments differed considerably in their organic carbon content (Table 2.1). The organic carbon content of sediments is known to influence the partitioning of contaminants between environmental compartments (McGroddy *et al.*, 1996), and it may also affect the uptake of hydrophobic compounds by plastics (Teuten *et al.*, 2007, 2009). Although the samples used in this work were obtained 48 hours following the spiking of sediments with

microplastics, phenanthrene may associate with polyethylene already within 24 hours of exposure (Teuten *et al.*, 2007). Additionally, the organic carbon content of sediments has been found to influence the composition of microbial communities within the seafloor (Sapp *et al.*, 2010), potentially impacting on the formation and structure of plastic-associated biofilms that may interfere with the micro-FT-IR spectra of plastics (Ojeda *et al.*, 2009; Webb *et al.*, 2009; Harrison *et al.*, 2011; Lobelle and Cunliffe 2011).



**Figure 2.9.** The reflectance micro-FT-IR spectra of A) granule-shaped and B) square-shaped LDPE fragments within retentates of vacuum-filtered offshore sediments (UK). Sampling sites are indicated above the spectra and correspond to locations shown in Figure 2.1. Absorbance is recorded as arbitrary units (a.u.).

**Table 2.1.** Organic carbon (OC) content of offshore sediments (UK). Site locations are shown in Figure 2.1.

Site	Mean OC $\pm$ SE (%) <sup>a</sup>
1	1.30 $\pm$ 0.08
2	0.07 $\pm$ 0.01
3	0.52 $\pm$ 0.06
4	0.41 $\pm$ 0.03
5	0.80 $\pm$ 0.14
6	0.59 $\pm$ 0.12
7	0.03
8	Data unavailable
9	0.37 $\pm$ 0.02
10	0.21 $\pm$ 0.01
11	0.72 $\pm$ 0.05
12	0.79 $\pm$ 0.06
13	0.65 $\pm$ 0.04
14	0.09 $\pm$ 0.02
15	0.08 $\pm$ 0.01

<sup>a</sup> Measured for size fractions of <2

mm ( $n = 5$ ). Values reported with

reference to sediment dry weight.

Although no spectral interference was detected due to the signal originating from organic materials when analysing PE fragments that had been manually added to sediments across all seventeen sampling sites in this study, digestion by a dilute mineral acid has been suggested as a potential method for the removal of organic impurities from polymer surfaces where necessary (Andrady, 2011). However, the spectra of synthetic plastics are frequently possible to distinguish from those of biological

materials because the former are characterised by the presence of sharp, highly specific and well-defined absorbance due to their repetitive structural units, as compared with the broader and more complex range of signals exhibited by other organic materials.

Despite variation in the organic carbon content of sediments across sampling sites, the present protocol for the micro-FT-IR analysis of microplastics in sediment retentates (see Section 2.3) provided consistent measurements. The results are also promising with reference to the molecular mapping experiment described in this study (Section 2.4.3), supporting these attempts to provide an experimental basis for the development of an optimised method for the reflectance micro-FT-IR analyses of microplastics. These results represent the first important step towards understanding the applicability of reflectance micro-FT-IR spectroscopy for the routine analysis of microplastics across different geographic locations and environmental matrices.

## **2.5. Conclusions**

This work has described the development and experimental evaluation of a protocol for the micro-FT-IR analysis of artificially added microplastics in vacuum-filtered retentates of marine sediments. Whilst the method produced reliable measurements across a broad range of geographic locations, micro-FT-IR analyses in reflectance mode must account for the potential for sample morphology to influence the spectra of microplastics. Where necessary, the employment of alternative spectroscopic techniques (*e.g.* ATR-FT-IR spectroscopy) may increase our ability to interpret the spectra of irregularly shaped microplastics in environmental samples. Additionally, this study has provided the first evidence for the ability of molecular mapping to successfully detect microplastics in sediment retentates, as based on their spectral characteristics and without the need for visual selection of fragments for characterisation. Despite the

inherent limitations of mapping techniques, microplastics were successfully detected both in the presence of manually added microplastics and in a non-spiked sediment retentate.

In conclusion, the present research provides a foundation for research into the development of improved FT-IR-based methodologies for the detection of microplastics in complex matrices. Specifically, FPA-based imaging may be used to rapidly scan the entire surface of the retentate, increasing the sensitivity and spatial resolution of the present protocol. Moreover, since these results were obtained by scanning small surface areas and typically by spiking sediments with high concentrations of polyethylene, there is a need to further verify the utility of spectral visualisation techniques with reference to untreated field samples and other polymer types. Finally, since spectroscopic analyses of microplastics ultimately depend on methods of sample collection, this study highlights the immediate need to develop more efficient and reproducible protocols for the separation of these pollutants from sediments.

## **2.6. Acknowledgements**

Sediments from offshore sampling sites (see Fig. 2.1) were collected and provided by the Centre for Environment, Fisheries and Aquaculture Science (Cefas). Measurements of sediment organic carbon content were performed by Cefas.

# **Chapter 3**

## **Rapid Bacterial Colonisation of Low-density Polyethylene Microplastics in Coastal Marine Sediments**

Aspects of this research have been submitted for publication as:

Harrison, J.P., Schratzberger, M., Sapp, M. and Osborn, A.M. Rapid bacterial colonization of low-density polyethylene microplastics in coastal marine sediments.

(Submitted) *FEMS Microbiology Ecology*.



### 3.1. Abstract

Synthetic microplastics ( $\leq 5$ -mm fragments) are now recognised as widespread environmental contaminants, with their ecological impact and fate only beginning to be revealed. Understanding of the interactions between microplastics and microorganisms that provide essential life-support functions in natural ecosystems is negligible. In this study, a laboratory microcosm experiment was used to investigate the colonisation of low-density polyethylene (LDPE) microplastics by bacteria within three types of coastal marine sediment from Spurn Point, Humber Estuary, UK. Scanning electron microscopy and terminal-restriction fragment length polymorphism (T-RFLP) analysis demonstrated rapid selection of LDPE-associated bacterial assemblages within the plastic-sediment interface, whose structure and taxonomic composition differed significantly from those in surrounding sediments. Moreover, T-RFLP analysis revealed successional convergence of the LDPE-associated communities from the different sediments over the 14-day experiment. Sequencing of cloned 16S rRNA genes revealed that these LDPE-associated communities were dominated after 14 days by location-specific populations of the genera *Arcobacter* and *Colwellia* (84-93% of sequences). These results represent the first culture-independent assessment of the potential for microplastics to function as sites for bacterial colonisation in coastal sediments. As such, this study opens an avenue for further microbial research into the formation of plastic-associated biofilms in marine sediments, and the environmental impacts of microplastic particles in the benthic marine environment.

### 3.2. Introduction

Plastic debris is globally distributed across estuarine and marine ecosystems (Carpenter and Smith, 1972; Colton *et al.*, 1974; Morris and Hamilton, 1974; Wong *et al.*, 1974; Moore *et al.*, 2001; Law *et al.*, 2010; Browne *et al.*, 2011; Hidalgo-Ruz *et al.*, 2012), reflecting the success of synthetic polymers as both consumer and industrial products, and their persistence in the environment (Barnes *et al.*, 2009). Worldwide manufacture of thermoplastics has increased from less than two million tonnes in 1950 to between 230 and 245 million tonnes during the last decade (Andrady and Neal, 2009). There is now widespread public concern about the ecological impacts of plastic waste on marine organisms. Whilst the physical impacts caused by plastic debris (for example, entanglement and suffocation of wildlife) are well-recognised (Gregory, 2009), the rapid proliferation of microplastics ( $\leq 5$ -mm fragments) in marine habitats (Colton *et al.*, 1974; Thompson *et al.*, 2004; Hidalgo-Ruz *et al.*, 2012) is leading to a re-evaluation of the potential detrimental effects of plastic litter (Andrady, 2011). Microplastics represent both a physical and chemical threat to the ecological integrity of our seas and oceans (Barnes *et al.*, 2009) due to their high bioavailability and capacity to transport persistent organic pollutants (*e.g.* polynuclear aromatic hydrocarbons) and plastic additives into marine food webs (Carpenter *et al.*, 1972; Teuten *et al.*, 2009). This is particularly true for coastal and intertidal sediments that represent sinks for the accumulation of plastic litter (Thompson *et al.*, 2004; Reddy *et al.*, 2006; Barnes *et al.*, 2009; Browne *et al.*, 2011; Claessens *et al.*, 2011; Cole *et al.*, 2011), where microplastic concentrations may reach up to 81 parts per million by mass (Reddy *et al.*, 2006) and constitute as much as 80% of plastic debris within the seafloor (Browne *et al.*, 2007).

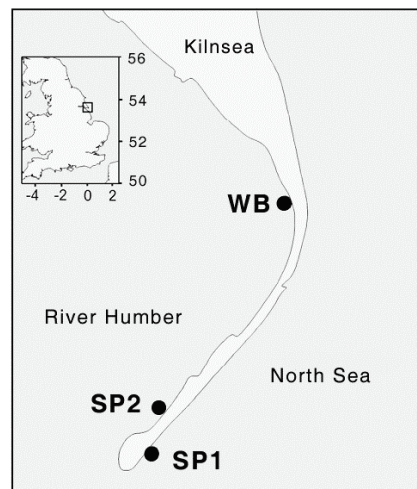
Most previous research into the environmental impacts of microplastics has focused either on their abundance and distribution (Barnes *et al.*, 2009; Hidalgo-Ruz *et al.*, 2012) or on their potential detrimental effects on higher organisms (Derraik, 2002). In marine environments, microorganisms function as pioneering surface colonists and drive critical ecosystem processes including primary production, biogeochemical cycling and the biodegradation of anthropogenic pollutants (Dang and Lovell, 2000; Dang *et al.*, 2008). However, ecological interactions between marine microorganisms and microplastics have received scant attention, with our understanding of this topic being limited primarily to pelagic habitats (Harrison *et al.*, 2011). Initial observations of the microbial colonisation of microplastics in seawater have reported the isolation of ‘rod-shaped Gram-negative bacteria’ from ~0.5-mm polystyrene spherules (Carpenter *et al.*, 1972) and the presence of diatoms on plastic fragments in the Sargasso Sea (Carpenter and Smith, 1972). Culture-based seawater microcosm studies have also demonstrated microbial attachment to polyethylene terephthalate (Webb *et al.*, 2009) and polyethylene plastic bags (Lobelle and Cunliffe, 2011). Moreover, experiments using molecular fingerprinting and 16S rRNA gene clone sequencing analyses have identified *Roseobacter* spp. and other Alphaproteobacteria as key colonists of acryl, polyurethane, poly(methyl methacrylate) and polyvinylchloride surfaces, following up to 72-hour exposures of these materials in coastal waters (Dang and Lovell, 2000; Dang *et al.*, 2008; Lee *et al.*, 2008). Bacterial association with plastic surfaces has also been reported in engineered ecosystems, including drinking water distribution systems (Assanta *et al.*, 2002; Batté *et al.*, 2003). Despite these initial reports there remains a lack of knowledge concerning microbial colonisation of (micro)plastic debris within marine environments, and in particular, there is an absence of any information concerning microbial-plastic interactions and colonisation processes within marine sediments (Harrison *et al.*, 2011; Lobelle and Cunliffe, 2011).

Hence, in this study, a 14-day laboratory microcosm experiment was used to investigate the potential for microplastics to function as sites for attachment of naturally occurring bacterial assemblages within coastal marine sediments. Specifically, this research aimed firstly to investigate variation in the structure and diversity of plastic-colonising bacterial assemblages over time and across both sandy and silty sediment types, and secondly to identify the predominant bacterial genera within the plastic-sediment interface. Low-density polyethylene (LDPE) was chosen as the model polymer, due to its importance both as a commercial product and as a widely documented component of marine plastic debris (Andrady and Neal, 2009; Barnes *et al.*, 2009; Browne *et al.*, 2011; Hidalgo-Ruz *et al.*, 2012).

### **3.3. Materials and Methods**

#### **3.3.1. Sediment sampling and characterisation**

Sediments were collected on the 25<sup>th</sup> of April 2010 from three sampling sites (SP1, SP2 and WB) within the Humber Estuary (UK) (Figure 3.1). Samples were obtained from the surface top centimetre of the sediment. Sediments were stored either overnight in darkness at 4 °C prior to use in laboratory microcosms, or at -20 °C for sedimentological analysis. Samples for sedimentological analysis were dried at 105 °C and analysed for loss on ignition (LOI) (%) and particle size distribution (PSD). The LOI measurements were based on changes in mass following heating at 425 °C for 18 hours. Sediment size fractions of <1 mm were analysed for PSD using a LA-950 particle sizer (Horiba Instruments Ltd, Northampton, UK), using 0.1 M sodium hexametaphosphate as a dispersant (Langford *et al.*, 2010). The sediments were predominantly comprised of fine sand (SP1), medium sand (SP2) and silt (WB) (Table 3.1).



**Figure 3.1.** Locations of field sites used for sediment sampling at Spurn Point, Yorkshire, UK. The regional location of the sampling sites within the UK is shown in the inset together with eastern latitude and northern longitude ( $^{\circ}$ ), as indicated.

**Table 3.1.** Particle size distribution (PSD) and loss on ignition (LOI) for coastal marine sediments from three sites (SP1, SP2 and WB) at Spurn Point, UK. Values are given in duplicate for the <1-mm fraction. The dominant particle size fraction for each sediment type is highlighted in bold.

Site	Sieved fractions (%)		<1 mm fraction (%)*					LOI (%)
	> 2 mm	1 – 2 mm	Coarse sand	Medium sand	Fine sand	Silt	Clay	
SP1	1.8	2.1	1.8 / 2.3	31.1 / 31.6	<b>66.8 / 65.8</b>	0.4 / 0.3	Absent	1.0
SP2	1.4	1.0	24.4 / 21.7	<b>65.1 / 64.7</b>	10.2 / 13.4	0.3 / 0.2	Absent	1.7
WB	Absent	Absent	0.5 / 0.6	9.5 / 9.8	24.8 / 25.6	<b>57.0 / 59.6</b>	8.1 / 4.4	10.1

\* <1-mm fraction comprised >96% of sediment particles from all sampling sites

### 3.3.2. Sediment-LDPE microcosms

Sediments were homogenised by stirring and microcosms were established by weighing 5 – 7 ml of homogenised sediment into sterile, triple-vented 55-mm Petri dishes. Each microcosm was spiked with six fragments ( $1 \times 5 \times 5$  mm) of LDPE (Goodfellow Cambridge Ltd, Huntingdon, UK) that had been sterilised with 70% ethanol. The sediments were submerged in sterile artificial seawater (ASW) (ZM Systems, Winchester, UK). The salinity of the ASW was based on *in situ* measurements (33 at SP1 and 30 at SP2 and WB) taken using a Portasal<sup>TM</sup> 8410A salinometer (Guildline,

Smiths Falls, Canada). Microcosms were incubated in darkness at 4 °C (based on water temperature at the sites at the time of sediment sampling). Sacrificial sampling of plastic fragments was performed in triplicate at seven intervals (immediately, at 6 hours and after 1, 2, 4, 7 and 14 days). The plastics were suspended, rinsed in ASW and centrifuged for five seconds (up to  $2038 \times g$ ). LDPE fragments were stored at -80 °C for molecular analyses or fixed overnight in 2% (v / v) formaldehyde at 4 °C for scanning electron microscopy. Following fixation, fragments were rinsed with ASW and 96% ethanol, and stored at -20 °C. Sediment samples were taken for molecular analysis from microcosms sampled at four time intervals (immediately and after 4, 7 and 14 days), and stored at -80 °C.

### **3.3.3. Scanning electron microscopy**

Following an initial fixation step using 2% (v / v) formaldehyde (Section 3.3.2), LDPE fragments were secondarily fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for two hours at room temperature. The fragments were washed twice for 30 minutes with 0.1 M sodium cacodylate buffer and post-fixed in 2% osmium tetroxide for one hour. Samples were dehydrated by a graded series of 15-minute exposures to ethanol, involving (1) 75%, (2) 90% and (3, 4) absolute ethanol, and (5) absolute ethanol over anhydrous copper sulphate. LDPE fragments were then placed in a 50 / 50% (v / v) mixture of absolute ethanol and hexamethyldisilazane for 30 minutes, followed by a 30-minute immersion in hexamethyldisilazane (Robards and Wilson, 1993). The samples were coated with ~25 nm of gold using an Edwards S150B sputter coater. Images were obtained with an XL-20 scanning electron microscope (Philips / FEI, Cambridge, UK) at an accelerating voltage of 20 kV.

### 3.3.4. DNA isolation and PCR amplification of 16S rRNA genes

DNA was isolated from either six pooled LDPE fragments (total surface area of 4.2 cm<sup>2</sup>) or from 0.5 g of sediment using a Powersoil<sup>®</sup> DNA isolation kit (MO BIO, Carlsbad, CA), and eluted in either 50 µl or 100 µl of sterile nuclease-free water (LDPE and sediment, respectively) (Ambion, Austin, USA). PCR was used to amplify 16S rRNA gene sequences for T-RFLP analyses (Liu *et al.*, 1997; Osborn *et al.*, 2000) and for the construction of 16S rRNA gene clone libraries. For T-RFLP fingerprinting, the primers FAM-63F (5'- CAG GCC TAA CAC ATG CAA GTC -3') (Marchesi *et al.*, 1998) and 1389R (5'- ACG GGC GGT GTG TAC AAG -3') (Osborn *et al.*, 2000) were used. For library construction, primers 27F (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1492R (5'- TAC CTT GTT ACG ACT T -3') were used (Lane, 1991). Each PCR contained 1 - 2 µl of template DNA, 1 × PCR buffer containing 1.5 mM of MgCl<sub>2</sub>, 2 × Q Solution (Qiagen, Crawley, UK), 0.25 mM of each deoxyribonucleoside triphosphate (dNTP), 0.4 µM of each primer and 2.5 U of *Taq* polymerase (Qiagen) made up to a total volume of 50 µl with sterile nuclease-free water. For T-RFLP analyses, PCR cycling conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 57 °C for 45 s, 72 °C for 90 s and a final extension step at 72 °C for 10 min. For amplification of 16S rRNA genes prior to clone library construction, PCR cycling conditions were 94 °C for 2 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 45 s, 72 °C for 2 min and a final extension step at 72 °C for 10 min. PCR products were visualized following electrophoresis on 0.8% (w / v) agarose gels. PCR products used for T-RFLP analyses were purified using QIAquick columns (Qiagen).

### 3.3.5. Terminal-restriction fragment length polymorphism (T-RFLP) analysis

Purified PCR products (5 – 10 µl) were digested with 20 U of AluI and 1 × restriction enzyme buffer (Roche, Burgess Hill, UK) in a total volume of 15 µl at 37 °C for three

hours. Digestion products (5  $\mu$ l) were desalted using 0.2 mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  and 5  $\mu$ g glycogen (20 mg  $\text{ml}^{-1}$ ) (Bioline, London, UK) in 70% ethanol. Desalted digests (1 – 4  $\mu$ l) were denatured with formamide containing 0.5% GeneScan™ 500 ROX™ internal size standard (Applied Biosystems, Warrington, UK) in a total volume of 10  $\mu$ l and incubated at 94 °C for 3 min prior to electrophoresis using an ABI 3730 PRISM® Genetic Analyser (Applied Biosystems), with injection times of 5 or 10 s and an injection voltage of 2 kV. Capillary electrophoresis was conducted at 15 kV for 20 minutes.

### 3.3.6. 16S rRNA gene clone library construction and sequencing

Purified PCR products were ligated into the pCR4®-TOPO® TA cloning vector and transformed into One Shot® chemically competent *Escherichia coli* TOP10 cells (Invitrogen, Paisley, UK). Transformants were selected on Luria-Bertani (LB) agar plates containing ampicillin (50  $\mu$ g  $\text{ml}^{-1}$ ) and X-gal (80  $\mu$ g  $\text{ml}^{-1}$ ). Insert DNA from white colonies was amplified using the vector primers T7 (5'- TAA TAC GAC TCA CTA TAG G-3') and T3 (5'- AAT TAA CCC TCA CTA AAG G -3'). Each PCR contained 1  $\mu$ l of overnight culture, 1  $\times$  PCR buffer, 1.5 mM of  $\text{MgCl}_2$ , 0.25 mM of each dNTP, 0.4  $\mu$ M of each primer and 2.5 U of *Taq* polymerase (Bioline), and was made up to a final volume of 25  $\mu$ l with sterile nuclease-free water (Ambion). The PCR was performed as described for the primers 27F and 1492R (see Section 3.3.4), using 25 cycles. PCR products were purified using the SureClean PCR purification kit (Bioline). Sequencing analysis was performed using 0.5  $\mu$ M of primer 27F, a BigDye® Terminator v3.1 cycle sequencing kit and an ABI 3730 PRISM® Genetic Analyser (Applied Biosystems).



### 3.3.7. Bioinformatics and multivariate analyses

T-RFLP profiles were analysed using Genemapper<sup>®</sup> (version 3.7, Applied Biosystems). Sizes of terminal restriction fragments (T-RFs) (50 - 500 nucleotides) were estimated using the Local Southern method (Southern, 1979). Peaks represented by fluorescence intensities of <100 units were excluded from further analyses. Remaining T-RFLP profiles were aligned using the software T-Align (<http://inismor.ucd.ie/~talign/>) (Smith *et al.*, 2005). The relative abundance of each T-RF was calculated as a proportion (%) of the total peak area within each profile. Peaks with relative areas of <0.5% were excluded. Square root-transformed Bray-Curtis similarity matrices based on the T-RFLP data were analysed using the PRIMER statistical package (version 6.1.13) (Clarke and Gorley, 2006) for non-parametric multidimensional scaling ordinations, analyses of similarity (ANOSIM) (Clarke, 1993), and Shannon's diversity (Shannon, 1948). A two-way ANOSIM was performed with 'time' (of exposure of LDPE microplastics within sediment) and 'substrate type' (sediments and plastic-sediment interface) as factors. One-way ANOSIMs were conducted with 'sediment type' or 'time' (of exposure of LDPE microplastics within sediment) as the factors. One-way ANOVAs for comparing Shannon's diversity were performed using the R statistical package (version 2.12.0) (R Development Core Team, 2010), with 'exposure time' as the factor.

DNA sequences of cloned 16S rRNA genes were edited using ChromasPro software (version 1.5; <http://www.technelysium.com.au>). Multiple alignments were constructed using ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), with chimeric sequences excluded using Mallard (version 1.02) (Ashelford *et al.*, 2006) and Bellerophon (Huber *et al.*, 2004). Alignments were inspected for anomalous reads and trimmed to a universal read length with Mothur (version 1.17.2) (Schloss *et al.*, 2009).

Taxonomic assignments were performed using the Ribosomal Database Project Classifier (version 6) (Wang *et al.*, 2007). Sequences were compared to the GenBank database using the Basic Local Alignment Search Tool for nucleotides (BLASTn) (Altschul *et al.*, 1990). Neighbour-joining trees (Saitou and Nei, 1987) were constructed using MEGA (version 5.03) (Tamura *et al.*, 2011). Evolutionary distances were calculated using the Kimura 2-parameter method (Kimura, 1980). Rate variation among sites was modelled by a gamma distribution with tree-specific shape parameters, as based on the maximum-likelihood fits of different nucleotide substitution models. Positions containing gaps and missing data were eliminated. Confidence levels for the tree topology were assessed by bootstrap analysis (1000 replicates).

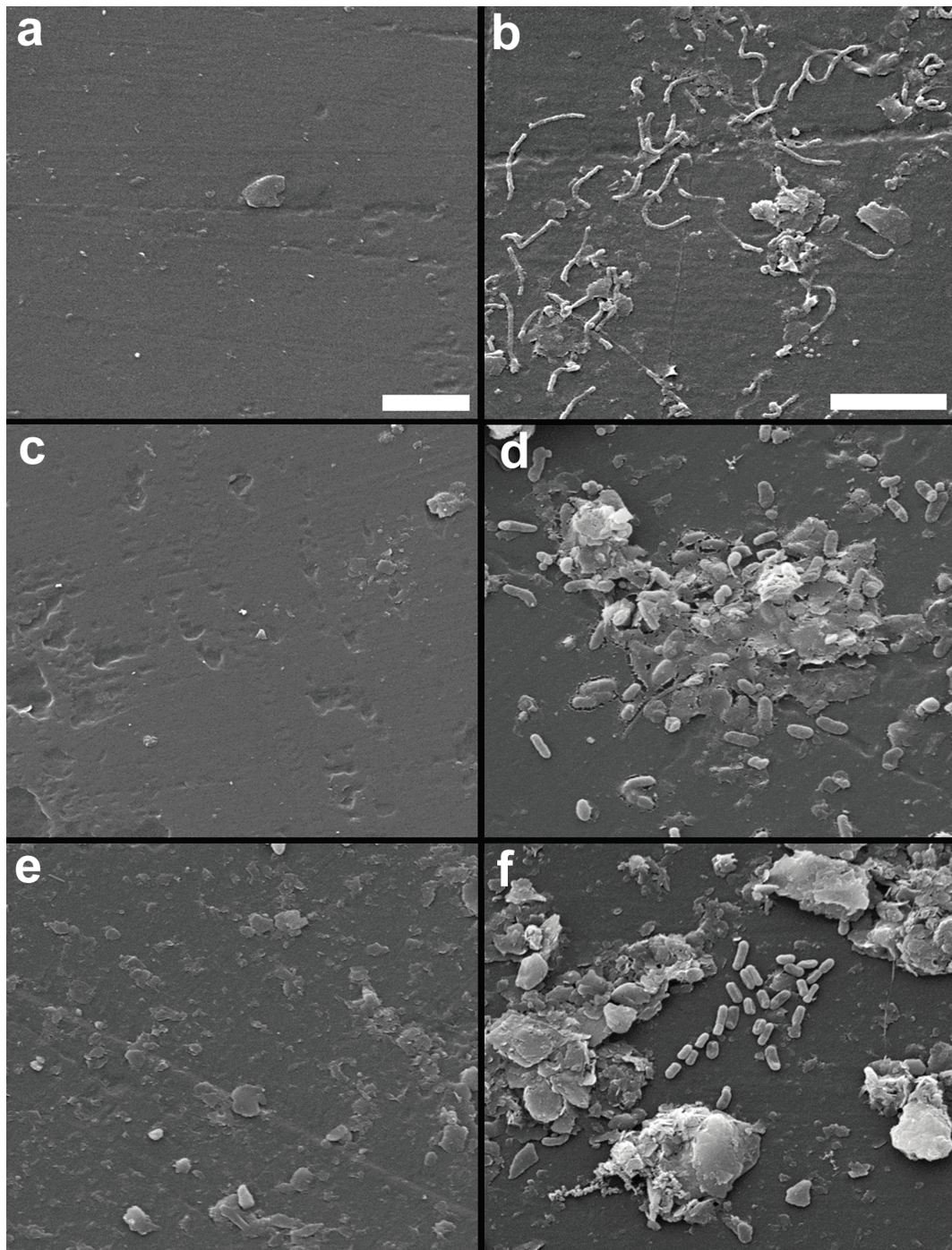
### **3.3.8. Accession numbers**

16S rRNA gene sequences have been submitted to the GenBank Database under accession numbers JF928573 to JF928823.

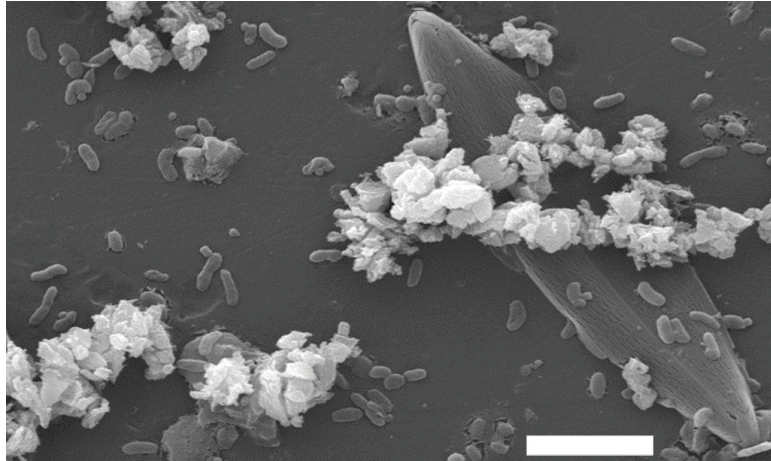
## **3.4. Results**

### **3.4.1. The structure and diversity of LDPE-associated bacterial communities**

Scanning electron microscopy was used to examine microplastics obtained from sediment-LDPE microcosms either immediately or following 14 days of exposure to either sandy or silty sediment types (sites SP1, SP2 and WB; see Table 3.1). Attachment of morphologically diverse prokaryotic cells (rod- and spirilla-shaped) was observed onto both the LDPE surface and also within the plastic-sediment interface in all sediment types (Figure 3.2), with additional attachment by pennate diatoms (Figure 3.3).



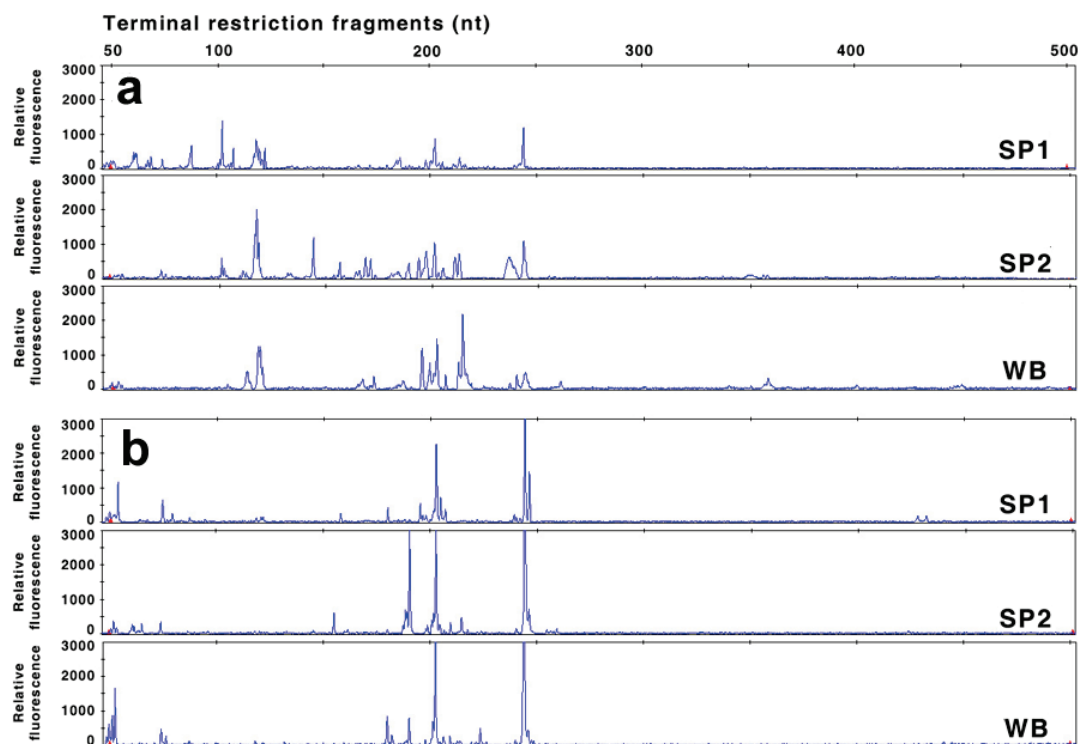
**Figure 3.2.** Scanning electron micrographs showing prokaryotic attachment onto the LDPE-sediment interface. Images were taken of LDPE microplastic fragments sampled from microcosm experiments containing coastal marine sediment from three sites: SP1 (a and b), SP2 (c and d) and WB (e and f) at Spurn Point (UK), sampled either immediately or after 14 days (left-hand and right-hand panels, respectively). The scale bars are 5  $\mu\text{m}$ .



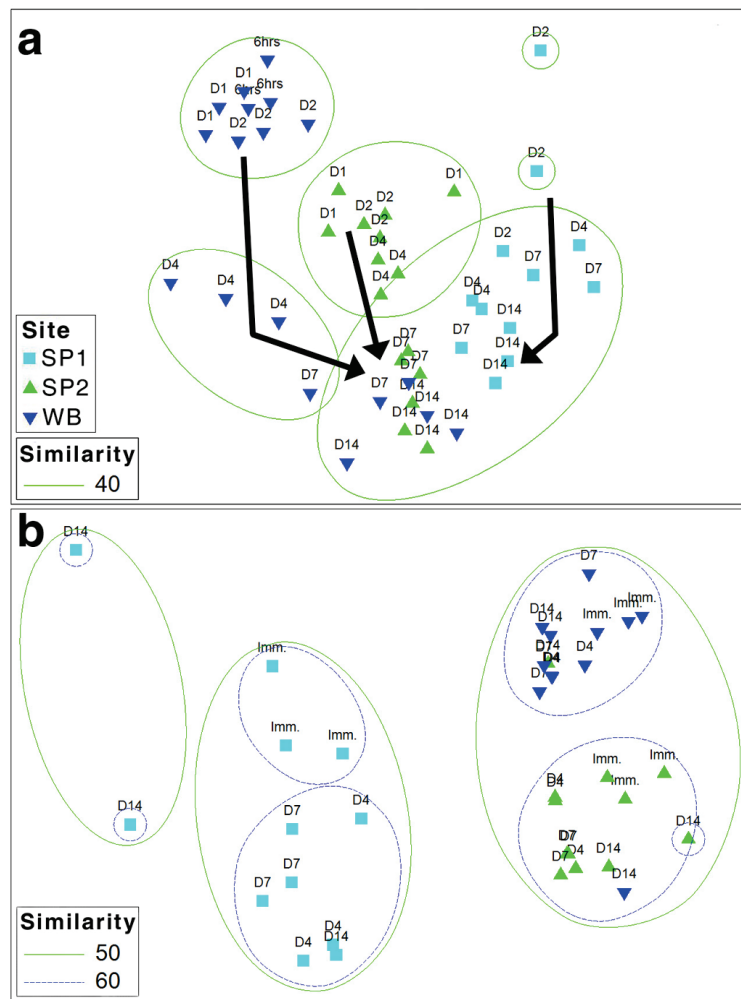
**Figure 3.3.** Scanning electron micrograph showing attachment by prokaryotic cells and an unidentified pennate diatom onto the LDPE-microplastic surface. The image is of LDPE sampled after 14 days from a microcosm experiment containing coastal marine sediment from Spurn Point, UK (site SP2). The scale bar is 5  $\mu\text{m}$ .

Bacterial communities associated with LDPE surfaces differed significantly from those within the sediments, as demonstrated by terminal-restriction fragment length polymorphism (T-RFLP) analysis of 81 AluI-digested PCR products derived from DNA isolated from individual sediment-LDPE microcosms (two-way ANOSIM, global  $R = 0.71$ ,  $p < 0.001$ ; see Figure 3.4). Initially (*i.e.* following two days of exposure to sediments), sediment type-specific bacterial communities were found within the LDPE-sediment interface (one-way ANOSIM, global  $R = 0.67$ ,  $p = 0.04$ ; Fig. 3.5a). Subsequently, significant variation was observed in the structure of the LDPE-associated bacterial communities during the 14-day experiment (Table 3.2). Specifically, there were significant shifts in the structure of LDPE-associated bacterial communities by Days 7 and 14 of the experiment, with notable convergence in the structure of these communities across the three sediment types (Fig. 3.5a). One-way ANOSIM  $R$  decreased from  $R = 0.67$  ( $p < 0.001$ ) to  $R = 0.01$  (not significant), when comparing differences between LDPE-associated communities at Day 2 vs. Day 14 and Day 7 vs. Day 14, respectively (Table 3.2). In contrast, sediment bacterial communities

from each site remained significantly different from each other throughout the 14-day experiment (one-way ANOSIM, global  $R = 0.72$ ,  $p < 0.001$ ; Fig. 3.5b). Moreover, no significant temporal variation was observed in the structure of the sediment bacterial communities (Table 3.2). Bacterial communities within the LDPE-sediment interface became significantly less diverse over time (one-way ANOVA of Shannon's diversity,  $F = 4.69$ ,  $p = 0.008$ ,  $d.f. = 3, 32$ ) (see Table 3.3). In contrast, no significant temporal shifts were observed in the diversity of sediment bacterial communities (one-way ANOVA of Shannon's diversity,  $F = 2.12$ ,  $p = 0.117$ ,  $d.f. = 3, 32$ ) (Table 3.2).



**Figure 3.4.** Representative T-RFLP electropherograms of bacterial communities in a) coastal marine sediments and b) within the LDPE-sediment interface. T-RFLP profiles were generated following PCR amplification of bacterial 16S rRNA genes amplified from DNA isolated from sediment-LDPE microcosms from three sampling sites (SP1, SP2 and WB, as indicated), sampled after 14 days.



**Figure 3.5.** Non-metric multidimensional scaling (nMDS) ordinations of sediment and LDPE-associated bacterial communities in sediment microcosms over time. The ordinations were derived from a Bray-Curtis resemblance matrix calculated from square-root-transformed terminal restriction fragment (T-RF) relative abundance data. Data are shown for bacterial communities within a) the LDPE-sediment interface (stress = 0.16) and b) sediments (stress = 0.13). Labels correspond to samples taken over time [immediately (Imm.), after 6 hours, and on days (D) 1, 2, 4, 7 and 14] from microcosms containing coastal marine sediments from three sites (SP1, SP2 and WB, as indicated). Similarity thresholds (%) are based on group-average clustering. Arrows indicate the temporal trajectory of bacterial community succession with the LDPE-sediment interface.

**Table 3.2.** Pairwise comparisons of the structure of bacterial communities within the LDPE-sediment interface and sediments over time. Global one-way ANOSIM R values for the factor ‘exposure time’ are derived from T-RFLP datasets generated following PCR amplification of bacterial 16S rRNA genes amplified from DNA isolated from sediment-LDPE microcosms from three sampling sites (SP1, SP2 and WB). Values are shown only for sampling intervals for which data were available for all three sites.

Exposure time	LDPE-sediment interface			Sediment		
	2 days	4 days	7 days	Immediate	4 days	7 days
4 days	0.10 *			0.08 (N.S.)		
7 days	0.46 *	0.07 (N.S.)		0.12 (N.S.)	0.09 (N.S.)	
14 days	0.67 *	0.38 *	0.01 (N.S.)	0.12 (N.S.)	0.00 (N.S.)	0.03 (N.S.)

\*  $P < 0.001$  N.S. = not significant

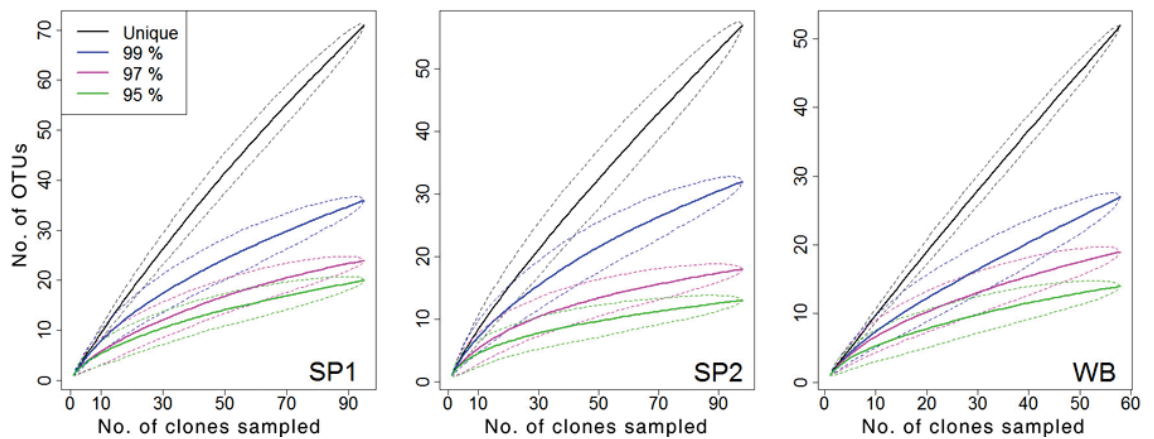
**Table 3.3.** Shannon’s diversity indices ( $H'$ ) for bacterial assemblages within sediments and the LDPE-sediment interface over time. The values are derived from T-RFLP datasets generated following PCR amplification of bacterial 16S rRNA genes amplified from DNA isolated from sediment-LDPE microcosms from three sampling sites (SP1, SP2 and WB). The data are given as mean  $\pm$  SE ( $n = 3$ ).

Substrate	Sampling interval				
	Immediate	2 days	4 days	7 days	14 days
Sediment	3.41 $\pm$ 0.05	N/A **	3.23 $\pm$ 0.07	3.26 $\pm$ 0.05	3.31 $\pm$ 0.04
LDPE-sediment interface	N/A *	3.26 $\pm$ 0.11	3.05 $\pm$ 0.10	2.88 $\pm$ 0.09	2.80 $\pm$ 0.08

\* Data not available for all sampling sites \*\* No samples collected

### 3.4.2. 16S rRNA gene sequence analyses of LDPE-associated bacterial communities

Clone libraries were constructed of PCR-amplified 16S rRNA genes from LDPE fragments from sites SP1, SP2 and WB, following fourteen days of exposure to either sandy or silty sediment types (see Table 3.1). A total of 251 sequences were generated across the three libraries. Rarefaction curves (Figure 3.6) displayed a tendency for curvilinearity when using operational taxonomic unit (OTU) designations based on 95, 97 or 99% sequence similarity. Moreover, Good’s coverage estimates of  $>75\%$  were typically obtained for OTU designations at these levels of sequence identity (Table 3.4).



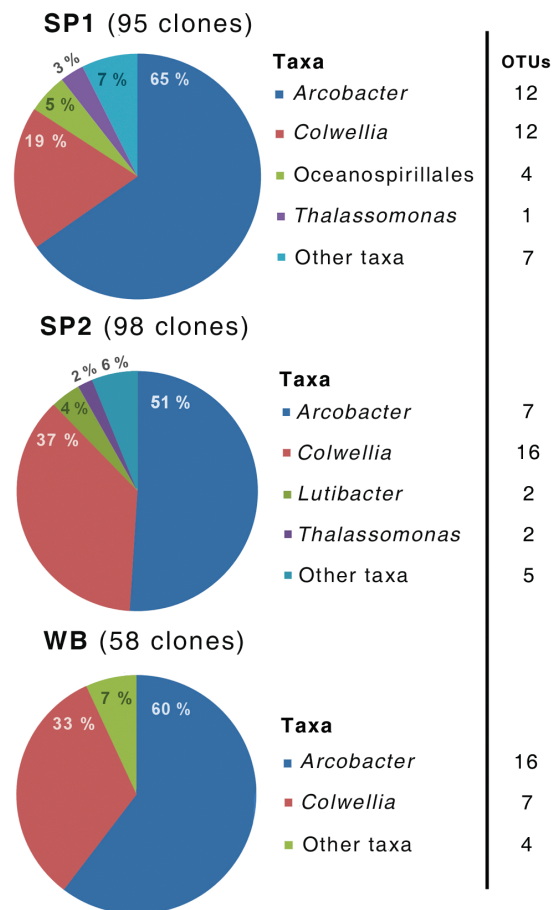
**Figure 3.6.** Rarefaction curves for bacterial 16S rRNA gene clone libraries of LDPE-associated assemblages. Clone libraries were generated following PCR amplification of bacterial 16S rRNA genes amplified from DNA isolated from sediment-LDPE microcosms from three sampling sites (SP1, SP2 and WB, as indicated), sampled after 14 days. Rarefaction curves are shown for operational taxonomic unit (OTU) designations for unique sequences and for OTUs based on similarity cut-off thresholds ranging from 99 to 95%, following removal of chimeric sequences. The dashed lines represent 95% confidence intervals.

**Table 3.4.** Good's coverage estimates for 16S rRNA gene clone libraries. Values are given for operational taxonomic unit (OTU) designations for unique sequences and for OTUs based on similarity cut-off thresholds ranging from 99 to 95%, following removal of chimeric sequences. 16S rRNA gene clone libraries were generated from the LDPE-sediment interface following 14-day laboratory microcosm experiments in coastal marine sediments from three sites (SP1, SP2 and WB).

Site	Number of clones	Coverage estimates (%) for different OTU designations			
		Unique sequences	99%	97%	95%
SP1	95	38.9	76.8	87.4	88.4
SP2	98	51.0	81.6	91.8	93.9
WB	58	15.5	63.8	82.8	87.9

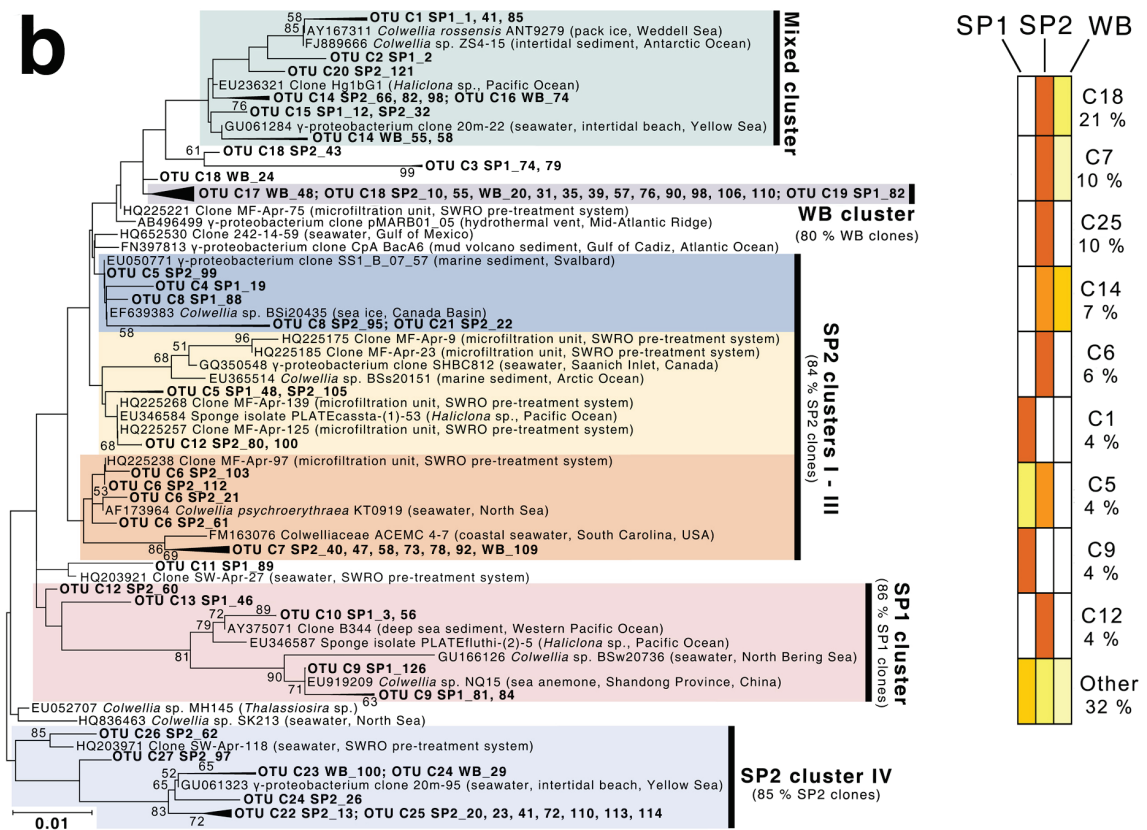
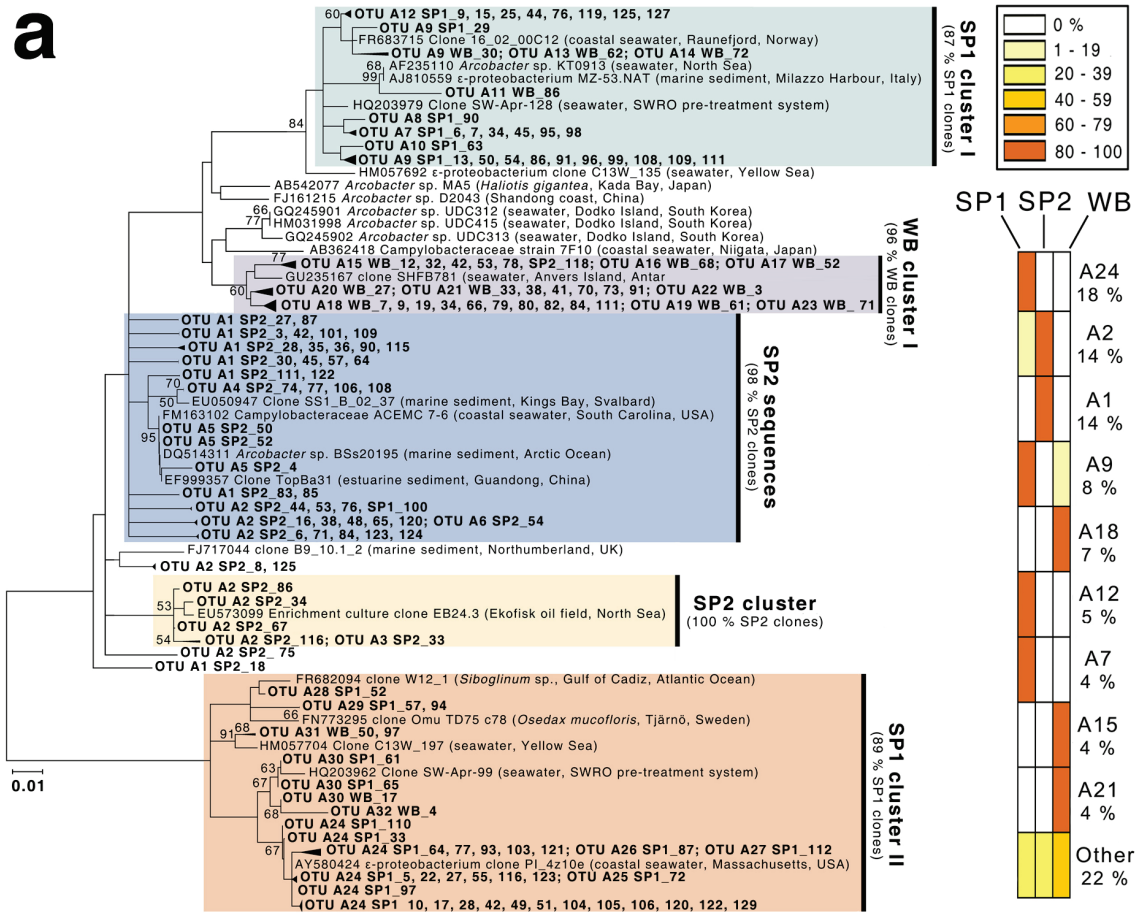


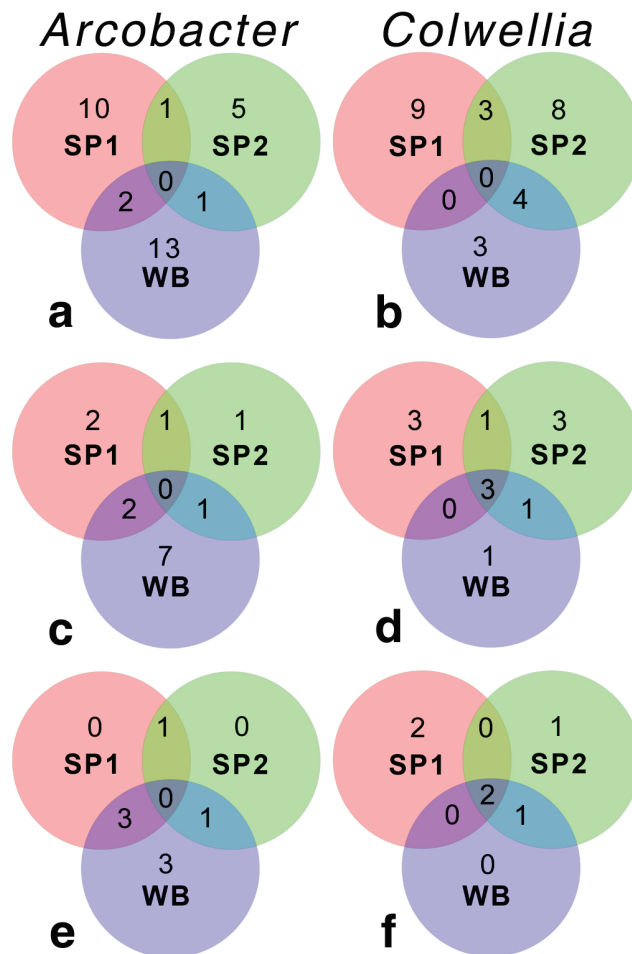
16S rRNA gene sequences from the genera *Arcobacter* (Epsilonproteobacteria) and *Colwellia* (Gammaproteobacteria) were found to dominate the LDPE-associated bacterial assemblages, comprising between 84 and 93% of sequences from the three sites (Figure 3.7). Neighbour-joining phylogenetic trees revealed a high degree of sediment-specific clustering within each genus, with 80 – 100% of the sequences within individual populations originating from a given sediment type (Figure 3.8). OTU-based analyses also showed sediment-specific clustering of these sequences, with 80 – 100% of the dominant OTUs within each genus typically originating from a single sampling site (Figure 3.8; see Figure 3.9). Moreover, the LDPE-affiliated communities from each site contained significantly different *Arcobacter* spp. populations, as assessed using LIBSHUFF (with Bonferroni correction,  $p < 0.05$ ). Site-specific *Colwellia* spp. populations were also found. However, for this genus, differences between sites SP2 and WB were barely significant ( $p = 0.05$ ), in agreement with the high similarities observed between the structures of the overall LDPE-associated bacterial communities at these two sites as determined by T-RFLP analysis (Figure 3.5).



**Figure 3.7.** Taxonomic composition and relative abundance (%) of LDPE-associated bacterial assemblages. Clone libraries were generated following PCR amplification of bacterial 16S rRNA genes amplified from DNA isolated from LDPE microplastics sampled after 14 days from sediment-LDPE microcosms from three sampling sites (SP1, SP2 and WB, as indicated). Clones were assigned to operational taxonomic units (OTUs) based on a similarity cut-off threshold of 99%, with numbers of individual OTUs within each taxon indicated.

**Figure 3.8. (next page).** Neighbour-joining phylogenetic trees and heat maps showing taxonomic affiliation of bacterial 16S rRNA gene sequences from the genera a) *Arcobacter* and b) *Colwellia* within the LDPE-sediment interface. Sequences (in bold) were obtained from LDPE microplastics sampled after 14 days from sediment-LDPE microcosms from three sites (SP1, SP2 and WB, as indicated). Most-closely related GenBank database sequences are included in the trees. Tree branches are collapsed according to OTU designations using a 99% similarity cut-off threshold. Highlighted regions indicate predominant sediment-specific populations. Bootstrap values of  $\geq 50\%$  are shown adjacent to nodes; scale bar represents 1% sequence divergence. Corresponding heat maps display the contributions (%) of the most dominant OTUs within the LDPE-sediment interface for each genus at each site. Overall contributions (%) of each OTU within each genus (*i.e.* across the three sites) are shown beneath each OTU label.





**Figure 3.9.** Frequencies of LDPE-associated *Arcobacter*- and *Colwellia*-affiliated operational taxonomic units (OTUs) within and across different sediment sampling sites. Values are shown for OTUs based on similarity cut-off thresholds of 99% (a and b), 97% (c and d) and 95% (e and f). Clone libraries were generated following PCR amplification of bacterial 16S rRNA genes amplified from DNA isolated from LDPE microplastics sampled after 14 days from sediment-LDPE microcosms from three sampling sites (SP1, SP2 and WB, as indicated).

### 3.5. Discussion

In this study, a 14-day laboratory microcosm experiment has shown that bacteria present in coastal marine sediments can rapidly colonise low-density polyethylene (LDPE) microplastics. Molecular analysis revealed that these LDPE-associated bacterial communities were structurally and taxonomically distinct from those found in the surrounding sediment environment. Scanning electron microscopy visually confirmed the direct attachment of primarily rod-shaped prokaryotic cells onto LDPE fragments

within both sandy and silty sediment types (sampling sites SP1, SP2 and WB; see Table 3.1). Terminal-restriction fragment polymorphism (T-RFLP) analysis demonstrated significant time-dependent shifts in the structure of bacterial assemblages within the LDPE-sediment interface, in particular, by Days 7 and 14 of the experiment, with successional convergence occurring across all three sediment types examined. In contrast, the structures of bacterial communities within sediments from the three sites remained significantly different from each other over the duration of the experiment, and no significant temporal patterns were observed in the structure of the sediment bacterial communities. Despite recognition of the role and importance of sediments as a sink for the accumulation of plastic debris (Thompson *et al.*, 2004; Reddy *et al.*, 2006; Barnes *et al.*, 2009; Browne *et al.*, 2011; Claessens *et al.*, 2011), previous research into the interactions between marine microorganisms and plastic debris has focused on investigation of microbial interactions with polymers in the water column (Carpenter *et al.*, 1972; Dang and Lovell, 2000; Dang *et al.*, 2008; Lee *et al.*, 2008; Webb *et al.*, 2009; Harrison *et al.*, 2011; Lobelle and Cunliffe, 2011). Moreover, prior culture-independent investigation into the bacterial colonisation of plastics within the water column has only investigated communities following exposure of polymers in seawater for up to 72 hours, representing initial colonisation events (Dang and Lovell, 2000; Dang *et al.*, 2008; Lee *et al.*, 2008). The present research has demonstrated that over longer time periods (up to 14 days), successional shifts in the structure of LDPE-affiliated bacterial assemblages occur, highlighting the need to undertake analyses over varying timescales to fully understand microbial biofilm colonisation processes on microplastics (Harrison *et al.*, 2011).

By the end of the 14-day experiment, the LDPE-sediment interface was primarily colonised by location-specific populations of *Arcobacter* spp.

(Epsilonproteobacteria) and *Colwellia* spp. (Gammaproteobacteria), as shown by 16S rRNA gene sequencing. Both rarefaction analysis and Good's coverage estimates for operational taxonomic units (OTUs) supported strong representation of the overall taxon diversity within the plastic-associated bacterial communities (see Figure 3.6 and Table 3.4, respectively). Interestingly, previous studies characterising bacterial-plastic interactions within USA, China and Korea coastal waters identified *Roseobacter* spp. and other Alphaproteobacteria as the primary colonists of acryl, polyurethane, poly(methyl methacrylate) and polyvinylchloride surfaces within 24 hours of exposure (Dang and Lovell, 2000; Dang *et al.*, 2008; Lee *et al.*, 2008). In contrast, Epsilonproteobacteria were not detected on any of these polymer types. Moreover, the relative abundance of Gammaproteobacteria found attached to polymers in seawater was repeatedly found to significantly decrease after 24 hours of exposure (Dang and Lovell, 2000; Dang *et al.*, 2008; Lee *et al.*, 2008). These differences in taxon composition between bacterial assemblages colonising polymers within either water or sediment may be attributable to several factors, including variation in the structure, composition and activities of bacterial communities between environmental compartments, differences in experimental conditions, polymer types and durations of exposure (Dang and Lovell, 2000; Bakker *et al.*, 2004; Dang *et al.*, 2008; Lee *et al.*, 2008; Harrison *et al.*, 2011). Despite these uncertainties, the present research strongly suggests that processes involved in the bacterial primary colonisation of microplastic debris are likely to differ between sediment habitats and the marine water column. It may also be hypothesised that the plastic-sediment interface represents an anthropogenic parallel to other types of microhabitats within natural ecosystems, including organic aggregates such as 'clay hutches' (Lünsdorf *et al.*, 2000) and marine snow within the water column (Rath *et al.*, 1998).

Whilst the LDPE-affiliated *Arcobacter* 16S rRNA gene sequences reported in this study were closely related to those from isolates and / or clones from marine environments (Figure 3.8), the ecological roles of this genus are poorly understood. Although *Arcobacter* spp. are increasingly found within marine environments including coastal habitats and sediments (Fera *et al.*, 2004; Collado *et al.*, 2008; Collado and Figueras, 2011), prior research has primarily focused on their role as clinical and animal pathogens (reviewed in Collado and Figueras, 2011). However, Assanta *et al.* (2002) have demonstrated attachment of *Arcobacter butzleri* to polyethylene pipe surfaces used in water distribution systems. *Colwellia* spp. identified in this study were most closely related to those in both polar and sub-tropical marine habitats (Fig. 3.8). Interestingly, whilst *Colwellia* spp. are considered as psychrophilic and have predominantly been found within polar environments (Methé *et al.*, 2005), members of this genus have also been identified as minor components of an acryl-colonising bacterial assemblage within seawater near the coast of Korea (Lee *et al.*, 2008). Furthermore, *Colwellia* spp. are known to produce extracellular polymeric substances (EPS) (Huston *et al.*, 2004) that may enhance biofilm formation on plastic surfaces. Whilst research into the direct detection of EPS and other bacterial metabolites on LDPE fragments was beyond the scope of this investigation, bacterial-surface interactions during primary colonisation are known to exert a significant influence on the composition and further successional recruitment of microorganisms on plastic surfaces (Dang and Lovell, 2000; Dang *et al.*, 2008).

Although the ecological roles of the LDPE-colonising sediment bacteria described in this study are unknown, both *Arcobacter* (Voordrouw *et al.*, 1996; Watanabe *et al.*, 2000; Yeung *et al.*, 2011) and *Colwellia* are additionally present in hydrocarbon-rich environments, with *Colwellia* spp. having previously been affiliated

with hydrocarbon contaminant mineralisation in cold ecosystems (Powell *et al.*, 2004; Yakimov *et al.*, 2004; Valentine *et al.*, 2010). To advance our understanding of microbial-plastic interactions and their implications for research into the environmental impacts and / or fate of plastic litter in the marine environment, more work is needed to characterise the ability of microplastic-associated bacteria to mediate breakdown of plastic co-contaminants, additives and/or of the petroleum hydrocarbon-derived polymers themselves (Harrison *et al.*, 2011). Wider investigation is also required in order to determine whether the structure, taxonomic identities and metabolic functions of plastic-affiliated microbial consortia vary across different *in situ* environmental conditions and polymers retrieved from both benthic and pelagic habitats (Dang and Lovell, 2000; Bakker *et al.*, 2004; Dang *et al.*, 2008; Lee *et al.*, 2008).

In conclusion, this study has demonstrated the capacity for rapid attachment of microorganisms onto LDPE microplastics within coastal marine sediments and the development, succession and selection of LDPE-associated bacterial communities dominated by sediment-specific populations of *Arcobacter* and *Colwellia* spp. These results represent the first demonstration of the potential for microplastics in marine sediments to function as sites for microbial colonisation and biofilm formation. Whilst the metabolic activities of the LDPE-associated bacterial assemblages reported in this study are unknown, the present results provide a starting point for research into the formation, ecology and functions of plastic-associated biofilms in benthic marine habitats, and into the ecological impacts of microplastic-associated contaminants and/or microplastics within sediment systems.



### **3.6. Acknowledgements**

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# **Chapter 4**

## **Quantification and Visualisation of Bacteria**

### **Attaching to Low-density Polyethylene**

### **Microplastics in Coastal Marine Sediments**

Aspects of this research have been submitted for publication as:

Harrison, J.P., Sapp, M., Schratzberger, M. and Osborn, A.M. Rapid bacterial colonization of low-density polyethylene microplastics in coastal marine sediments.

(Submitted) *FEMS Microbiology Ecology*.

#### 4.1. Abstract

Synthetic microplastics ( $\leq 5$ -mm fragments) are now globally ubiquitous in marine sediments. Whilst little is known about the ability of microorganisms to associate with microplastics in sediments, research into this topic is fundamental to understand the environmental fate of marine plastic debris. This study employed a 14-day sediment microcosm experiment and quantitative PCR to enumerate 16S rRNA genes as a proxy for the abundance of bacteria present on low-density polyethylene (LDPE) surfaces over time and across three sediment types, ranging from sand to silt. Log-fold increases in the abundance of 16S rRNA genes from LDPE-associated bacteria occurred within 7 days with bacterial numbers differing significantly across sediment types. Catalysed reporter deposition fluorescence *in situ* hybridisation (CARD-FISH) demonstrated attachment of bacteria, and specifically *Colwellia* spp., onto LDPE within sediments. This research provides the first quantitative assessment showing that polyethylene microplastics can function as sites for rapid bacterial attachment in coastal marine sediments.

#### 4.2. Introduction

Synthetic microplastics ( $\leq 5$ -mm fragments) are globally distributed persistent pollutants that are accumulating particularly in marine sediments (Browne *et al.*, 2011; Claessens *et al.*, 2011; Cole *et al.*, 2011). Due to their high bioavailability and potential to transport organic contaminants (*e.g.* polynuclear aromatic hydrocarbons) and additives into marine food webs, microplastics represent both a physical and chemical threat to marine organisms (Andrady, 2011). Whilst the interactions between plastics and microorganisms (bacteria, archaea and microbial eukaryotes) remain understudied, research into the microbial colonisation of microplastics is fundamental to understanding of the longer-term potential for biodegradation of plastic-associated

contaminants and/or the plastics themselves (Harrison *et al.*, 2011). Bacterial colonisation of plastics within the marine environment was first reported in the 1970s, whereby rod-shaped Gram-negative bacteria were shown to be present on a polystyrene fragment in the Sargasso Sea (Carpenter *et al.*, 1972). More recently, several studies have highlighted the ability of marine microorganisms to rapidly colonise and persist on several types of synthetic polymers in seawater (Sudhakar *et al.*, 2007a, 2007b; Webb *et al.*, 2009; Lobelle and Cunliffe, 2011). However, in contrast, very little is known about microbial-plastic interactions within sediments that serve as sinks for the accumulation of microplastics in the marine environment (Browne *et al.*, 2011).

Consequently and in order to investigate microbial biofilm formation on microplastics within marine sediments, a sediment microcosm experiment was employed (see Chapter 3) to demonstrate colonisation of low-density polyethylene (LDPE) microplastics by naturally occurring bacteria in coastal sediments. Briefly, bacterial assemblages present at the LDPE-sediment interface were found via molecular analysis to be structurally and taxonomically different from those in bulk sediments, and these communities exhibited successional convergence over a 14-day exposure period. The majority of bacteria within these communities were found to be members of the genera *Arcobacter* and *Colwellia*. Whilst this work initiated research into microbial-plastic interactions in marine sediments, there remains a continuing lack of fundamental ecological data that could inform research into microbial colonisation processes on plastic pollutants within the marine environment and on the wider environmental impacts of plastic debris within marine sediments, and ultimately on the potential microbial mineralisation of polymer-associated contaminants and/or of the plastics themselves (Harrison *et al.*, 2011). For example, there is a paucity of both quantitative data and visual information on early-stage microbial colonisation of microplastics in

sediment habitats. Moreover, most prior research into microbial-plastic interactions has been culture-based. Since only 1% or fewer of environmental microbial taxa are typically culturable in the laboratory (Amann *et al.*, 1995), research employing culture-independent molecular analyses is essential to inform understanding of the ecological interactions between microorganisms and plastic litter.

In the present study, a microcosm experiment in combination with molecular analyses was used to investigate the early-stage bacterial colonisation of microplastics added into coastal marine sediments. Specifically, this research aimed to quantify attachment of naturally occurring bacteria onto LDPE fragments over time in both sandy and silty sediments, and to visually confirm the colonisation of LDPE surfaces by sediment bacteria. LDPE was used as a reference material due to the high abundance of this plastic type within the marine environment (Barnes *et al.*, 2009). Bacterial 16S rRNA gene numbers on LDPE fragments were quantified as a proxy of the relative abundance of plastic-colonising bacteria using a quantitative real-time PCR (Q-PCR) assay (Becker *et al.*, 2000; Suzuki *et al.*, 2000; Smith *et al.*, 2006; Smith and Osborn, 2009). Catalysed reporter deposition in situ hybridisation (CARD-FISH) (Pernthaler *et al.*, 2002; Wendeberg *et al.*, 2010) was then used to visualise bacterial cells and specifically *Colwellia* spp. (Gammaproteobacteria) attached on LDPE surfaces.

### **4.3. Materials and Methods**

#### **4.3.1. Sediment sampling and sediment-LDPE microcosms**

Sediment sampling was performed as described in Section 3.3.1. Sediment-LDPE microcosms were established and LDPE fragments were sampled as outlined in Section 3.3.2. Sediment samples for the construction of a Q-PCR standard curve were collected from the microcosms following 7 days of exposure. LDPE fragments and sediments for

Q-PCR analysis were stored at -80 °C. LDPE fragments for CARD-FISH analysis were fixed overnight in 2% (v / v) formaldehyde at 4 °C, then rinsed with ASW and 96% ethanol, and stored at -20 °C until use.

#### **4.3.2. DNA isolation and Q-PCR amplification of bacterial 16S rRNA genes**

DNA was isolated both from sediments and LDPE fragments as described in Section 3.3.4. Bacterial 16S rRNA genes were amplified by PCR from DNA extracted from sediments from sites SP2 and WB using the universal bacterial primers EUB338 (5'-ACT CCT ACG GGA GGC AGC AG -3') and EUB518 (5'- ATT ACC GCG GCT GCT GG -3') (Fierer *et al.*, 2005). No PCR amplification product could be obtained from sediment from site SP1 (data not shown). Each PCR contained 1 µl of template DNA, 1× PCR buffer containing 1.5 mM of MgCl<sub>2</sub>, 0.25 mM of each deoxyribonucleoside triphosphate (dNTP), 0.3 µM of each primer and 2.5 U of *Taq* polymerase (Qiagen) made up to a total volume of 25 µl with sterile nuclease-free water (Ambion). PCR cycling conditions were 94 °C for 3 min, followed by 40 cycles of 94 °C for 30 s, 50 °C for 45 s and 72 °C for 30s and a final extension step at 72 °C for 7 min. The resulting PCR products (~200 bp) were purified with a QIAquick<sup>®</sup> gel extraction kit (Qiagen) and used to construct a standard curve to quantify 16S rRNA gene numbers on LDPE surfaces across the three sites, over a range of  $1.0 \times 10^6$  to  $1.0 \times 10^9$  amplicons of target DNA per mm<sup>2</sup> of LDPE.

Q-PCR analysis was performed on a single assay plate (Smith *et al.*, 2006). Each Q-PCR reaction contained 1 µl of template DNA, 5x QuantiFast<sup>®</sup> SYBR<sup>®</sup> Green PCR Mastermix (Qiagen) (Wittver *et al.*, 1997) and 0.3 µM of each primer, made up to a total volume of 25 µl with sterile nuclease-free water (Ambion). Primers and cycling conditions were as described for end-point PCR amplifications, with the exception of

omitting the final extension step (see above). No-template controls (NTCs) ( $n = 3$ ) were included. Measurements were performed in triplicate for each sampling interval and sediment type, using a CFX96<sup>TM</sup> Real-Time PCR Detection System (Bio-Rad, Hemel Hempstead, UK) and quantified by CFX Manager<sup>TM</sup> software (Bio-Rad). Mean cycle threshold ( $C_t$ ) values (*i.e.* the number of cycles required for the fluorescence signal to exceed the background) were estimated (Smith and Osborn, 2009). PCR product specificity was confirmed by melting curve analysis (data not shown) (Ririe *et al.*, 1997). Statistical analyses of the Q-PCR data were performed using the R statistical package (version 2.12.0) (R Development Core Team, 2010).

#### **4.3.3. CARD-FISH analysis of LDPE-associated bacteria**

An existing catalysed reported deposition fluorescence *in situ* hybridisation (CARD-FISH) protocol (Wendeberg *et al.*, 2010) was employed in conjunction with universal bacterial oligonucleotide probes (EUB338 I-III) and a negative control probe (NON338) (Thermo Fisher Scientific or Biomers, Ulm, Germany) (Table 4.1), using hybridisation conditions listed in Table 4.1. A probe targeting *Colwellia* spp. (PSA184) (Biomers) was also used (Table 4.1). Catalysed reporter deposition was performed using a TSA<sup>TM</sup> Cyanine 3 Tyramide Reagent Pack (Perkin-Elmer, Buckinghamshire, UK). Following hybridisation and catalysed reporter deposition, the LDPE fragments were counterstained by 4',6-diamidino-2-phenylindole (DAPI) (Wendeberg *et al.*, 2010). Bacteria were visualised using an Olympus IX71 epifluorescence microscope equipped with a 100X / NA 1.3 objective. All hybridisations were performed using LDPE fragments exposed to sediments from site SP2 for 14 days.

**Table 4.1.** Oligonucleotide probes and hybridisation conditions used for CARD-FISH analysis of bacteria attached to LDPE fragments, retrieved from microcosms following 14 days of exposure to sediment from sampling site SP2 (Humber Estuary, UK).

Probe	Nucleotide sequence (5' – 3')	Probe target	% FA <sup>a</sup>	°C <sup>b</sup>	References
NON338	ACT CCT ACG GGA GGC AGC	Negative control	55	35	Wallner <i>et al.</i> (1993)
EUB338 I	GCT GCC TCC CGT AGG AGT	Most Bacteria	55	35	Amann <i>et al.</i> (1990)
EUB338 II	GCA GCC ACC CGT AGG TGT	Planctomycetales	55	35	Daims <i>et al.</i> (1999)
EUB338 III	GCT GCC ACC CGT AGG TGT	Verrucomicrobiales	55	35	Daims <i>et al.</i> (1999)
PSA184	CCC CTT TGG TCC GTA GAC	<i>Pseudoalteromonas</i> , <i>Colwellia</i>	30	31	Eilers <i>et al.</i> (2000)

<sup>a</sup> Per cent (v / v) formamide (FA) in hybridisation buffer, based on Sekar *et al.* (2003) (NON338, EUB probes) or Eilers *et al.* (2000) (PSA184)

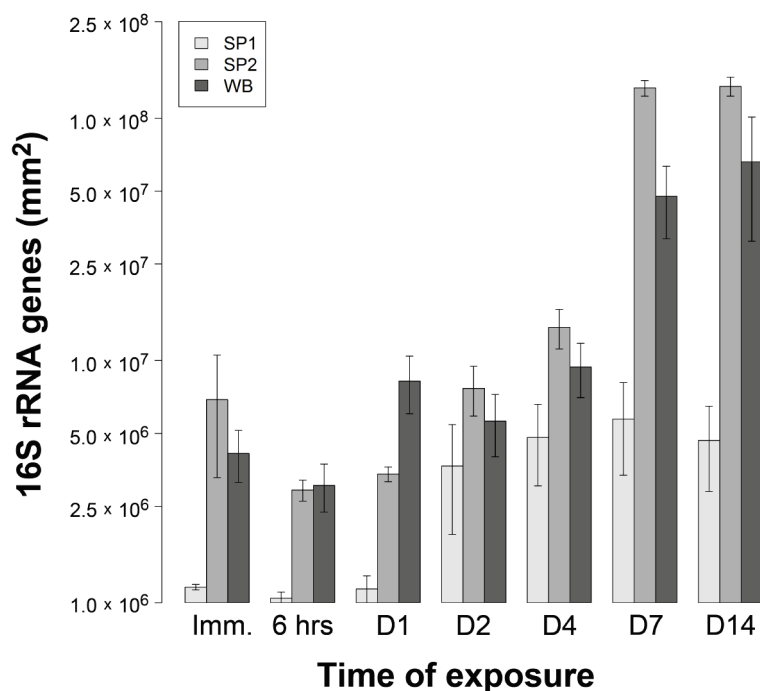
<sup>b</sup> Hybridisation temperature, based on Sekar *et al.* (2003) (NON338, EUB probes) or a modification of Pernthaler *et al.* (2002) (PSA184)



#### 4.4. Results and Discussion

In order to obtain meaningful Q-PCR results, it is necessary to separate the DNA template amplification signal from background fluorescence (Smith *et al.*, 2007; Smith and Osborn, 2009). It is considered that gene number estimates with mean  $C_t$  values less than 3.3 cycles lower than those corresponding to NTCs (*i.e.* less than a log-fold difference in gene numbers) are potentially influenced by background interference (Smith and Osborn, 2009). For the three sites (SP1, SP2 and WB), this was the case for LDPE fragments sampled prior to Day 4 and additionally for site SP1 at Days, 4, 7 and 14. Consequently, estimates of bacterial 16S rRNA gene numbers for these samples were excluded from statistical analysis.

For LDPE fragments retrieved from microcosms containing sediments from sites SP2 and WB, the abundance of 16S rRNA genes differed significantly both as a function of time of exposure to sediments and to sediment type (two-way ANOVA:  $F_{2,12} = 16.50$ ,  $p < 0.001$  and  $F_{1,12} = 14.65$ ,  $p < 0.01$ , respectively). 16S rRNA gene numbers quantified following 7 and 14 days of exposure to sediments from sites SP2 and WB were approximately a log-fold higher than after four days of exposure (Tukey multiple comparison test for factor ‘exposure time’,  $p < 0.01$  and  $p < 0.001$ , respectively) (Figure 4.1). There was no significant difference between numbers of 16S rRNA genes amplified from DNA extracted from LDPE fragments sampled after 7 and 14 days of exposure to sediments. Following seven days of exposure to sediments, mean 16S rRNA gene numbers on LDPE surfaces corresponded to a ratio of 2.8:1.0 between sites SP2 ( $\bar{x} = 1.3 \times 10^8 \pm 9.9 \times 10^6$  SE) and WB ( $\bar{x} = 4.8 \times 10^7 \pm 1.6 \times 10^7$  SE), respectively (Figure 4.1).



**Figure 4.1.** Variation in the relative abundance of bacterial 16S rRNA genes amplified from DNA extracted from LDPE fragments in sediments sampled over time. LDPE fragments were sampled in triplicate over time [immediately (Imm.), after 6 hours (hrs) and on days (D) 1, 2, 4, 7 and 14] from microcosms containing sediments from three sites (SP1, SP2 and WB, as indicated). Abundances are expressed as 16S rRNA genes per mm<sup>2</sup> of LDPE. Error bars represent one standard error ( $n = 3$ ). Gene numbers were calculated from the following standard curve:  $r^2 = 0.979$ ,  $y$  intercept = 42.6, slope = -4.64,  $E$  (amplification efficiency) = 64%, and  $C_t$  cut-off of 28.7.

For LDPE fragments exposed to sediments from site SP1, a nearly log-fold increase in the abundance of bacterial 16S rRNA genes on the plastic surfaces was observed over the duration of the experiment. Whilst the data relating to site SP1 were omitted from statistical analysis due to the estimated gene copy numbers for this site being near the detection limit of the Q-PCR analysis, this result is in agreement with the corresponding estimates of approximately log-fold increases in gene numbers on LDPE fragments analysed following exposure to sediments from sites SP2 and WB (Figure 4.1). Therefore, despite the potential influence of background fluorescence on gene

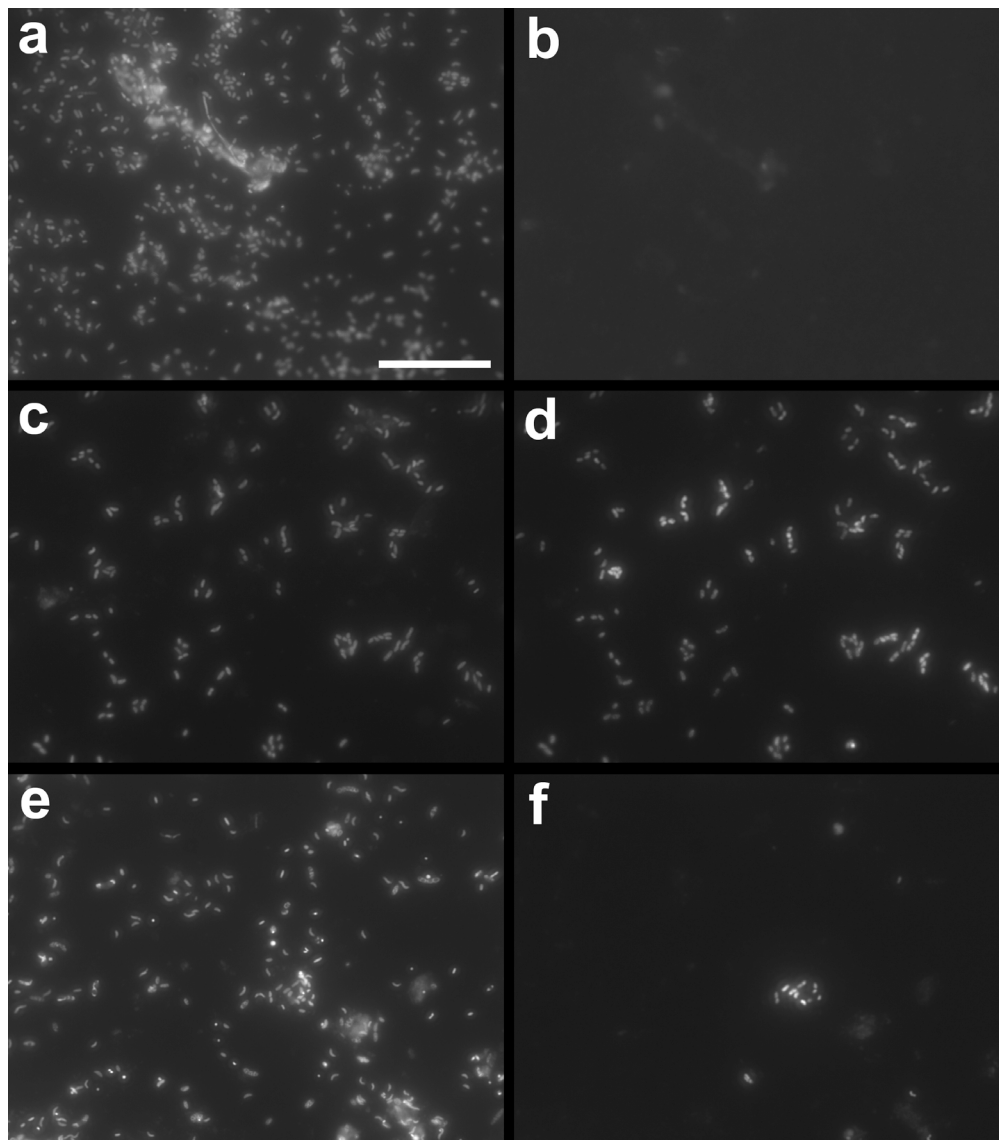
copy numbers calculated for site SP1 (see above), the results presented for this site are indicative of bacterial primary colonisation of LDPE fragments.

The Q-PCR data reported herein provide the first quantitative evaluation of the potential for both time-dependent and sediment-specific bacterial attachment onto LDPE fragments within coastal marine sediments. The observed log-fold increases in the abundance of bacterial 16S rRNA genes on LDPE surfaces within 7 days of exposure to sediments corroborate existing evidence for rapid colonisation of plastics by naturally occurring bacterial assemblages (Chapter 3; see Lobelle and Cunliffe, 2011). Although these results were obtained by culture-independent molecular analyses, they are consistent with previous culture-based research reporting a significant increase in the number of polyethylene-associated bacteria following three weeks of exposure in seawater (Lobelle and Cunliffe, 2011), and demonstrating colonisation of high-density polyethylene surfaces by cultures of *Arthrobacter* sp. and *Pseudomonas* sp. isolated from marine sediments in the Gulf of Mannar, India (Balasubramanian *et al.*, 2010).

Although universal bacterial primers were used for the present Q-PCR analysis, prior research (Chapter 3) demonstrated the successional development of bacterial communities within the LDPE-sediment interface that were both structurally and taxonomically distinct from those in bulk sediments. 16S rRNA gene clone library analyses using ‘Day 14’ LDPE samples indicated that plastics within each of the three sediment types were primarily associated with members of the genera *Arcobacter* (Epsilonproteobacteria) and *Colwellia* (Gammaproteobacteria). However, there is limited microscopy-based evidence confirming the attachment to and taxonomic identities of naturally occurring bacteria on microplastics within marine sediments. Therefore, CARD-FISH analysis (Section 4.3.3; Table 4.1) was employed to determine

whether bacterial cells and specifically *Colwellia* spp. attach directly to LDPE fragments exposed to sediments. This taxon is of potential interest to research into the environmental impacts and fate of microplastic-associated pollutants and/or the plastics themselves, due to its prior affiliation with hydrocarbon contaminant degradation (Powell *et al.*, 2004; Yakimov *et al.*, 2004; Valentine *et al.*, 2010). LDPE fragments exposed to sediment from site SP2 were chosen for CARD-FISH analysis on the basis of the high abundance of bacterial 16S rRNA genes (see Fig. 4.1) and due to the prevalence of LDPE-affiliated *Colwellia* 16S rRNA gene sequences found previously for this site (Chapter 3).

Bacterial cells were found to be directly attached onto the LDPE surfaces, as shown by CARD-FISH analysis performed with the oligonucleotide probes EUB338 I-III (Figure 4.2c-d). Bacteria typically constituted the majority of prokaryotic cells observed on the LDPE fragments (Figure 4.2d). Moreover, CARD-FISH analysis using the PSA184 probe (Table 4.1) demonstrated the presence of *Colwellia* spp. on the LDPE surface following 14 days of exposure to sediments (Fig. 2f), as previously indicated by sequencing of 16S rRNA gene clone libraries (Chapter 3). As such, this information provides the first insight into the spatial localisation of members of *Colwellia* spp. within the plastic-sediment interface.



**Figure 2.** Epifluorescence micrographs showing bacterial attachment to LDPE surfaces following 14 days of exposure to coastal sediment (sampling site SP2) in experimental microcosms. Micrographs corresponding to staining by 4',6-diamidino-2-phenylindole (DAPI) are displayed in the left-hand panels (a, c and e). Micrographs corresponding to staining by CARD-FISH are displayed on the right-hand panels, as shown for the oligonucleotide probes NON338 (b), EUB338 I – III (d) and PSA184 (f). The scale bar is 20  $\mu\text{m}$ .

Collectively, this study provides the first quantitative and culture-independent assessment of the ability of LDPE fragments in coastal marine sediments to function as sites for rapid bacterial attachment. Whilst only a single previous study has demonstrated the potential for bacteria originating from marine sediments to colonise plastic surfaces (Balasubramanian *et al.*, 2010), the present data are in broad agreement

with existing observational and culture-based research demonstrating microbial attachment to plastic debris in the water column (Carpenter *et al.*, 1972; Carpenter and Smith, 1972; Sudhakar *et al.*, 2007b; Webb *et al.*, 2009; Lobelle and Cunliffe, 2011). However, microbial-plastic interactions in the marine environment remain poorly understood in comparison with those in terrestrial ecosystems (Section 1.4.2; Lobelle and Cunliffe, 2011). For this reason, there is a particular need for longer-term and *in situ* exposure experiments to characterise microbial attachment and biofilm formation on microplastics within sediment habitats. Moreover, further experiments utilising both culture-independent and traditional microbiological methods are required to characterise the formation and ecological functions of plastic-associated biofilms in different marine habitats, as well as inform wider microbial research with regard to the environmental impacts and fate of marine microplastic debris.

#### **4.4. Acknowledgements**

Drs. Sonja Oberbeckmann and Sekar Raju provided technical support with the CARD-FISH analysis.

# **Chapter 5**

## **Concluding Discussion**

## 5.1. Summary of Main Findings

This study aimed to provide fundamental knowledge concerning the abundance, ecological impacts and fate of microplastics within coastal benthic environments that function as sinks for the accumulation of these rapidly emerging pollutants (Chapter 1). Research was initiated into the development of improved FT-IR spectroscopy methods to detect, image and quantify polyethylene microplastics within marine sediments (Chapter 2). Moreover, a 14-day laboratory microcosm experiment and culture-independent analyses were used to evaluate the potential for bacterial colonisation of LDPE microplastics within three sediment types (ranging from sand to silt) collected from the Humber Estuary, UK. The structure, diversity and taxonomic identities of LDPE-associated bacterial assemblages were determined by scanning electron microscopy, T-RFLP analysis and 16S RNA gene clone library sequencing (Chapter 3). Additionally, bacterial attachment to LDPE surfaces in sediments was quantified and visually confirmed using a Q-PCR assay and CARD-FISH, respectively (Chapter 4).

Individual overviews of the experimental findings reported in this study are provided as part of earlier chapters (Sections 2.1, 3.1 and 4.1). Briefly, whilst ATR-FT-IR spectroscopy produced similar spectra of both regularly and irregularly shaped LDPE fragments within sediment retentates, reflectance micro-FT-IR analysis of irregularly shaped plastics was susceptible to refractive error. Therefore, the reflectance spectra of unevenly shaped fragments must be interpreted with caution. Using several types of spectroscopy (including ATR-FT-IR spectroscopy) may assist the identification of microplastic particles (Hidalgo-Ruz *et al.*, 2012). Despite this potential for spectral distortion, reflectance micro-FT-IR spectra of LDPE fragments in polymer-spiked sediments were shown to be consistent across a total of 17 sampling sites, demonstrating the wider suitability of this method between different environmental



substrates. Additionally, molecular mapping detected PE microplastics in both polymer-spiked sediments and in a non-spiked sediment retentate, irrespective of the inherent biases of this technique and challenges associated with the separation of microplastics from sediments. Whilst additional work is now required to assess the wider applicability of spectral imaging to detect microplastics in marine ecosystems, these results represent the first important step towards developing new techniques for the quantitative and high-throughput spectral visualisation of microplastics.

Bacteria were found to rapidly colonise microplastics deposited into both sandy and silty sediment types, exhibiting successional convergence across all three sampling sites. Both the structure and diversity of the LDPE-associated bacterial assemblages differed significantly from those within bulk sediments. Following 14 days of exposure, these LDPE-colonising bacterial assemblages were primarily comprised of members of the genera *Arcobacter* (Epsilonproteobacteria) and *Colwellia* (Gammaproteobacteria). Whilst no previous studies have examined the colonisation of plastics by bacteria in marine sediments, plastic-associated Epsilonproteobacteria have not been detected during prior research characterising bacterial attachment onto several different polymer types within coastal waters (Dang and Lovell, 2000; Dang *et al.*, 2008; Lee *et al.*, 2008). Additionally, the present evidence for positive successional selection of *Colwellia* spp. within the LDPE-sediment interface is in contrast with previous studies reporting rapid declines in the relative abundance of plastic-associated Gammaproteobacteria in seawater (Dang and Lovell, 2000; Dang *et al.*, 2008; Lee *et al.*, 2008). Therefore, the results obtained in this study strongly suggest that patterns of bacterial primary colonisation of plastics in the marine environment are likely to differ between sediments and the water column.

Log-fold increases in the relative abundance of LDPE-associated bacteria were observed within 7 days of exposure to sediments. The abundance of plastic-affiliated bacteria also differed significantly between the three sediment types. For example, mean bacterial 16S rRNA gene numbers on LDPE surfaces following 7 days of exposure to sediments corresponded to a ratio of 2.8:1.0 in relation to sites SP2 and WB, respectively. These results represent the first quantitative assessment of the ability of LDPE fragments in coastal sediments to function as sites for bacterial attachment. The present evidence for site-specific variation in the relative abundance of LDPE-associated bacteria was also qualitatively supported by scanning electron microscopy and T-RFLP analysis. For example, T-RFLP profiles of bacterial communities within the plastic-sediment interface following less than 2 days of exposure to sediment from site SP1 typically exhibited low fluorescence intensities (data not shown), in agreement with the comparatively low 16S rRNA gene numbers estimated for this site (see Figure 4.1). Moreover, the presence of bacterial cells and specifically of *Colwellia* spp. onto the LDPE surfaces was confirmed independently of PCR-based methods by CARD-FISH analysis.

Collectively, the results of the microcosm experiment reported herein provide a fundamental insight into the time-dependent colonisation of LDPE microplastics by bacteria within different types of marine sediments. Intriguingly, the ecological roles of these plastic-associated bacteria are presently unknown.

## **5.2. Recommendations for Further Research**

Several recommendations for research into the environmental impacts of microplastics were proposed as a theoretical foundation to the present study (see Section 1.5). Recommendations for further investigation into topics highlighted by the main results

obtained via this research have also been provided within each experimental chapter (Chapters 2 – 4). In particular, considerations for future work involving the spectroscopic detection and imaging of microplastics in marine habitats are discussed in detail within Chapter 2 (Section 2.4.3.3). The current section expands upon these previous suggestions and outlines several examples of key areas for additional investigation at the interface between spectroscopy, microbial ecology and research into the environmental impacts of marine microplastics on higher organisms.

### **5.2.1. Spectroscopic characterisation of microbial-plastic interactions**

FT-IR spectroscopy is considered the most reliable technique for identifying synthetic microplastics in the marine environment (Hidalgo-Ruz *et al.*, 2012). Therefore, this study has aimed to advance research into developing improved FT-IR spectroscopy methods for detecting and imaging these pollutants in sediment habitats (Chapter 2; research recommendations discussed in Section 2.4.3.3). Whilst limitations associated with our ability to detect microplastics in marine ecosystems represent an important constraint to our understanding of the extent of marine plastic pollution (GESAMP, 2010b), FT-IR spectroscopy may also be useful for characterising interactions between microplastics and marine microorganisms. Reflectance micro-FT-IR spectroscopy has already been used to determine the chemical composition of microbial biofilms on thick and opaque materials including steel surfaces (Schmitt and Flemming, 1998; Ojeda *et al.*, 2009). Therefore, it is possible that this method could also be used to obtain novel information concerning microbial attachment to plastic fragments and microbial-microplastic interactions within both marine habitats and higher organisms (Figure 1.4; Section 5.2.8). For example, although the formation of conditioning films and secretion of extracellular polymeric substances (EPS) by LDPE-adhering bacteria were not examined as part of this present study, reflectance micro-FT-IR spectroscopy has

previously been applied to characterise the adsorption of EPS onto solid surfaces (Omoike and Chorover, 2006). FT-IR spectroscopy may also aid research into the colonisation of plastics by marine microorganisms by providing data concerning *in situ* biofilm composition and the specific bonding mechanisms involved in microbial adherence onto surfaces (Ojeda *et al.*, 2009).

In addition to FT-IR spectroscopy, other forms of vibrational spectroscopy may also be useful to both molecular and culture-based research into microbial-plastic interactions in marine ecosystems. For example, Raman microspectroscopy has previously been employed to differentiate genetically identical microbial populations in environmental samples based on their phenotypic, physiological and metabolic states (Huang *et al.*, 2010). This technique has also been used to identify macromolecules involved in cellular attachment to surfaces (Andrews *et al.*, 2010) and to resolve temporal patterns related to the formation and chemical structure of bacterial colonies (Choo-Smith *et al.*, 2001). Moreover, Raman microspectroscopy is compatible with several other analytical approaches (*e.g.* fluorescence *in situ* hybridisation and stable isotope probing), enabling direct investigation of the ecological functions of naturally occurring microbial communities at the scale of single cells (Huang *et al.*, 2010). As such, this method is of considerable interest to research aimed at identifying microbial activities influencing both the long-term fate and potential biodegradation of microplastics in marine ecosystems (Section 5.2.7).

### **5.2.2. Laboratory experiments under a wider range of environmental conditions**

To date, investigation of microbial-plastic interactions in marine ecosystems has been restricted to aerobic habitats (see Table 1.1). Therefore and due to practical reasons, the microcosm experiment employed in this study focused on elucidating bacterial-plastic

interactions under aerobic conditions and with reference to a single exposure scenario (based on measurements of *in situ* water temperature and salinity) (Chapters 3 and 4). Within natural ecosystems, the penetration depth of oxygen in coastal marine sediments is likely to be limited to a range of milli- and/or centimetres (Cai and Sayles, 1996; Köster *et al.*, 2008). This decline in oxygen content is accompanied by a stratification of redox conditions that corresponds to marked differences in both the composition and activities of resident microbial communities (Edlund *et al.*, 2008; Köster *et al.*, 2008). Although the present study demonstrated striking similarities in the taxonomic identities of LDPE-colonising bacterial assemblages across several sediment types supporting structurally distinct bacterial communities (Chapter 3), it remains probable that processes of microbial colonisation on microplastics are influenced by several environmental parameters including oxygen availability and the prevailing redox state. Similarly to microorganisms residing in pelagic habitats (Fuhrman *et al.* 2006; Kan *et al.* 2007; Sapp *et al.* 2007), the community structure of microorganisms inhabiting coastal sediments is known to fluctuate seasonally (Köster *et al.* 2008). Therefore, further laboratory experiments are now required to characterise microbial attachment to microplastics under a wide range of environmental conditions and across several seasons. This research may also be combined with field studies of microplastic-associated microorganisms (Section 5.2.4).

Due to the propensity of microplastics to associate with organic contaminants (*e.g.* polynuclear aromatic hydrocarbons) (see Section 1.3), it is also necessary to note that the spiking of sediments with LDPE microplastics in this study was performed in the absence of other artificially added pollutants. Therefore, it is of particular interest to future laboratory-based research into microbial-plastic interactions to determine how the presence of plastic co-contaminants impacts upon the structure, composition and

particularly the ecological activities of microplastic-associated microbial communities (Section 5.2.7).

### **5.2.3. Colonisation of microplastics by microbial eukaryotes and archaea**

Attachment by microbial eukaryotes (*e.g.* diatoms, dinoflagellates and fungi) onto synthetic polymer surfaces has previously been observed within aquatic habitats and the marine water column (Carpenter and Smith, 1972; Masó *et al.*, 2003; Sudhakar *et al.*, 2007b; Shah *et al.*, 2008; Pramila and Ramesh, 2011). Interestingly, the present study also demonstrated attachment by pennate diatoms onto LDPE fragments deposited into coastal marine sediments (Figure 3.3). Despite this and whilst microbial eukaryotes have frequently been affiliated with plastic biodegradation in terrestrial habitats (*e.g.* Shah *et al.*, 2008; Russell *et al.*, 2011), no research has been conducted into elucidating successional patterns of microbial eukaryotic colonisation on plastic litter within marine ecosystems. Additionally, whilst archaea have previously been shown to adhere both to steel surfaces in seawater (Dang *et al.*, 2011) and polyvinylidene fluoride membranes within a wastewater treatment facility (Calderón *et al.*, 2011), the presence of these organisms on marine plastic debris has not been reported. Since the potential ecological interactions between microplastics, microbial eukaryotes and archaea and in the marine environment (see Figure 1.4) remain poorly understood, there is a need for fundamental laboratory- and field-based research into this topic.

### **5.2.4. *In situ* studies of microbial-plastic interactions within coastal sediments**

The present research employed sacrificial sediment-LDPE microcosms as a model system to obtain an understanding of the ability of microplastic particles to facilitate bacterial attachment within coastal sediments (Chapters 3 and 4). Whilst microcosm experiments represent an invaluable tool to ecological research due to their simplicity,

reproducibility and potential to reveal biological processes that are of importance in natural ecosystems, the reliability of results obtained by laboratory-based studies must ultimately be verified under field conditions (Teuben and Verhoef, 1992; Ahl *et al.*, 1995; Kampichler *et al.*, 2001). Sediment mesocosm systems are likely to represent a useful and experimentally controlled counterpart to laboratory studies that could enable elucidation of microbial colonisation processes on microplastic surfaces under *in situ* environmental conditions. Due to their partially enclosed nature and direct contact with both organic and inorganic fluxes in the wider environment (Kampichler *et al.*, 2001), field-based mesocosm experiments may also allow for longer-term investigation of successional processes on polymer surfaces than enclosed microcosm experiments.

Although combining microcosm experiments with mesocosm studies will undoubtedly advance our understanding of microbial-plastic interactions in sediment habitats, it must be noted that no previous research has been conducted into determining the structure, composition and activities of microbial communities associated with microplastics retrieved directly from sediments within different sampling sites. Therefore, further research is required to characterise microbial biofilms adhering to plastic fragments obtained by field surveys focusing on both coastal and offshore locations (Section 5.2.6).

#### **5.2.5. Isolation of key microbial taxa associated with microplastics**

Molecular analyses of rRNA genes are highly useful to research into the structure, diversity and composition of naturally occurring microbial communities because they enable direct examination of phylogenetically diverse assemblages without the involvement of a culturing step (Amann *et al.*, 1995). However, the isolation of environmental microorganisms is of continuing relevance to microbial ecology due to

the ability of culture-based experiments to provide valuable information concerning the physiology and life histories of selected microbial taxa (e.g. Orphan *et al.*, 2000). In this current study, both *Arcobacter* spp. and *Colwellia* spp. were identified as key LDPE-colonising bacterial taxa within coastal marine sediments (Chapter 3). However, the degree to which the attachment of these taxa to microplastics is facilitated by the properties of the polymer, ambient environmental conditions and/or the presence of other plastic-colonising microbial taxa is unknown. For this reason, attempts are now required to isolate these key plastic-colonising bacterial taxa and to characterise their potential to associate with LDPE surfaces using culture-based experiments. Such experiments are particularly likely to be of importance to elucidating the ecological functions of plastic-associated microorganisms in marine habitats (see Section 5.2.7).

#### **5.2.6. Microbial-plastic interactions in coastal versus open-water habitats**

The results presented in this study highlight the likelihood that ecological interactions between marine microorganisms and synthetic microplastics will differ between environmental compartments, including sediments and the water column (Chapters 3 and 4). As such, studies comparing successional patterns of microbial colonisation on plastic surfaces in both sediments and seawater should constitute a research priority. Attention has also been directed towards the need for wider research into characterising patterns of microbial colonisation of microplastic debris across a broad range of environmental conditions and habitat types (Sections 5.2.2 and 5.2.4).

Although coastal environments are of particular significance to microplastics research due to their role as primary sites for marine biogeochemical processes and because of their high risk of exposure to plastic contamination (Halpern *et al.*, 2008; Barnes *et al.*, 2009; Browne *et al.*, 2010; Browne *et al.*, 2011; Cole *et al.*, 2011),



microbial-plastic interactions in these habitats are poorly understood. Given the additional and global-scale accumulation of microplastic debris in open-ocean environments including the North Atlantic Subtropical Gyre (Law *et al.*, 2010), additional research is needed in order to understand whether and how colonisation processes and activities of plastic-associated microorganisms vary between coastal and offshore environments. Expeditions of marine ecosystems across the globe have previously taken place in order to enable the molecular fingerprinting and high-throughput metagenomic sequencing analyses of microbial communities within pelagic environments (Venter *et al.*, 2004; Rusch *et al.*, 2007; Fuhrman *et al.*, 2008; Simon and Daniel, 2011). Next-generation metagenomic analyses have also become increasingly affordable and have recently been applied to assess the functional diversity of microbial communities in natural ecosystems (Simon and Daniel, 2011; Desai *et al.*, 2012). The presence of microplastics in the marine environment is already being investigated globally (Barnes *et al.*, 2009; Browne *et al.*, 2011). As such, high-throughput screening of metagenomic libraries may also enable research into characterising the diversity and metabolic potential of plastic-associated microbial assemblages worldwide.

#### **5.2.7. Functional analyses of microplastic-associated microbial assemblages**

In this study, DNA-based molecular analyses were used to advance our understanding of microbial-microplastic interactions within coastal marine sediments (Chapters 3 and 4). However, the ecological functions of microplastic-associated microbial taxa remain unknown and currently only indirect inferences may be made about this topic, based on examination of the phylogenetic relationships of these organisms as shown by 16S rRNA gene sequencing analyses (see Figure 3.8). Therefore, and due to the extensive reliance of previously published research into microbial-plastic interactions on culture-based methods (Section 1.4.2), there is a particular lack of culture-independent research

into the metabolic activities, process rates and patterns of functional gene expression by plastic-associated microorganisms in the marine environment.

Where sufficient information is available concerning the likely microbially mediated ecological functions within a given environment, both the abundance and expression of selected functional genes may be rapidly quantified using Q-PCR analysis (Becker et al., 2000; Suzuki et al., 2000; Smith *et al.*, 2007; Smith and Osborn, 2009). For example, *Colwellia* spp. were identified as key colonisers of LDPE surfaces in the present study (Chapter 3), and members of this genus are known to possess mono- and dioxygenase genes potentially involved hydrocarbon contaminant degradation (Méthé *et al.*, 2005). However, the expression of these genes within the plastic-sediment interface has not been studied. Therefore, Q-PCR analysis targeting these genes is of interest to future research into microbial-plastic interactions, breakdown of plastics and/or plastic-associated contaminants, and the environmental fate of marine microplastic debris.

Future investigation into microbial-plastic interactions and the breakdown of microplastics and their co-contaminants may also be facilitated by molecular techniques including stable isotope probing (SIP) and microbial transcriptomics. These methods enable culture-independent identification of taxa responsible for key metabolic activities and functional gene expression in natural environments (Radajewski *et al.*, 2000; Radajewski *et al.*, 2003; Madsen, 2005; Poretsky *et al.*, 2005; Poretsky *et al.*, 2009; Gilbert *et al.*, 2010; Vila-Costa *et al.*, 2010). For example, SIP may be used to trace the assimilation of a  $^{13}\text{C}$ -labelled carbon source by specific microbial populations within natural ecosystems (Radajewski *et al.*, 2000). This technique is also compatible with Raman microspectroscopy (Section 5.2.1). As such, SIP could be utilised to determine the potential for  $^{13}\text{C}$ -labelled plastics and plastic-associated compounds to function as a

source of carbon for microbial metabolism (Lucas *et al.*, 2008). Additionally, transcriptomic profiling is likely to provide useful insights into the metabolic activities of microplastic-associated microbial communities in the absence of previous research into this topic (Poretsky *et al.*, 2005). This method has previously been applied to reveal the presence of previously undetected functional genes and to elucidate temporal patterning in several aspects of microbial metabolism within the marine environment (Poretsky *et al.*, 2005; Poretsky *et al.*, 2009; Gilbert *et al.*, 2010).

In addition to culture-independent functional analyses of microplastic-associated microbial communities, culture-based research using isolates of key plastic-colonising taxa (Section 5.2.5) is required to fully understand the physiology and activities of plastic-colonising microorganisms in marine ecosystems. Future laboratory experiments could be employed to identify and characterise microbially produced compounds (*e.g.* biosurfactants and enzymes) (Satpute *et al.*, 2011) facilitating biofilm formation and/or catalysing metabolic reactions performed by plastic-colonising microorganisms. For example, extracellular polymeric substances (EPS) produced by *Colwellia psychrerythraea* strain 34H have already been shown to become retained within artificial sea ice, likely enabling direct cellular attachment to ice within pelagic marine habitats (Ewert and Deming, 2011). Moreover, analysis of the extracellular aminopeptidase ColAP purified from this strain has revealed several structural features conferring cold adaptation and protein metabolism at low temperatures (Huston *et al.*, 2004). Concurrently to investigating the role of microbially produced macromolecules in both the formation and ecology of plastic-associated biofilms, further work is required to determine the ability of selected microbial taxa to grow on microplastics as the only carbon source (*e.g.* Gilan *et al.*, 2004; Russell *et al.*, 2011). This type of research could be performed both in the presence and absence of artificially added plastic co-

contaminants (Teuten *et al.*, 2009), in order to broaden our understanding of how these compounds are likely to influence microbial-plastic interactions within natural habitats.

#### **5.2.8. Microbial-plastic interactions in contact with higher organisms**

Although this research has focused on characterising the ecological relationship between microplastics and microorganisms within marine sediments, the potential for ingestion of these pollutants by higher organisms is widely documented (Section 1.3). As such, microplastics may also represent a vector for the transport of plastic-associated microorganisms into higher organisms (Figure 1.4). Additionally, microbial taxa within the gut may also interact with microplastics and/or microplastic-associated microbial assemblages originating from the wider environment. However, no research has been conducted into these topics, and there is a need for future experimentation to understand the ability of microplastics to influence higher organisms from a microbiological viewpoint. Additionally, due to the potential negative impacts of microplastics and plastic-associated contaminants upon higher organisms (Section 1.3), the presence of microplastic debris may indirectly influence microbially mediated biogeochemical processes in marine ecosystems. For example, burrowing activities by bioturbating invertebrates are known to exert a significant impact upon the structure, composition and activities of sediment microbial communities (Laverock *et al.*, 2010). Therefore, and since sediment-dwelling organisms are highly susceptible to ingestion of microplastics (Section 1.3), future research must address whether the potential deleterious impacts of microplastic particles on these organisms could also influence the maintenance of vital ecosystem processes by naturally occurring microbial communities.

### **5.3. Outlook**

The findings reported in this study provide both a novel and significant contribution toward improving our ability to detect marine microplastic fragments and to understand the potential of these pollutants to facilitate formation of plastic-associated biofilms. In addition to initiating research into both the development of improved spectroscopic protocols for the detection of microplastics and microbial colonisation of microplastics in marine sediments, this work has identified a broad range of key subjects for future research. The results of this research will be of particular use to developing robust and standardised methods for quantifying microplastics, and as an experimental foundation to performing laboratory-based and *in situ* microbiological investigations aimed at characterising the ecological impacts and long-term residence times of microplastic debris within the global marine environment.

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