The survival, persistence, and ecology of microbial pathogens on the surfaces of environmental plastic pollution.

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Abstract

Plastics in the environment become rapidly colonised by microbial biofilm. These 'plastisphere' communities can support, or even enrich human pathogens, which can be transported, disseminated and transferred to humans e.g., through direct exposure at beaches or bathing waters. With the continued pollution of plastics in the environment, the human exposure to pathogen colonised plastic pollution will rise, and increasing our understanding of the survival, transfer and characterisation of these potentially harmful pathogens will help in assessing the potential implications to human health. Therefore, this thesis aimed to quantify the colonisation, survival and pathogenicity of bacterial and fungal pathogens on environmental plastic pollution in freshwater, marine, and beach environments. Field and mesocosm studies utilising both microbiological and molecular methods were used to address the following key objectives: (1) evaluate the load of potential bacterial and fungal pathogens colonising different types of environmental plastic pollution; (2) quantify the survival dynamics of potential pathogens on plastics during the transfer through different environments; (3) characterise antimicrobial resistance, thermotolerance, and pathogenicity of pathogens in the plastisphere. The Results presented here demonstrate that bacterial and fungal pathogens colonise environmental plastic pollution (e.g., microplastic beads, wet wipes). Pathogens could survive in in the plastisphere for up to 15 weeks and continue to persist during the movement through the freshwater-marine continuum, which could allow them to disseminate into areas where they are more likely to come into contact with humans, e.g., at the beach. Species recovered from the plastisphere included the faecal indicator organisms (FIOs), *E. coli* and intestinal enterococci, *Vibrio* spp. and pathogenic species of the yeast *Candida*. FIOs were more often associated with plastic pollution compared with natural materials (e.g., seaweed), suggesting that plastics provide an additional risk in addition to those natural materials already in the environment. Potential pathogens also showed evidence of antimicrobial resistance (AMR), thermotolerance and pathogenicity, indicating that they could be more likely to cause disease in humans. Together this research highlights the heightened public health risk that pathogen colonised plastics can provide. This signifies the importance of reducing the presence of plastic pollution in the environment, through improved environmental regulation, monitoring and management, together with public awareness and involvement programmes.

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List of Common Abbreviations

AMR: Antimicrobial resistance

ANOVA: Analysis of variance test

ARGs: Antimicrobial resistance genes

CFU: Colony forming unit

CSOs: Combined sewer overflow events

EC: Electrical conductivity

FIOs: Faecal indicator organisms

FTIR: Fourier Transform Infrared Spectrometry

IE: Intestinal enterococci

ITS: Internal transcribed spacer region

LB: Luris-Bertani broth

MGEs: Mobile genetic elements

MIC: Minimum inhibitory concentration

MPs: Microplastics

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PSU: Practical salinity units

WHO: World Health Organisation

WWTPs: Wastewater treatment plants

Plastic polymers:

HDPE: High density polyethylene

LDPE: Low density polyethylene

PBT: Polybutylece terephthalate

PE: Polyethylene

PET: Polyethylene terephthalate

PP: Polypropylene

PS: Polystyrene

PU: Polyurethane

PVC: Polyvinyl chloride

Agar:

BSA: Bismuth sulfite agar

CA: *Campylobacter* blood free agar

KCA: *Klebsiella* ChromoSelect agar

MLGA: Membrane lactose glucuronide agar

PA: *Pseudomonas* base agar

SB: Slantez and Bartley agar

SGA: Sabouraud glucose agar

TCBS: Thiosulfate citrate bile salts sucrose agar

YPD: Yeast extract peptone dextrose

Publications arrising from this thesis

- **Metcalf, R.,** Oliver, D.M., Moresco, V. and Quilliam, R.S., (2022) 'Quantifying the importance of plastic pollution for the dissemination of human pathogens: The challenges of choosing an appropriate 'control' material.' *Science of the Total Environment*, *810*, p.152292.
- **Metcalf, R.,** White, H.L., Moresco, V., Ormsby, M.J., Oliver, D.M. and Quilliam, R.S., (2022) 'Sewage-associated plastic waste washed up on beaches can act as a reservoir for faecal bacteria, potential human pathogens, and genes for antimicrobial resistance.' *Marine Pollution Bulletin*, *180*, p.113766.
- **Metcalf, R.,** White, H.L., Ormsby, M.J., Oliver, D.M. and Quilliam, R.S., (2023) 'From wastewater discharge to the beach: Survival of human pathogens bound to microplastics during transfer through the freshwater-marine continuum.' *Environmental Pollution*, *319*, p.120955.
- **Metcalf, R.,** Messer, L.F., White, H.L., Ormsby, M.J., Matallana-Surget, S. and Quilliam, R.S., (2024) 'Evidence of interspecific plasmid uptake by pathogenic strains of Klebsiella isolated from microplastic pollution on public beaches.' *Journal of Hazardous Materials*, *461*, p.132567.
- **Metcalf, R.,** Fellows, R., White, H.L., Quilliam, R.S., (in review in Marine Pollution Bulletin) 'Persistence of 'wet wipes' in beach sand: an unrecognised reservoir for localise *E. coli* contamination'.
- **Metcalf, R.,** Akinbobola, A., Quilliam, R.S., (in review in Journal of Hazardous Materials) 'Screening environmental isolates of human pathogenic *Candida* spp. colonising plastic pollution'.

1. General Introduction

1.1 Plastic pollution

Plastics are now ubiquitous, with an array of plastic products and applications. Plastic production continues to increase, with 400 million tonnes being produced in 2022 (Plastics Europe, 2023). Global plastic production is expected to exceed 500 million metric tons by 2050 (Sardon and Dove, 2018), and by that time, unless disposal is effectively controlled it is predicted that 12,000 million metric tonnes of plastic will have been discarded, either in landfills or the in natural environment (Geyer *et al.,* 2017). Globally, less than 10% of plastics are recycled, resulting in large volumes ending up in the environment (Mazhandu, 2019). Plastics enter the environment either directly or indirectly through a variety of pathways (Fig. 1.1), e.g., from plastic supply chains, wastewater treatment plants (WWTPs), agriculture, and littering (Ryberg et al., 2019; Chaudhary et al., 2021; Liu et al., 2023; Sa'adu et al., 2023). Despite stricter legislation and fines, plastics are increasingly entering the aquatic environment through illegal wastewater discharge and overflow events; in 2022, untreated sewage was released over 1,000 times a day in the UK (Ross *et al.,* 2023). Importantly however, plastics are highly resistant to environmental degradation, which allows them to persist and accumulate in terrestrial, freshwater, and marine ecosystems (Karbalaei *et al.,* 2018).

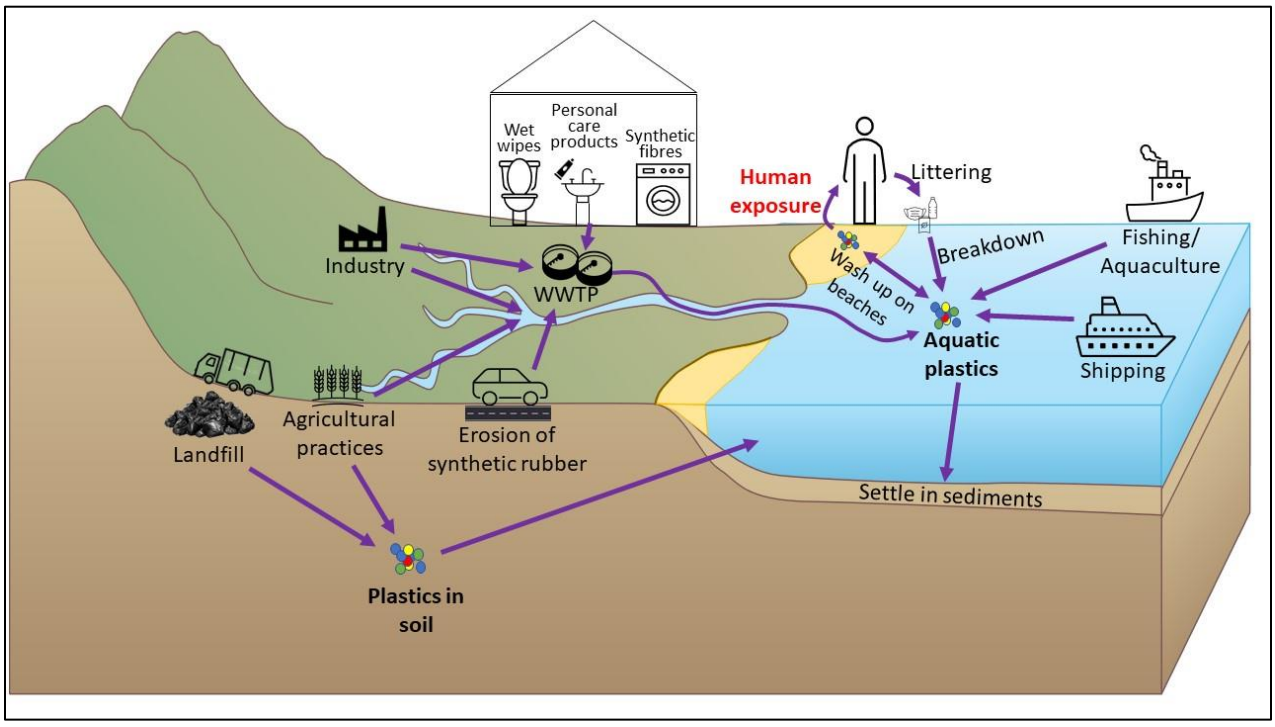

Figure 1.1 Sources and dispersal routes of plastic pollution. WWTPs: Wastewater treatment plants.

Plastics include a diversity of polymer types, colours, and shapes (McCormick *et al.,* 2016), but are often classified by size, with the typical categories being macroplastic (>20 mm diameter), mesoplastic (5-20 mm), microplastic (<5 mm) and nanoplastic (<1000 nm) (Napper and Thompson, 2020). A large proportion of plastics in the environment are microplastics, which include plastic fragments, fibres and beads (Cole *et al.*, 2011). They can either be manufactured as microplastics (primary microplastics such as virgin preproduction pellets, or fibres used in fabrics) or be derived from degradation and fragmentation of larger plastic debris (secondary microplastics) (Karbalaei *et al.,* 2018). Due to their insufficient containment during waste water treatment, millions of microplastics are released from WWTPs everyday (Krishnan *et al.,* 2023). Other plastic pollutants frequently discharged from WWTPs include wet wipes, cotton bud sticks and sanitary products, which are composed in part or fully of plastic polymers (Briain *et al.,* 2020). Morales-Caselles *et al.* (2021) collated information on the nature of plastic pollutants in aquatic environments and identified the following top five products: plastic bags (14%), plastic bottles (12%), food containers (9%), fishing related items (8%) and synthetic ropes (8%); highlighting the variety of other plastic pollutants found in the environment.

1.2 Plastisphere pathogens

Plastic pollutants are unsightly and negatively impact ecosystems, e.g., through entanglement and ingestion by wildlife, release of toxic chemicals, and the transport of non-native species (Bucci *et al.,* 2020). Plastic properties, including hydrophobicity and density, make them suitable surfaces for microbial colonisation and biofilm formation, with the distinct microbial communities colonising plastic debris being termed 'plastisphere' communities (Zettler, 2013). Microbes within the plastisphere benefit from enhanced dispersal and protection from environmental stressors and predation (Li *et al.,* 2021). Importantly, plastisphere communities can contain or even enrich human pathogens which can then be dispersed within environments and pose a risk to human health (Kaur *et al.,* 2022; Metcalf et al., 2022; Li et al., 2024). WWTPs are hotspots for both human pathogens and plastics (Liu *et al.,* 2021), with a high likelihood of plastics becoming colonised by human pathogens as they pass through the WWTP. Once released into the environment, the durability of plastics provides the opportunity for the continued re-colonisation by pathogens (Ormsby *et al.,* 2024).

Plastic properties, including buoyancy and durability, enable plastics to be dispersed over large distances and transfer pathogens into areas where they are more likely to come into contact with humans (e.g., bathing water beaches). Large volumes of plastics wash up onto our beaches, with plastic accounting for 81.5% of all litter collected in the Great British Beach Clean in 2022 (Marine Conservation Society, 2023). Due to recreational use of beaches occurring year-round (e.g., walking, swimming, sunbathing, water sports), beaches are one of the main human exposure routes to pathogen colonised plastics; however, there are also several other human exposure routes to pathogen colonised plastic pollution (Fig. 1.2), including the ingestion of food items containing plastics (Mortensen *et al.,* 2021), and the inhalation of airborne plastics (Amato-Lourenco *et al.,* 2020).

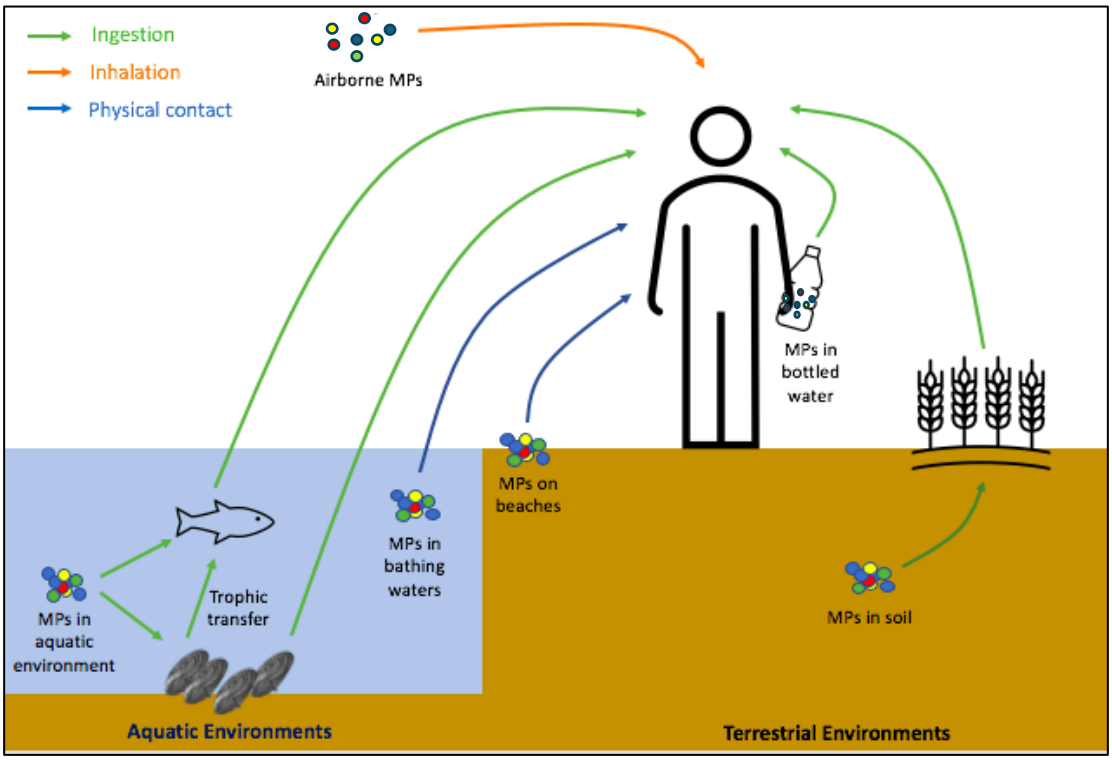

Figure 1.2 Human exposure routes to pathogen carrying microplastics.

1.3 Recent progress in the field of plastisphere research

Metcalf *et al*. (2022) summarised all the published studies that had detected human bacterial pathogens in the plastisphere of environmental plastic pollution. Since this paper has been published, the field of research has advanced. Most studies identified in that review had used molecular techniques, sequencing a fragment of the 16S rRNA gene to identify potential pathogens. This method is unable to identify bacteria down to species level and does not provide information on viability.

Therefore, culture-based methods have been used to further quantify the viable potential of human pathogens in the plastisphere (e.g., Liang *et al.,* 2023). Ormsby et al., (2023) highlighted how most research on plastisphere pathogens has focussed on bacteria, although there are an increasing number of studies focusing on pathogenic fungi (Akinbobola *et al.,* 2024), and viruses (Moresco *et al.,* 2021; Witsø *et al.,* 2023).

Importantly, it has been highlighted that identifying pathogens in the plastisphere to species level is insufficient and that it is necessary to identify the presence of virulence genes to determine actual pathogenesis (Beloe *et al*., 2022). Consequently, studies have begun to incorporate sequencing methods to determine the presence of virulence genes (e.g., Ni *et al.,* 2023; Wang *et al.,* 2023). However, just because virulence genes are present does not mean that they will be expressed and cause disease. To overcome this, some studies have used qPCR to quantify the expression of virulence genes (e.g., Wang *et al.,* 2023), and several studies have also included a *Galleria melonella* model of infection to determine whether these virulence genes are being expressed and causing disease and mortality (e.g., Lear *et al.,* 2022; Ormsby *et al.,* 2024).

Antimicrobial resistance (AMR) is now considered a chronic global public health challenge, with 10 million deaths as a result of AMR predicted by 2050 (Tang *et al.,* 2023). Microplastics and their plastisphere communities co-occur in environments with drug-resistant bacteria, antimicrobials and antimicrobial resistance genes (ARGs), driving the selection of antimicrobial resistance in the plastisphere (Stevenson *et al.,* 2023). ARGs are frequently present in the plastisphere, where they can be up to 5000 times more abundant compared to the surrounding water (Zhang *et al.,* 2020; Liang *et al.,* 2023). Plastisphere pathogens have been found to contain a wide range of ARGs, which confer resistance against common antibiotics including tetracycline and fosfomycin (Kaur *et al.,* 2022; Lear *et al.,* 2022), meaning they are likely to be harder to treat and could pose a greater risk to human health. Therefore, in order to fully assess the implications to human health, there is a need to determine the presence and survival of pathogens in the plastisphere, but also quantify whether they are virulent and are expressing any ARGs.

1.4 Research rationale, aim and objectives

In order to fully understand the potential implications to human health, there needs to be an increased understanding of the survival, transfer and characterisation of potentially harmful pathogens colonising plastic pollution. This will also enable appropriate environmental management and policy recommendations to be developed. Therefore, the overarching aim of this thesis is to quantify the **colonisation**, **survival**, and **pathogenicity** of bacterial and fungal pathogens on environmental plastic pollution in freshwater, marine, and beach environments. At the start of the project, a detailed review of the subject area was carried out, followed by fieldwork and experimental mesocosm studies focused on the following primary objectives:

- Evaluate the load of potential bacterial and fungal pathogens **colonising** different types of environmental plastic pollution.
- Quantify the **survival dynamics** of potential pathogens on plastics during the transfer between different environmental matrices.
- Characterise the AMR, thermotolerance, and **pathogenicity** of potential pathogens.

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2. Quantifying the importance of plastic pollution for the dissemination of human pathogens: understanding the challenges of choosing an appropriate 'control' material

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- **Vanessa Moresco:** Writing – review & editing.
- **Richard S. Quilliam:** Writing – review & editing, Supervision,

Abstract

Discarded plastic wastes in the environment are serious challenges for sustainable waste management and for the delivery of environmental and public health. Plastics in the environment become rapidly colonised by microbial biofilm, and importantly this so-called 'plastisphere' can also support, or even enrich human pathogens. The plastisphere provides a protective environment and could facilitate the increased survival, transport and dissemination of human pathogens and thus increase the likelihood of pathogens coming into contact with humans, e.g., through direct exposure at beaches or bathing waters. However, much of our understanding about the relative risks associated with human pathogens colonising environmental plastic pollution has been inferred from taxonomic identification of pathogens in the plastisphere, or lab experiments on the relative behaviour of plastics colonised by human pathogens. There is, therefore, a pressing need to understand whether plastics play a greater role in promoting the survival and dispersal of human pathogens within the environment compared to other substrates (either natural materials or other pollutants). In this paper, we consider all of the published studies that have detected human pathogenic bacteria on the surfaces of environmental plastic pollution and critically discuss the challenges of selecting an appropriate control material for plastisphere experiments. Whilst it is clear there is no 'perfect' control material for all plastisphere studies, understanding the context-specific role plastics play compared to other substrates for transferring human pathogens through the environment is important for quantifying the potential risk that colonised plastic pollution may have for environmental and public health.

Keywords: Biofilm; Environmental risk; Experimental design; Human health; Microplastics;

2.1 Introduction

Plastics are inexpensive, lightweight, strong and durable, making them the ideal material for a diverse array of products and applications with widespread societal benefits (Thompson, 2006; Andrady and Neal, 2009). This has resulted in an increase in the global production of plastics, from 35 million tonnes in the 1950s to 335 million tonnes in 2019 (Plastics Europe, 2020). However, less than a fifth of plastic is recycled globally and large amounts of plastic are continuously released into the environment either directly or indirectly via multiple pathways, e.g., from wastewater treatment plants (WWTPs), agriculture (e.g. mulching film or seed coating) and littering (Ivleva *et al.*, 2017; Ren *et al.*, 2021; Woodward *et al.*, 2021), and due to their longevity, can persist and accumulate in terrestrial, freshwater and marine ecosystems (Thompson, 2006; Ivleva *et al.*, 2017; Karbalaei *et al.*, 2018). Plastics in the environment can have multiple negative impacts, including blocking drains, wildlife entanglement, the accumulation of toxins (e.g. PCBs, DDTs and HCHs) and the transport of non-native species (e.g. mussels, barnacles and diatoms) (Wang *et al.*, 2018; Napper and Thompson, 2020; Welden, 2020).

2.2 Biofilms on environmental plastics

Intrinsic properties of plastics and microplastics (defined as plastic particles < 5 mm), such as their hydrophobicity, density, and high surface area to volume ratio, can promote microbial colonisation and biofilm formation (Harrison *et al.*, 2014; Frere *et al.*, 2018; Cai *et al.*, 2019). Organic matter, nutrients and biomolecules can also rapidly adsorb to plastic surfaces in the environment, forming a unique ecocorona that further attracts microbial colonisers (Bhosle *et al.*, 2005; Galloway *et al*, 2017; De Carvalho, 2018). Many microorganisms prefer being attached to a surface rather than remaining planktonic; and biofilms can provide several benefits, including the capture of nutrients, protection from environmental stressors and predation, and enhanced dispersal (Lee *et al.*, 2008; De Carvalho, 2018; Santos *et al.*, 2018). Consequently, biofilms are composed of a dynamic array of successional microbial communities, including the bacterial groups Gammaproteobacteria (during the initial 24 hours), Alphaproteobacteria (from 24 hours) and Bacteroidetes (Dang and Lovell, 2000; Oberbeckmann *et al.*, 2015; Wright *et al.*, 2021), together with a diverse range of fungi, diatoms, algae, and viruses (Audrézet *et al.*, 2020; Gkoutselis *et al.*, 2021; Moresco *et al.*, 2021).

Biofilms can form on any surface, including living tissues, indwelling medical devices, water system piping, wood and plastics (Donlan, 2002; Lobelle and Cunliffe, 2011; De Carvalho, 2018).

However, Zettler et al (2013) coined the term 'plastisphere' to describe the distinct microbial communities that colonise environmental plastic debris. Plastisphere communities are highly variable, diverse and genetically different from the free-living communities that surround them, implying that plastic provides a novel ecological habitat (Kirstein *et al.*, 2019; Wu *et al.*, 2020; Li *et al.*, 2021). Importantly, biofilms on plastics can also support, or even enrich, microbial communities, including human pathogens (Oberbeckmann and Labrenz, 2020; Sun *et al.*, 2020; Wu *et al.*, 2020).

Although an increasing number of studies have found human pathogens within the plastisphere, the relative risk to human health has not yet been quantified (Noventa et al., 2021); thus, there is a pressing need to increase our understanding of the dynamics of human pathogens colonising environmental plastic pollution. However, we argue that in order to determine whether plastics play a greater role in enhancing the survival and dispersal of human pathogens within the environment compared to other substrates (either natural materials or other pollutants), future experiments need to incorporate a 'control material' as a direct comparison. Very few published studies have attempted to compare the behaviour of human pathogens in the plastisphere with human pathogens in the biofilm on a control substrate. Therefore, in this paper, we summarise all of the published studies that have detected human pathogenic bacteria on the surfaces of environmental plastic pollution and critically discuss the challenges of selecting an appropriate control material for plastisphere experiments.

2.3 Human pathogens in the plastisphere

Human pathogens (and potential pathogens) have been identified within the plastisphere of several different plastic polymers, including polyethylene, polypropylene, and polystyrene (Table 2.1). Pathogens that are frequently detected within plastisphere communities include *Vibrio* spp.; although not all vibrios are pathogenic, some human pathogen species have been found colonising marine plastics, particularly in summer months, e.g., *Vibrio parahaemolyticus*, *V. cholerae* and *V. vulnificus* (Kirstein *et al.*, 2016; Silva *et al.*, 2019; Laverty *et al.*, 2020; Rasool *et al.*, 2021). Such pathogens can cause diarrhoea, cellulitis and septicaemia in humans and are responsible for significant levels of mortality, particularly in developing sub-Saharan African and Southeast Asian countries where medicines and resources are less readily available (Ali *et al.*, 2015; Heng *et al.*, 2017). Due to the benefits of living within the biofilm, the infectiousness of pathogens such as *V. cholerae,* can be increased (Lyons *et al.*, 2010; Bowley *et al.*, 2020; Wu *et al.*, 2020). Other human pathogens identified within the plastisphere, include *Escherichia coli*, *Providencia rettgeri* and *Salmonella* spp., which can cause

diarrhoea, gastrointestinal, urinary tract and eye infections (El-Liethy *et al.*, 2020; Moore *et al*, 2020; Shi *et al.*, 2021). Human pathogens are often identified within the plastisphere by sequencing or culture-base approaches, therefore, testing for virulence genes is required to determine their actual pathogenesis (Wright *et al.*, 2020).

Plastics in the environment have the potential to facilitate the dispersal of human pathogens and transport them large distances through terrestrial, freshwater, and marine environments (Debroas *et al*, 2017), particularly as certain species of human pathogen have been detected colonising plastics in more than one environmental matrix (Table 2.1). The potential for human pathogens in the plastisphere to survive, persist and be transported between different environments, could increase exposure routes and opportunities for coming into contact with humans, e.g., through direct exposure at beaches or bathing waters (Keswani *et al.*, 2016; Rodrigues *et al.*, 2019), or via the consumption of shellfish or water (Cox et al., 2019; Bowley et al., 2020; Fabra et al., 2021). The survival dynamics of pathogens colonising environmental plastic pollution is yet to be fully understood, although it has been suggested that *Staphylococcus aureus* is able to survive on dry plastic for up to three years (Chaibenjawong and Foster, 2011); which highlights the highl potential for pathogens to be transported large distances during this time.

Colonisation and persistence of human pathogenic bacteria in the plastisphere are probably influenced by the plastic polymer type, with recent studies suggesting that the highest diversity of human bacterial pathogenic species were found colonising polyethylene (Appendix A); although some species, e.g., *Vibrio*, have been found colonising nearly all plastic polymer types (Appendix A). Bacterial pathogens are also detected on the surfaces of non-plastic 'control' materials, e.g., glass and wood (Table 2.1), with *Vibrio* spp. detected on the majority of these materials. This suggests that both the plastic polymer and the control material can influence the pathogens which bind to it. There is also evidence to suggest that the properties of the bacterial species themselves can influence which materials they preferentially attach to. For example, some strains of pathogenic *E. coli* and *Enterococcus faecalis* have high surface free energy, resulting in weaker adhesion forces to hydrophobic surfaces such as plastics, whereas *Salmonella typhimurium* and *Pseudomonas putida*, have low surface free energy and preferentially attach to more hydrophobic surfaces (Zhang et al., 2015; Song et al., 2020). Importantly, most pathogenic species colonising plastic surfaces have not been simultaneously tested with a non-plastic control material, which makes it difficult to discern the comparative risk of environmental plastic pollution for disseminating human pathogens.

Table 2.1. Potential human pathogenic bacteria detected in the plastisphere of environmental plastic pollution and on the surfaces of control materials.

Plastic Type*	Control Material**	Pathogenic bacteria	Environment	Method	Reference
PVC, PA, PE, PS	-	*Burkholderia* sp. *Enterococcus faecium* *Klebsiella pneumoniae* *Listeria monocytogenes* *Mycobacterium*	Terrestrial	16S rRNA	(Zhu *et al.,* 2021)
PP, PS, PET, PE, PVC	-	*Klebsiella pneumoniae* *Vibrio cholerae*	Terrestrial	Selective media, 16S rRNA	(Rasool *et al.*, 2021)
PS	-	*Pseudomonas aeruginosa* *Escherichia coli*	Terrestrial	16S rRNA	(Shi *et al.*, 2021)
PE, PP, PS, PET, PAN	-	*Coxiella* sp. *Legionella* *Streptococcus* sp.	Freshwater	16S rRNA	(Galafassi *et al.,* 2021)
PE, PP, PS	-	*Arcobacter* sp. *Campylobacteraceae* *Enterobacteriaceae* *Klebsiella pneumoniae* *Moraxcellaceae* *Pseudomonas* spp.	Freshwater (WWTP)	16S rRNA	(Kelly *et al.,* 2021)
LDPE, HDPE, PP, PC, PS	Glass	*Vibrio cholerae* *Vibrio parahaemolyticus* *Vibrio vulnificus*	Freshwater	16S rRNA	(Laverty *et al.*, 2020)
Undetermined	-	*Arcobacter* sp. *Pseudomonas* spp. *Campylobacteraceae*	Freshwater	16S rRNA	(McCormick *et al.*, 2014)
PE, PP, PS	-	*Arcobacter* sp. *Campylobacteraceae*	Freshwater	16S rRNA	(McCormick *et al.,* 2016)
PE	-	*Enterobacter* spp. *Helicobacter* spp. *Arcobacter sp.* *Clostridium perfringens* *Escherichia coli*	Freshwater	16S rRNA	(Murphy *et al.*, 2019)
PE, PS	Sand	*Raoultella ornithinolytica* *Stenotrophomonas maltophilia*	Freshwater	16S rRNA	(Pham *et al*, 2021)
PVC, PE	-	*Legionella* *Mycobacterium* *Neisseria* *Arcobacter*	Freshwater (Sewage)	16S rRNA	(Wang *et al.*, 2021)
PBT, PE, PP, PS	-	*Pseudomonas* spp.	Freshwater	16S rRNA	(Xue *et al.*, 2020)
PE, PP, PET, PS, PU	-	*Vibrio* spp. *Pseudomonas* spp. *Bacillus spp.*	Freshwater	16S rRNA	(Zhang *et al.*, 2021c)

		Rhodococcus spp.			
PVC	-	*Mycobacterium* *Legionella spp.* *Rhodoccocus* spp.	Freshwater	16S rRNA	(Zhao *et al.*, 2021b)
PS, PP, PE, PET, PVC	-	*Bacillus sp.* *Mycobacterium sp.*	Estuary	16S rRNA	(Guo *et al.*, 2018)
LDPE	-	*Arcobacter sp.*	Estuary	16S rRNA	(Harrison *et al.*, 2014)
PS, PP, PE	-	*Pseudomonas* spp. *Vibrio* spp.	Estuary	16S rRNA	(Jiang *et al.*, 2018)
Undetermined	-	*Vibrio* spp. *Shewanella sp.*	Estuary	Selective media	(Li *et al.*, 2019)
Undetermined	-	*Escherichia coli* *Enterococci*	Estuary	Selective media	(Pazos *et al.*, 2020)
PE, PP, PS, PET, PU	-	*Pseudomonas* spp.	Estuary	16S rRNA	(Wu *et al.*, 2020)
HDPE, LDPE, PP	-	*Francisella* *Rickettsia*	Marine	16S rRNA	(Barral *et al.*, 2018)
PE, PP, PS	-	*Mycobacterium* *Staphylococcus* spp.	Marine	Microscopy, 16S rRNA	(Basili *et al.*, 2020)
Undetermined	-	*Pseudomonas alcaligenes* *Vibrio* spp.	Marine	16S rRNA	(Curren and Leong, 2019)
PE	-	*Vibrio* spp.	Marine	16S rRNA	(De Tender *et al.*, 2015)
LDPE, OXO, PHBV	-	*Staphylococcus aureus* *Vibrio* spp.	Marine	16S rRNA	(Dussud *et al.*, 2018)
PET, PP, PE	-	*Acinetobacter oleivorans* *Escherichia coli* *Vibrio fischeri* *Vibrio splendidus*	Marine	16S rRNA	(Hou *et al.,* 2021)
PS	Glass, Chitin	*Arcobacter sp.*	Marine	16S rRNA	(Kesy *et al.*, 2016; 2017)
PE, PS	Wood	*Vibrio* spp.	Marine	16S rRNA	(Kesy *et al.*, 2019; 2021)
PE, PP	-	*Vibrio parahaemolyticus*	Marine	Selective media	(Kirstein *et al.*, 2016)
PS	-	*Shewanella sp.*	Marine	16S rRNA	(Lagana *et al.*, 2019)
PE, PET	-	*Vibrio* spp. *Pseudomonas* spp. *Streptococcus* spp.	Marine	16S rRNA	(Li *et al.*, 2020)

Undetermined	-	*Providencia rettgeri*	Marine	Selective media	(Moore *et al.*, 2020)
PET	-	*Vibrio* spp.	Marine	16S rRNA, 18S rRNA	(Oberbeckmann *et al.*, 2016)
HDPE, PS	Wood	*Vibrio* spp.	Marine, Freshwater	16S rRNA	(Oberbeckmann *et al.*, 2018)
Undetermined	Sand, Seaweed	*Vibrio* spp.	Marine	Selective media	(Quilliam *et al.*, 2014)
PET	-	*Pseudomonas* spp. *Morganella morganii*	Marine	Whole genome sequencing	(Radisic *et al.*, 2020)
PVC	Metal	*Vibrio parahaemolyticus*	Marine	Selective media, 16S rRNA	(Rajeev *et al.*, 2019)
Undetermined	-	*Escherichia coli* *Vibrio* spp.	Marine	Selective media	(Rodrigues *et al.*, 2019)
Undetermined	-	*Vibrio* spp.	Marine	16S rRNA	(Schmidt *et al.*, 2014)
Undetermined	-	*Escherichia coli* *Vibrio cholerae*	Marine	16S rRNA	(Silva *et al.*, 2019)
Undetermined	Feather	*Vibrio* spp.	Marine	Microscopy	(Sun *et al.*, 2020)
Undetermined	-	*Escherichia coli* *Bacillus cereus*	Marine	NGS	(Van der Meulen *et al.*, 2015)
PP, PVC	-	*Vibrio* spp.	Marine	16S rRNA	(Xu *et al.*, 2019)
PP, PE	-	*Vibrio* spp.	Marine	16S rRNA	(Zettler *et al*, 2013)
Undetermined	-	*Vibrio* spp.	Marine	16S rRNA	(Zhang *et al.*, 2020)

* PP-polypropylene, PE-polyethylene, HDPE-high density polyethylene, LDPE-low density polyethylene, PET-polyethylene terephthalate, PS-polystyrene, PU-polyurethane, PVC-polyvinyl chloride, PC – Polycarbonate, PBT- Polybutylece terephthalate, OXO-Additivated PE with pro-oxidant, PHBV-poly(3-hydroxybutyrate-co-3-hydroyvalerate), PAN - polyacrylonitrile

** Only 8 of 45 studies included a control material and also detected human bacterial pathogens within their biofilms.

2.4 What is an appropriate 'control' substrate for plastisphere studies?

Although it is well documented that the community composition of the plastisphere is often significantly different to the surrounding environment due to planktonic/biofilm species preferences (Xue *et al.*, 2020; Martínez-Campos *et al.*, 2021), this does not provide a functional 'control' that gives us a narrative on the relative role of environmental plastic pollution. Natural organic materials, such as leaves and wood, and inorganic materials, such as glass and rubber, are also important materials for the transport of freshwater and marine organisms (Kesy *et al.*, 2019; Miao *et al.*, 2019), and human pathogens have previously been identified within the biofilms of materials such as glass, wood and feathers (Islam *et al.*, 2007; Sun *et al.*, 2020; Pham *et al.*, 2021). Although more recent studies have begun to include control materials (Kesy *et al.*, 2019; Sun *et al.*, 2020; Martínez-Campos *et al.*, 2021), for the majority of plastisphere studies, there is still a lack of comparison between microbial communities binding to plastic surfaces compared with the surfaces of other substrates in the environment (Table 2.2). This makes it difficult to draw useful conclusions on whether the composition and survival dynamics of pathogens and plastisphere communities differ from the biofilms colonising other substances, and whether environmental plastics play any more of a significant role in facilitating the survival and dispersal of human pathogens than other materials. The source and dispersal routes of plastic pollution often significantly differ from potential control materials. For example, there are several opportunities for plastics to encounter high concentrations of human pathogens, e.g., when they pass-through WWTPs or are exposed to hospital waste; in contrast, potential control materials are less likely to be exposed to these sources, and therefore, plastics may be entering the environment already colonised with significant populations of human pathogens.

Of the studies that reported pathogen enrichment on plastic surfaces, 62% showed higher pathogen abundances on plastic compared to control materials (Table 2.2), and often include species of *Vibrio* and *Pseudomonas* (Wu et al., 2019; Sun et al., 2020). Plastics in the environment can provide a novel niche, with distinct properties and characteristics that promote pathogen colonisation (Sun et al., 2020). Some pathogens, including *Vibrio*, are known to be secondary opportunistic colonisers dependent on primary colonisers already present in the biofilm (Datta et al., 2016; Foulon et al., 2016). As plastics support distinct microbial communities compared to control materials (Kirstein et al., 2019; Miao et al., 2019; Oberbeckmann and Labrenz, 2020), the

composition of the plastisphere plays an important role in the potential colonisation of pathogens (Wu et al., 2019).

Evidence on whether pathogens preferentially colonise plastics over other materials in the environment remains contradictory (e.g. Oberbeckmann and Labrenz, 2020; Song et al., 2020). These inconsistencies are likely due to the variable environmental conditions of each study, with environmental factors often having a stronger influence on plastisphere formation and diversity than the type of polymer (Basili et al., 2020; Kesy et al., 2021; Zhang et al., 2021a). Organic materials in the environment, e.g., seaweed and wood, can provide a more readily available source of nutrients compared with plastics (Takemura et al., 2014; Quilliam et al., 2014; Song et al., 2020), but the higher durability of plastics compared to organic materials, increases the potential for dissemination and transport of microbial colonisers. However, to understand the relative risk of pathogen persistence in the plastisphere, studies that include environmentally relevant control materials are urgently needed to determine whether plastic pollution really does increase the opportunity for pathogen transport and transmission in comparison to colonisation of other substrates in the environment.

A range of different organic and inorganic controls have been used in plastisphere studies (Table 2.2). The most commonly used organic control is wood (10 of 46 studies), whilst glass is the most commonly used inorganic control (23 of 46 studies), with the majority of studies preferring an inorganic material as a control substrate (35 of 46 studies). To ensure a similar available colonisation area, the control material needs to be of a very similar size, shape and texture as the plastic particle being quantified. Size, shape and colour of materials are important to control for because not only can they influence available surface area, buoyancy and transport, biofilm community structure and the abundance of potential pathogens, such as *Vibrio* and *Pseudomonas* (Mughini-Gras et al., 2021; Zhang et al., 2021b), but also any subsequent potential ingestion, e.g., by bivalve species (Bowley et al., 2020). Many plastisphere studies have reported higher microbial diversity on control substrates compared to plastic surfaces (Table 2.2), which implies that the availability of a surface to colonise is probably more important for driving microbial diversity than the composition of the surface itself.

To date, the majority of plastisphere research has focused on individual environmental matrices with a particular emphasis on the marine environment (e.g., Bowley et al., 2020). This conceptual compartmentalisation of the environment masks our understanding of how plastisphere

communities behave as they are transported between different environments within the landscape. The “plastic cycle” transports plastics between different abiotic (and biotic) compartments as they are transferred through terrestrial, freshwater, and marine environments (Bank and Hansson, 2019; Rochman, 2018). This needs to be considered when selecting an appropriate control as the transport mechanisms of both the plastic and the control material are likely to be affected by their interaction with the conditions in each specific environmental matrix. For example, stream and river ecosystems are characterised by continuous downstream movement, whilst marine ecosystems have varying tidal flows and currents; therefore, the specific behaviour of plastic and control materials is likely to vary in these contrasting environmental matrices (Boyle and Örmeci, 2020).

Although plastisphere communities differ between environmental matrices, there are relatively few studies that have considered plastisphere communities in both freshwater and marine environments (Kettner et al., 2017; Kettner et al., 2019; Oberbeckmann et al., 2018); and only one that has physically moved plastic particles between these two environments (Song et al., 2020). Whereas the transition of plastics between terrestrial and aquatic environments e.g., from runoff and erosion of contaminated soil or when plastics are washed up on beaches, has so far been ignored in the literature. The contrasting environmental conditions and surrounding autochthonous microbial communities of terrestrial vs aquatic (freshwater and marine) environments will strongly influence the composition and diversity of plastisphere and biofilm communities (e.g., in terms of the species involved with primary biofilm formation and subsequent succession of the community), before they are delivered to the new environmental matrix.

Environmental factors, such as nutrient availability, temperature, and salinity, impose differential selective forces and can significantly influence plastisphere composition and structure (Li et al., 2019; Pinto et al., 2019; Zhang et al., 2021a). For example, the higher nutrient concentrations found around WWTPs in freshwater environments can increase bacterial richness and diversity of biofilm communities of both plastic and wood compared to coastal environments (Oberbeckmann et al., 2018). Yet, relatively little is known about how pathogenic bacteria in the plastisphere are affected as they transition between environmental matrices. The survival and abundance of certain pathogenic species decrease as particles transition from freshwater to saltwater environments. Higher abundances of the taxonomic groups Enterobacteriaceae and *Vibrio* were found colonising plastics in freshwater locations compared to marine locations (Oberbeckmann et al., 2018), whilst the survival of *E. coli* decreased as plastic and control particles transitioned along a salinity gradient

(Song et al., 2020). Interestingly, Song et al. (2020) detected higher abundances of pathogens on the control particles (tyre wear and wood) compared to the HDPE plastic particles, suggesting that pathogenic bacteria were less likely to survive the transition between environmental matrices on plastics compared to other materials. Determining how the colonisation and persistence of pathogens in the plastisphere changes as it transitions between environmental compartments will help determine the risk of pathogen transfer and transmission on microplastics and other materials as they are transported within and between environments.

Several different organic and inorganic substrates have previously been used as control materials (Fig. 2.1). In addition to providing a surface to colonise, organic materials can also provide a nutrient source and are often associated with higher diversity and heterotrophic growth, whereas inert inorganic substrates are more associated with autotrophic growth (Tobias-Hünefeldt et al., 2021). This suggests that heterotrophic human bacterial pathogens are likely to be more abundant on organic substrates, which may explain why pathogen abundances can be enriched on control materials, such as wood and chitin (Table 2). However, organic materials are not always as buoyant as plastics (Fig. 1) and decompose more quickly (e.g., straw and coconut husks); thus, plastics have the potential to transport and disseminate microbes for longer and further than natural organic materials (Thiel and Gutow, 2005; Keswani et al., 2016; Laverty et al., 2020). Although plastic is the main constituent of anthropogenic litter, other inorganic materials, such as metal, glass and ceramics make up a proportion of the anthropogenic litter across all environments within the landscape (Addamo et al., 2017; Nelms et al., 2017). Importantly, the use of a single control material will not control for all variables of any particular plastic polymer; therefore, some studies have used both organic and inorganic controls, which gives more useful information by providing several comparisons (Ogonowski et al., 2018; Muthukrishnan et al., 2019; Tobias-Hünefeldt et al., 2021). Most potential control materials will be found within most environmental matrices, although there are some which are only found (or are much more abundant) in certain environmental compartments or geographical locations (e.g., seaweed, pumice). The selection of a control material needs to be environmentally relevant to the environmental matrices being studied, but the provenance of the material is also an important factor as in natural systems the material will have already been colonised in the preceding matrix.

Table 2.2 Recent plastisphere studies that have used a control material.

Plastic Type*	Size (mm)	Control	Size (mm)	Environment	Method	Experimental Design**	Higher Diversity	Difference in Community Composition		Pathogen enrichment on plastic	Reference
								Plastic vs. Control	Plastic vs. Matrix		
PVC, PA, PE, PS	0.03-1.5	Glass	0.03	Terrestrial (soil)	16S rRNA	Mesocosm, Field deployed	No difference	Difference	Difference	-	(Zhu *et al.,* 2021)
PE, PP	3-4	Cobblestone, Wood	30-50	Freshwater	16S rRNA	Mesocosm	Control	Difference	-	-	(Miao *et al.*, 2019)
PVC	3	Rock, Leaves	2-4	Freshwater	16S rRNA	Mesocosm	Plastic	Difference	Difference	Yes	(Wu *et al.*, 2019)
PE, PS	0.085 – 0.106	Sand	0.088 – 0.105	Freshwater	16S rRNA	Mesocosm	Plastic	Difference	-	Yes	(Pham *et al.,* 2021)
PLA, PHB, PCL, PET, POM, PS, LDPE	3-5	Glass	2-8	Freshwater	16S rRNA	Field deployed	No difference	Difference	Difference	-	(Martínez-Campos *et al.*, 2021)
PE, PS	-	Glass	-	Freshwater	16S rRNA	Mesocosm	Control	Difference	Difference	-	(Parrish and Fahrenfeld, 2019)
LDPE, HDPE, PP, PC, PS	-	Glass	-	Estuary	Selective media	Field collected; Field deployed	Plastic	No difference	Difference	Yes	(Laverty *et al.*, 2020)
PLA, LDPE	3-5	Glass	4	Estuary	Microscopy	Field deployed	-	-	-	-	(Richard *et al.*, 2019)
PC	-	Steel	20-50	Estuary	16S rRNA	Field deployed	-	Difference	Difference	-	(Jones *et al.*, 2007)
PS, PP, PVC, PCL	3-4	Wood	-	Estuary	Metagenomic	Field deployed	No difference	Difference	Difference	Yes	(Bhagwat *et al.*, 2021)
Undetermined	-	Cardboard, leaves, glass, aluminium, ceramic tiles	-	Marine	16S rRNA	Field collected	No difference	Difference	-	-	(Hoellein *et al.*, 2014)
PET, PHA	3-4	Ceramic	3-4	Marine	Metagenomics	Mesocosm, Field deployed	-	No difference	Difference	-	(Pinnell and Turner, 2019)
PA	-	Chitin	-	Marine	16S rRNA	Mesocosm	No difference	No difference	Difference	No	(Kesy *et al.*, 2017)
Undetermined	1-4	Feathers	1-4	Marine	Microscopy,	Field deployed	No difference	No difference	Difference	Yes	(Sun *et al.*, 2020)

				16S rRNA						
PP, PE, PLA		Glass		Marine	16S rRNA	Mesocosm	No difference	Difference	Difference	-	(Cheng *et al.*, 2021)
PVC	300	Glass	300	Marine	16S rRNA	Field deployed	Control	-	-	-	(Dang *et al.*, 2008)
LDPE	5x10	Glass	5x10	Marine	16S rRNA	Field deployed	No difference	Difference	Difference	-	(Erni-Cassola *et al.*, 2020)
PS	-	Glass	-	Marine	Microscopy	Field deployed	-	Difference	-	-	(Hung *et al.,* 2008)
PS	0.25-0.4	Glass	0.25-0.4	Marine	16S rRNA	Mesocosm	-	Difference	Difference	No	(Kesy *et al.*, 2016)
HDPE, LDPE, PP, PS, PET, PLA, SAN, PESTUR, PVC	50x50	Glass	50x50	Marine	16S rRNA, 18S rRNA	Mesocosm	-	Difference	-	-	(Kirstein *et al.*, 2018)
LDPE, HDPE, PP, PS, SAN, PESTUR, PLA, PET, PVC	-	Glass	-	Marine	16S rRNA	Mesocosm	Plastic	Difference	-	-	(Kirstein *et al.*, 2019)
PMMA	170x100	Glass, Steel	170x100	Marine	16S rRNA	Field deployed	-	Difference	Difference	-	(Lee *et al.*, 2008)
PET	-	Glass	-	Marine	16S rRNA	Field deployed	No difference	Difference	Difference		(Oberbeckmann *et al.*, 2014)
PET	-	Glass	-	Marine	16S rRNA, 18S rRNA	Field collected	-	No difference	Difference		(Oberbeckmann *et al.*, 2016)
PE, PP, PS	2-2.5	Glass, Cellulose	0.2/0.006 3 x 0.13	Marine	16S rRNA	Mesocosm	No difference	Difference	Difference	-	(Ogonowski *et al.*, 2018)
LDPE, HDPE, PP, PVC	40 x 40 x 0.5	Glass	10 x 10 x 1	Marine	16S rRNA	Field deployed	No difference	Difference	Difference	-	(Pinto *et al.*, 2019)
PS, PE, PVC	-	Glass	-	Marine	Microscopy	Laboratory	-	-	-	-	(Snoussi *et al.*, 2009)
PMMA	75x25	Glass, ceramic, wood	-	Marine	16S rRNA, 18S rRNA	Field deployed	Control	Difference	-	-	(Tobias-Hünefeldt *et al.*, 2021)
Undetermined	-	Glass, metal, fabric, rubber	-	Marine	16S rRNA	Field collected	Plastic	Difference	Difference	-	(Woodall *et al.*, 2018)
HDPE, PLA	3	Glass	18	Marine	Microscopy, 16S rRNA	Field deployed	No difference	No difference	Difference	Yes	(Zhang *et al.*, 2021a)

PE, PP, PS	5x5	Glass	5x5	Marine	Microscopy	Field deployed	-	-	-	-	(Zhao *et al.*, 2021a)
PS	60	Granite	300	Marine	16S rRNA	Field deployed	Control	-	-	-	(Chung *et al.*, 2010)
PP, HDPE, BDA	3x4	Gravel	3x4	Marine	16S rRNA	Field deployed	Control	Difference	Difference	-	(Agostini *et al.*, 2021)
LDPE, HDPE, PS, PP, PET, PU, PLA	-	Latex, rope, steel	-	Marine	16S rRNA	Mesocosm	Plastic	Difference	-	-	(Gerritse *et al.*, 2020)
PP, PA, PVC		Paint, Cellulose		Marine	16S rRNA	Field collected	-	Difference	Difference	-	(Tagg *et al.*, 2019)
Undetermined	-	Sand, Seaweed	-	Marine	Selective media	Field collected	-	-	-	No	(Quilliam *et al.,*2014)
PVC	100x50	Steel, Titanium	100x50	Marine	Selective media, 16S rRNA	Field deployed	-	-	Difference	Yes	(Rajeev *et al.*, 2019)
PVC	4	Steel, Silica	4	Marine	16S rRNA	Field deployed	Control	Difference	-	-	(Wang *et al.*, 2020)
PS	40x50	Volcanic pummice	-	Marine	Microscopy	Field deployed	Plastic	Difference	-	-	(Bravo *et al.*, 2011)
PE, PP, PET, PS	-	Wood	-	Marine	16S rRNA, 18S rRNA	Field collected	-	Difference	Difference	-	(Debroas *et al.,* 2017)
PE, PS	3	Wood	-	Marine	16S rRNA	Mesocosm	Control	No difference	Difference	Yes	(Kesy *et al.*, 2019)
HDPE, PS	3-5	Wood	-	Marine, Freshwater	18S rRNA	Field deployed	No difference	Difference	Difference	-	(Kettner *et al.*, 2017)
PE, PS	3-5	Wood	-	Marine, Freshwater	18S rRNA	Field deployed	Control	Difference	Difference	-	(Kettner *et al.*, 2019)
PET, PE	3	Wood, Steel	600 cm^2	Marine	16S rRNA	Field deployed	Control	Difference	Difference	-	(Muthukrishnan *et al.,* 2019)
HDPE, PS	3	Wood	-	Marine, Freshwater	16S rRNA	Field deployed	-	Difference	Difference	No	(Oberbeckmann *et al.,* 2018)
HDPE	4	Wood, Tyre wear	4	Marine, Freshwater	Selective media	Field deployed	-	-	-	No	(Song *et al.*, 2020)

* PP-polypropylene, PE-polyethylene, HDPE-high density polyethylene, LDPE-low density polyethylene, PS-polystyrene, PB-polybutylene, PVC-polyvinyl chloride, PLA-polylactic acid, PA-polyamide, BDA-HDPE with oxo-biodegradable additive BDA, PET-polyethylene-terephthalate, SAN-styrene-acrylonitryle, PESTUR-polyurethane-prepolymer, PHB-poly-3-hydroxybutyrate, PCL-polycaprolactone, POM-polyoxymethylene, PHA-polyhydroxyalkanoate, PMMA-poly(methyl methacrylate), PCL-polycaprolactone.

** Experimental design classifies studies depending on whether samples were collected from the field (field collected), placed out into the field under controlled settings (field deployed) or placed in a controlled mesocosm within the laboratory (mesocosm).

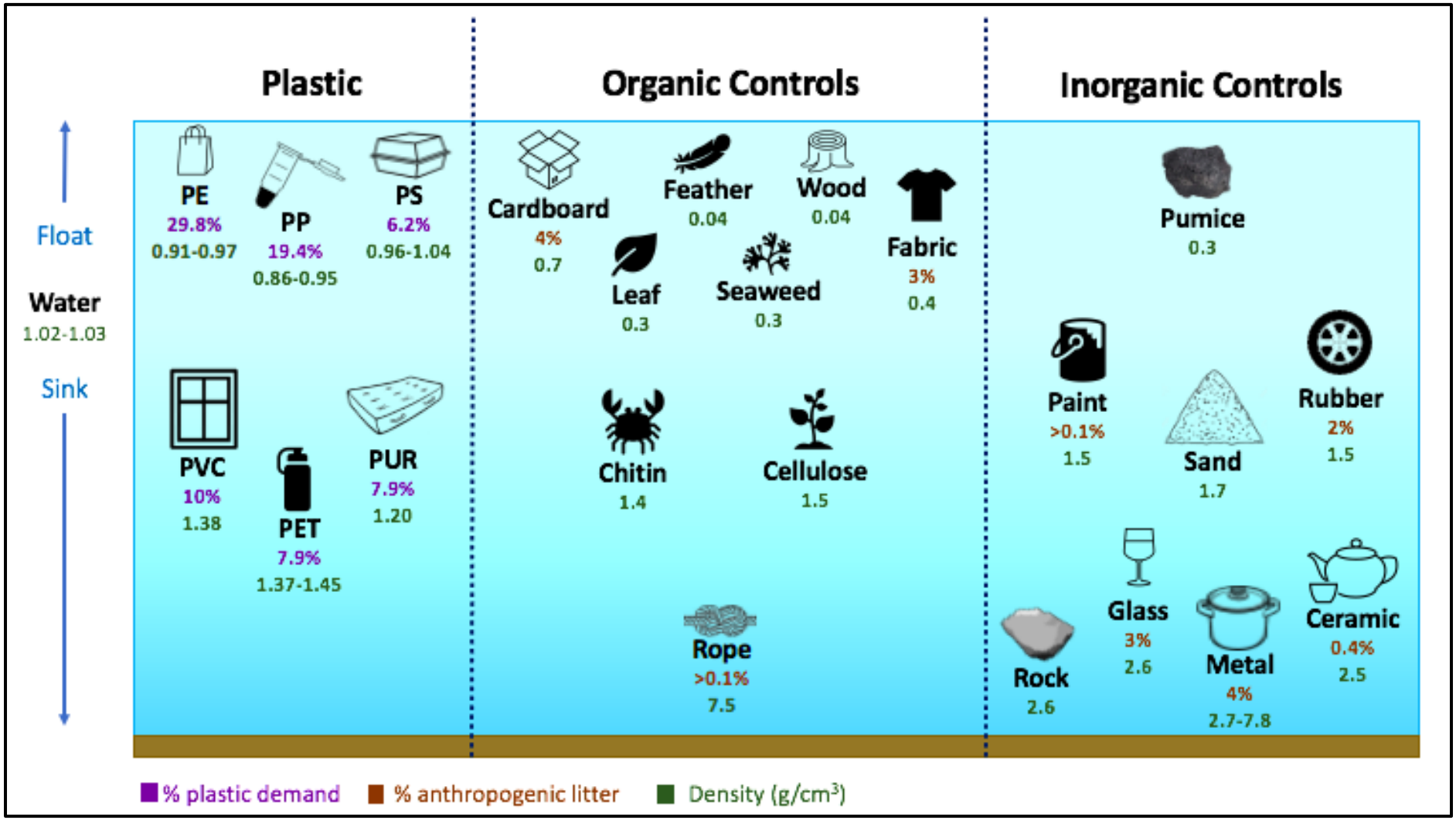

Figure 2.1 Potential organic and inorganic control materials for plastisphere studies. Plastic polymers: PE-polyethylene, PP-polypropylene, PS-polystyrene, PVC-polyvinyl chloride, PET-polyethylene terephthalate, PUR-polyurethane.

2.5. Material properties affecting microbial colonisation

The development and composition of biofilm communities is influenced by a range of biotic and abiotic driving factors (Harrison et al., 2018). Both physical and chemical differences between plastic polymers and potential control materials can influence microbial adhesion and community composition (Renner and Weibel, 2011). Physicochemical properties of surfaces are most influential during the primary stages of colonisation and the importance of these properties decreases as the biofilm matures (Datta et al., 2016; Ogonowski et al., 2018). Therefore, the intrinsic properties of the material supporting plastisphere communities are likely to be most influential at points where plastics are first released into the environment (Harrison et al., 2018). Virgin plastic polymers and other control materials, such as glass and ceramic, have smooth surfaces, whilst materials such as wood have rougher surfaces that increase the surface area and potential sites available for bacterial attachment, whilst also providing protection against shear forces (Bollen et al., 1996; Yoda et al., 2014; Zheng et al., 2021). As a result, increased surface roughness can enhance bacterial adhesion and diversity but is also likely to increase persistence of any attached bacteria due to increased protection. Surface roughness is highly variable, and differs between plastic polymers and control materials (Appendix B), but can also change rapidly as materials are colonised, for example, Bhagwat et al. (2021) demonstrated that the accumulation of biomolecules, together with the formation of conditioning films, increased the surface roughness of plastic surfaces over 24 h.

Different materials have varying levels of buoyancy, hydrophobicity, surface charge and roughness (Appendix B); all of which can influence microbial adhesion and biofilm formation, and subsequent transport and dissemination (Ogonowski et al., 2018; Cai et al., 2019; Gong et al., 2019). These properties need to be taken into consideration when deciding upon an appropriate control for plastisphere studies and will need to be relevant to the properties of the specific plastic polymer being used. Density is important for facilitating environmental transport and ultimately fate, e.g., by determining how particles disperse and/or sink in the aquatic environment (Horton et al., 2017; Erni-Cassola et al., 2019). Biofilm formation can increase the density of buoyant plastics and cause them to lose their buoyancy (Chubarenko et al., 2016; Lagarde et al., 2016; Wright et al., 2020); however, Amaral-Zettler et al. (2021) recently showed how there is a size tipping point, above which microbial colonisation alone fails to induce particle sinking. Therefore, even after microbial colonisation, most microplastics are likely to remain floating on or near the water surface. Three of the most abundant plastic polymers (polyethylene, polypropylene and polystyrene) are less dense than water and float on the surface leading to greater dissemination than plastic polymers which are less buoyant and become incorporated into the sediment. Consequently, there is a move to study

more buoyant plastic polymers, which have the ability to transport pathogens for longer and over further distances. Although some control materials can also float (Fig. 1), others are denser than plastic and so behave differently in water, e.g., glass and ceramics, and are perhaps less relevant for demonstrating the potential for long distance transport than a control material that floats. However, several studies have used metal cages to sink both the plastic and control samples (Sun et al., 2020; Martínez-Campos et al., 2021). Although this ensures that the materials remain at the same level within the water column and removes the effects of differing buoyancies, it does not replicate the actual movement and conditions experienced by the materials, which are unlikely to remain in one location over extended periods of time.

Hydrophobicity can also influence bacterial adhesion and community composition, with hydrophobic surfaces being more attractive to bacteria (Rummel et al., 2017; Ogonowski et al., 2018; Martínez-Campos et al., 2021). A contact angle (i.e., the angle at a solid-liquid interface) > 90° indicates a hydrophobic surface (Law, 2014), and plastics usually have a contact angle of between 83 and 93° (Cai et al., 2019), whilst control substances such as wood and glass have lower contact angles and are therefore often hydrophilic (Iglauer et al., 2014; Papp and Csiha, 2017). Thus, the hydrophobic surfaces of plastics are likely to be more attractive to bacteria than hydrophilic control surfaces. At neutral pH, most bacteria possess an overall negative charge due to the presence of peptidoglycan and therefore are more attracted to surfaces with a positive surface charge (Zhu et al., 2015; Kovačević et al., 2016; Guo et al., 2018). In addition to influencing initial bacterial attachment, surface charge can also affect subsequent bacterial attachment at later stages of biofilm development (Kao et al., 2017; Shen et al., 2020), which is important for secondary colonisers such as some pathogenic species of *Vibrio* (Datta et al., 2016). Many plastics have a negatively charged surface, with an average zeta potential of −10 mV (Cai et al., 2019), whereas potential control materials, including wood and glass, have a more negative zeta potential (Gu and Li, 2000; Muff et al., 2018). Zeta potential changes with pH (Xu et al., 2014), meaning bacterial attraction and adhesion will differ as a result of the material moving through the different environmental matrices of the landscape.

Once released into the environment, plastics become fragmented and degraded over time due to mechanical, photo-, chemical-, and biodegradation (Gewert et al., 2015), leading to increased surface roughness through the formation of pits and ridges (Zettler et al., 2013). Therefore, surface roughness is a property that not only differs between materials but can also spatially and temporally vary across a given surface. Surface roughness, chemical composition, colour, and the surface charge

of plastics change as they age (Liu et al., 2020; Luo et al., 2020; Su et al., 2021). These age-related properties will subsequently influence the persistence and potential dissemination of human pathogens within these plastisphere communities. Biofilm formation is greater on aged plastics (Rummel et al., 2017; Kaiser et al., 2017), with the pathogenic potential and abundance of antimicrobial resistance genes (ARGs) also enhanced on aged microplastics compared to virgin microplastics (Su et al., 2021). As plastics become more weathered, they begin to release additives, e.g., phthalates and Bisphenol A (Luo et al., 2020; Wu et al., 2021), which can either be used as a microbial nutrient source or can be toxic to plastisphere communities. However, this intrinsic physicochemical property of weathered plastic polymers in the environment is difficult to experimentally control for with the types of control materials discussed above (Table S2), and the effect of compounds leaching from plastics on the potential transport of pathogens within the plastisphere has not yet been considered.

Although there is no 'perfect' control for studies on human pathogens in the plastisphere, the trade-off for selecting which variables will be controlled for will be determined by the study's specific objectives and research question. Density is suggested as the most important factor to consider when selecting a control material, as it can significantly influence both the transport and environmental fate of materials. Glass is the most commonly used control material in plastisphere studies because like plastics, glass also persists in the environment, and is not an immediate source of nutrients. However, glass is perhaps not the most appropriate choice of control material: glass has very smooth surfaces compared to plastics, and its higher density, which causes it to sink, limits its ability to transport and transfer pathogens within aquatic environments. With buoyant environmental plastic pollution being the most abundant type of plastic, similarly buoyant control materials are perhaps most relevant as these materials have the potential to transport human pathogens for longer and over further distances.

2.6 Conclusion

With plastic demand continuing to grow and production expected to quadruple by 2050 (Suaria *et al.*, 2016), the volume of microplastics entering the environment will also rise, increasing the environmental surfaces available for colonisation by human pathogens, and therefore increasing the potential risk of exposure of humans and other species to harmful pathogens in the plastisphere. To fully understand this risk, it is essential that appropriate controls are included in future experiments and environmental surveys to determine whether plastic pollution does increase the opportunity for pathogen transport and transmission compared to binding to other substrates. However, it is clear there is no single control substrate relevant for all plastisphere studies, but it is

important to take into account a number of factors relating to the plastic polymer, the control material, and the environmental conditions before deciding on the most relevant control to use. With infectious diseases responsible for 22 % of annual deaths worldwide (Lozano *et al.*, 2012), we must continue to study the potential of plastics to act as novel vectors of disease (Wißmann *et al.*, 2021), particularly as the longevity of plastic in the environment could facilitate the increased persistence and dissemination of pathogens compared to more accepted environmental pathways.

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3. From wastewater discharge to the beach: Survival of human pathogens bound to microplastics during transfer through the freshwater-marine continuum

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ABSTRACT

Large quantities of microplastics are regularly discharged from wastewater treatment plants (WWTPs) into the aquatic environment. Once released, these plastics can rapidly become colonised by microbial biofilm, forming distinct plastisphere communities which may include potential pathogens. We hypothesised that the protective environment afforded by the plastisphere would facilitate the survival of potential pathogens during transitions between downstream environmental matrices and thus increase persistence and the potential for environmental dissemination of pathogens. The survival of *Escherichia coli, Enterococcus faecalis* and *Pseudomonas aeruginosa* colonising polyethylene or glass particles has been quantified in mesocosm incubation experiments designed to simulate, (1) the direct release of microplastics from WWTPs into freshwater and seawater environments; and (2) the movement of microplastics downstream following discharge from the WWTP through the river-estuary-marine-beach continuum. Culturable *E. coli, E. faecalis* and *P. aeruginosa* were successfully able to survive and persist on particles whether they remained in one environmental matrix or transitioned between different environmental matrices. All three bacteria were still detectable on both microplastic and glass particles after 25 days, with higher concentrations on microplastic compared to glass particles; however, there were no differences in bacterial die-off rates between the two materials. This potential for environmental survival of pathogens in the plastisphere could facilitate their transition into places where human exposure is greater (e.g., bathing waters and beach environments). Therefore, risks associated with pathogen-microplastic co-pollutants in the environment, emphasises the urgency for updated regulations on wastewater discharge and the management of microplastic generation and release.

Keywords: human health; plastic pollution; plastisphere; sewage discharge; wastewater management

Highlights:

- Faecal Indicator Organisms and *P. aeruginosa* can survive on plastics and glass
- Potential pathogens persisted for at least 25 days
  Viable bacteria still detected after transition between environmental matrices

3.1 Introduction

Three hundred and sixty-seven million tonnes of plastic were produced globally in 2020 (Plastics Europe, 2021), although poor disposal and low levels of recycling results in large volumes of plastic being released into the environment, where they persist and accumulate (Geyer *et al.,* 2017). Once in the environment, plastics can be transported through terrestrial, freshwater, and marine ecosystems (Rochman, 2018) where they become rapidly colonised by microbial biofilm (Zettler *et al.,* 2013). Such 'plastisphere' communities are diverse with their dynamics driven by the biotic and abiotic conditions and stressors inherent to the environmental matrix they are exposed to (Basili *et al.,* 2020; Zhang *et al.,* 2021; Delacuvellerie *et al.,* 2022).

Wastewater treatment plants (WWTPs) can discharge large amounts of plastic into aquatic environments (Mason *et al.,* 2016; Kay *et al.,* 2018; Okoffo *et al.,* 2019), including sanitary products such as disposable wet wipes, and microfibres from washing machine grey water (Napper and Thompson, 2016; Van Wezel *et al.,* 2016; Yaseen *et al.,* 2022). More than 2.2 x 10^7 microplastic particles (particle size < 5 mm) a day (e.g., from personal care products, cosmetics, and cleaning products) have been measured in discharge from a tertiary WWTP (Blair *et al.,* 2019). Importantly, microplastics in WWTPs can become colonised by human bacterial and viral pathogens and act as hotspots for anti-microbial resistance (Martinez-Campos *et al.,* 2021; Junaid *et al.,* 2022; Kelly *et al.,* 2021; Moresco *et al.,* 2022). Once discharged from the WWTP, microplastics often pass through different environmental matrices (freshwater, estuarine, marine) as they move downstream. As physicochemical conditions (e.g., salinity, pH) change through these different matrices the survival and composition of plastisphere communities is likely to be affected (Xue *et al.,* 2020). The weathering and stability of microplastics is also likely to change upon this transition between environmental matrices, changing physicochemical properties including colour, chemical leaching and surface morphology; this could subsequently influence biofilm formation and plastisphere communities (Duan *et al.,* 2021). Particles could eventually become so small that biofilm is unable to form on them. Although human pathogens can survive for variable amounts of time in the plastisphere (Li *et al*., 2019; Laverty *et al*., 2020), initial studies suggest that once released from WWTPs into the environment the survival and abundance of potential pathogens decreases as microplastic particles move between freshwater and marine environments, e.g., lower abundances of the taxonomic groups Enterobacteriaceae and *Vibrio* in marine compared to freshwater environments (Oberbeckmann *et al*., 2018).

Upon exposure to saltwater, pathogenic bacteria are simultaneously challenged by a combination of stress factors, including salinity, temperature, pH, and nutrient availability (Rozen and Belkin, 2001). Increasing salinity, and increased levels of UV irradiance can contribute to the die-off of planktonic faecal indicator organisms (FIOs), e.g., *E. coli* and intestinal enterococci (IE), in the marine environment (Campos *et al*., 2013; Song *et al.,* 2020; De Vilbiss *et al*., 2021). However, microbial biofilms can provide a level of tolerance or protection from environmental stressors compared to free-living planktonic communities (De Carvalho, 2018; Boni *et al*., 2021; Xue et al., 2020) and increase the potential for dispersal into new ecosystems (Sooriyakumar *et al.,* 2022). Plastics recovered from the environment often harbour potential pathogens and FIOs (Shi *et al.,* 2021; Rodrigues *et al.,* 2019; Kelly *et al.,* 2021; Metcalf *et al.,* 2022); although most of this evidence has come from 16S rRNA sequencing studies, which does not differentiate between live and dead bacteria (Li *et al.,* 2017).

Relatively little is known about how the transport of microplastics between different environmental matrices affects the survival of potential pathogens within plastisphere communities. The protective environment provided by the plastisphere is hypothesised to facilitate the survival of pathogens and FIOs during these transistions, and increase their persistence in the environment. Human exposure to plastics colonised by human pathogens, e.g., whilst swimming, or during recreational use of beaches, could increase the potential for pathogen transfer and subsequent implications for human health. Therefore, the aim of this study was to use a culture-dependent approach to determine the influence of time and downstream transfer, on the survival of viable FIOs and *Pseudomonas aeruginosa* in the biofilm colonising microplastic or glass particles. Two mesocosm incubation experiments were used, where; (1) microplastic and glass particles were incubated in WWTP effluent and then moved into either freshwater or seawater mesocosms for 25 days, e.g., simulating direct release into these environments; and (2) microplastic and glass particles were sequentially incubated in WWTP effluent, freshwater, estuary, seawater, and beach sand mesocosms to replicate the movement of particles downstream following discharge from the WWTP through the river-estuary-marine-beach continuum. We hypothesised that FIOs and *P. aeruginosa* would persist in the plastisphere of plastics in either freshwater or seawater mesocosms, or as they transitioned between different environmental matrices.

3.2 Materials and Methods

3.2.1 Experiment 1

This mesocosm experiment was used to replicate the direct discharge of plastic particles from the WWTP into either freshwater or seawater.

3.2.1.1 Mesocosm Set-up

Effluent and water samples were collected from three sampling sites within the Forth Catchment (Dunblane, Bridge of Allan and Kirkaldy) in Scotland, UK between 4th and 6th June 2022 (shown in map of Fig. 3.1), stored at 4°C, and used in the mesocosms within 24 h. Salinity, electrical conductivity (EC) and turbidity were measured with, a salinity refractometer (RGBS), HI2550 EC meter and HI88703 turbidimeter respectively (Hanna Instruments, UK). To determine background bacterial concentrations, water samples (100 ml, *n* = 3) were filtered through 0.45 µm cellulose acetate membranes (Sartorius Stedim Biotech., Gottingen, Germany). Membranes were transferred onto the surface of selective media (see description below) for the enumeration of *E. coli*, intestinal enterococci, and *Pseudomonas aeruginosa*.

Polyethylene beads (Goodfellow, UK) were used as a representative microplastic, and were compared with glass beads (Sigma Aldrich, USA); both the microplastic and glass beads were described by the manufacturer as being 2 mm diameter. Particles were characterised (Zeiss Stemi 305 with an Axiocam 208; Zen software, Zeiss; 0.8X magnification) to visualise differences in size, shape and surface characteristics (Appendix D). Spherical stainleess steel metal cages (45 mm high, 38 mm diameter, 1 mm pore size; Golf, China) were used to hold the particles in each mesocosm (*n* = 20, glass tank, 30 x 30 x 20 cm, 12 L volume, MB Store, UK). Each metal cage contained either 100 microplastic particles or 100 glass particles. All metal cages, containing the particles, were sterilised by autoclaving prior to being placed into each replicate mesocosm glass tank.

Overnight cultures of *E. coli*, *Enterococcus faecalis* and *P. aeruginosa* were grown in Luria-Bertani (LB) broth (Fisher Bio-reagents, UK) in an orbital shaking incubator at 37°C (120 rpm, IncuShake MIDI, SciQui, UK). 1 ml of each overnight culture was added to LB (19 ml) and grown in a shaking incubator (120 rpm) at 37°C for 5 h to ensure that cells were in their exponential growth

phase when added to the mesocosms. Cells were then centrifuged (4000 rpm for 10 min), the pellet washed in phosphate buffered saline (PBS) and resuspended in WWTP effluent; 160 ml of each bacterial resuspension was added to 50 L effluent and 4 L of this inoculated effluent added to each replicate mesocosm. Serial dilutions were carried out in PBS and plated onto selective media to determine the final concentration of the inoculant added to each replicate 'effluent mesocosm' (*E. coli*, 1.13 x 10^7 CFU/ml; *E. faecalis*, 8.11 x 10^5 CFU/ml; *P. aeruginosa*, 1.00 x 10^7 CFU/ml). The initial 48 h incubation in inoculated effluent aimed to replicate the potential concentrations of FIOs and *P. aeruginosa* that plastics would encounter within the WWTPs. After 48 h, all the metal cages were removed and added to new replicate tanks (n = 4) containing 4 L of either freshwater or seawater, where they remained for the rest of the experiment. Each replicate mesocosm tank contained 30 metal cages to allow multiple sampling points over time. All mesocosm tanks were kept in an orbital incubator (Innova 4900 multi-tier environmental shaker, New Brunswick Scientific, UK) at 15°C with shaking (30 rpm).

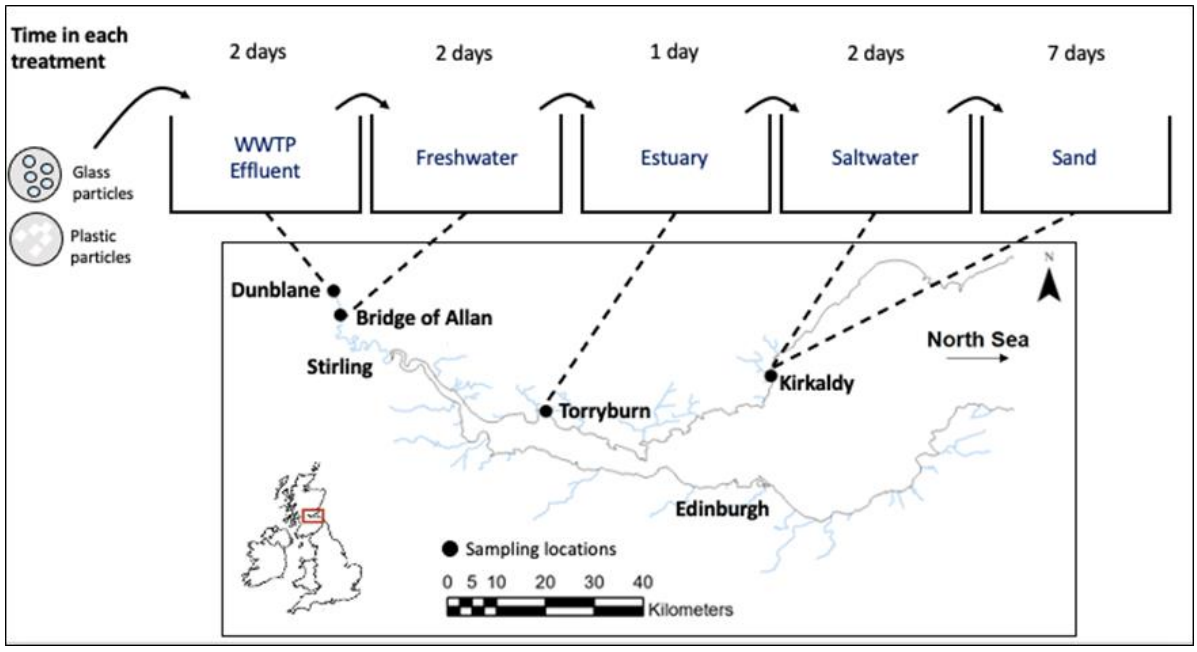

Figure 3.1. Map of water collection sites along the Forth Catchment, Scotland, and the mesocosm setup for Experiment 2 showing the simulated transfer through the effluent-freshwater-estuary-marine-beach continuum.

3.2.1.2 Sample processing

At each timepoint (1, 2, 3, 4, 5, 6, 7, 9, 12, 17, 22 and 27 days after being placed into the initial effluent mesocosms) one cage containing microplastic particles and one cage containing glass particles was removed from each replicate tank. From each cage 100 particles were transferred, using sterile forceps, to a 15 ml Falcon tube containing 5 ml sterile PBS, vortexed vigorously at 1,500 rpm for 3 min and the wash solution removed. Fresh PBS (5 ml) was added to the particles and

vortexed again; the two wash solutions were combined, and serially diluted in PBS for subsequent bacterial enumeration. Dilutions were filtered through 0.45 µm cellulose acetate membrane filters (Sartorius Stedim Biotech., Gottingen, Germany) and transferred onto the surface of selective media (membrane lactose glucuronide agar [MLGA] for *E. coli*, Pseudomonas base agar [PA] (plus CN supplement) for *P. aeruginosa*, and Slantez and Bartley agar [SB] for *E. faecalis* [Oxoid, UK]). All plates were inverted and incubated; MLGA plates were incubated at 37°C for 24 h, PA plates incubated at 35°C for 48 h and SB plates incubated at 44°C for 44 h. After incubation, colony forming units (CFU) were enumerated.

3.2.2 Experiment 2

To simulate the transport of microplastic particles downstream from effluent discharge to the beach environments, distinct water and sand samples were collected from four sites along the Forth Catchment (Dunblane, Bridge of Allan, Torryburn and Kirkaldy) between 12th and 16th April 2022 (Fig. 3.1). Salinity, EC, turbidity, and initial background concentrations of target bacteria in all water samples were quantified as described above.

3.2.2.1 Mesocosm Set-up

Using the same laboratory-controlled mesocosm set-up described above, metal cages containing microplastic or glass particles were placed into mesocosm glass tanks. The initial WWTP effluent was inoculated as described above, but with 40 ml of bacterial inoculum added to a total of 12 L effluent; and 3 L of this inoculated effluent was added to each mesocosm tank (n = 4). The final bacterial concentrations in each of the WWTP effluent mesocosms was: *E. coli*, 4.36 x 10^7 CFU/ml; *E. faecalis*, 6.84 x 10^6 CFU/ml; *P. aeruginosa*, 2.40 x 10^7 CFU/ml. Following the initial 48 hr incubation in the 'effluent mesocosms', the cages were sequentially moved through a series of mesocosm tanks containing river water, estuary water, seawater, and beach sand (Fig. 3.1) to simulate the potential downstream movement of microplastic particles. For the sand treatment, particles were emptied from the cages onto the surface of sand (40 g) in a petri dish, and incubated at 15°C.

3.2.2.2 Sample processing

At each timepoint, one cage of each material was removed from each replicate mesocosm tank. Crystal violet staining was used to determine the amount of biofilm colonising the surfaces of microplastic and glass particles. A sample of 40 particles from each cage were added into the wells

of a sterile 12-well plate (clear polyethylene plate with 12 x 245 mm deep wells, Greiner Bio-one, UK), rinsed twice with sterile distilled water to remove weakly attached bacteria, and incubated with 1 ml of 0.2 % crystal violet in distilled water (Sigma-Aldrich) at room temperature for 20 min. The particles were then washed three times in sterile distilled water and air dried overnight. Cell bound crystal violet on the surface of the particles was dissolved in 1 ml of 30 % acetic acid for 20 min. Absorbance at 550 nm was measured in a spectrophotometer (Infinite M200 plate reader, Tecan, Switzerland) to determine the concentration of crystal violet recovered; the amount of biofilm colonising the particles was considered proportional to the absorbance readings. Virgin plastic and glass particles were used as negative controls and 30 % acetic acid used as a blank.

To quantify target bacteria concentrations following incubation of the microplastic and glass particles in each mesocosm tank, the same vortexing method was used as described above: 40 particles per cage were vortexed and washed twice in PBS and serial dilutions filtered through 0.45 µm cellulose acetate membrane filters. To remove bacteria associated with the sand samples, 10 g sand was added to 10 ml sterile PBS and vortexed (1500 rpm, 10 min). Serial dilutions of the supernatant were filtered through 0.45 µm cellulose acetate membranes, which were aseptically transferred onto the surface of selective media. Following incubation, CFUs were enumerated to quantity the concentration of each target microorganism (100 ml^{-1} water or 100 g^{-1} dry weight sand).

3.2.3 Statistical analysis

CFU counts were normalised by transforming to log_{10} CFU per 40 particles. Non-linear and linear regression analysis, carried out in Minitab version 18 (Minitab Inc.; State College, PA, USA), were used to establish relationships describing the pattern of bacterial decline, and subsequently used to determine the die-off characteristics of bacteria under the different treatments using the same approach as Afolabi *et al.,* (2020).

In Experiment 1, bacterial concentrations declined rapidly before reaching a stable population level after 15 days; therefore, an exponential model was fitted to each resulting time-series of bacterial die-off associated with the different treatments for the first 15 days. The asymptotic exponential model fitted to the log_{10} transformed data is described by Equation (1):

$$Log_{10}(C) = A + Be^{-\lambda t} \qquad \text{(Eq. 1)}$$

Where C is the cell concentration (CFU/40 particles), λ is the exponential rate of decline (d^{-1}) that governs the decay of the die-off rate constant over time, B is the difference in cell numbers between experiment start and finish (log_{10} CFU/40 particles), A is the final level of bacterial population stability (log_{10} CFU/40 particles), and t is time (d). The % decrease in 'bacterial concentration per unit time' decays with time rather than being constant.

In Experiment 2, the decrease in bacterial concentrations followed a linear decline over time, meaning the asymptotic exponential model was an inappropriate fit for the data. Therefore, a log-linear regression model was fitted to the log_{10} transformed data and is described by Equation (2):

$$Log_{10}(C) = Log_{10}(C_0) - kt \qquad \text{(Eq. 2)}$$

Where C_0 is the cell concentration at t = 0 and k is a die-off rate constant (d^{-1}). Using the log-linear model, the % decrease in bacterial concentration per unit time is constant. Decimal reduction times (*D*-values; the number of days to reduce viable bacteria by 90%) were calculated based on the decline rates for populations following a log-linear die-off profile.

Other statistical analyses were conducted using R Studio version 3.3.2 (R Studio Team, 2016). Student's *t*-tests and analysis of variance (ANOVA) with Tukey's posthoc test were used to test for differences in bacterial die-off characteristics, and to compare treatments. All data were tested for distribution and homogeneity of variances (Shapiro-Wilk and Levene's) before parametric tests were used. Where assumptions were not met, data was either log transformed, or non-parametric Scheirer Ray Hare tests (Scheirer *et al.,* 1976), followed by Tukey's posthoc test was used. Data is reported as the mean ± standard error. P values < 0.05 were considered significant.

3.3 Results

Experiment 1

3.3.1.1 Physicochemical characteristics

FIO and *P. aeruginosa* concentrations were highest in the effluent samples at the time of sampling (Table 3.1). The initial EC in the freshwater (ranging from 0.27 to 0.31 mS) was significantly lower than the seawater (55.5 to 56.5 mS). EC initially decreased in the mesocosms before remaining constant throughout the experiment; turbidity remained low in the freshwater mesocosms compared to a slight increase in the seawater mesocosms (data not shown).

Table 3.1. Background bacterial and physiochemical properties of water types used in Experiment 1. The mean was calculated from 3 replicates, ± the standard error.

	Bacteria concentration (log_{10} CFU/100 ml)			Physicochemical characteristics		
	E. coli	Intestinal enterococci	*Pseudomonas aeruginosa*	Salinity (‰)	Electrical conductivity (mS)	Turbidity (NTU)
Effluent	4.50 ± 2.82	3.72 ± 2.27	2.07 ± 1.85	0	0.27 ± 0.01	2.43 ± 0.09
Freshwater	2.79 ± 1.89	1.64 ± 1.23	0.95 ± 0.60	0	0.31 ± 0.01	1.78 ± 0.12
Seawater	1.52 ± 0.30	1.56 ± 0.30	0.78 ± 0.78	3.13 ± 0.03	56.00 ± 0.29	1.60 ± 0.11

3.3.1.2 Bacterial die-off in freshwater and seawater

Both microplastic and glass particles became colonised by *E. coli*, *E. faecalis* and *P. aeruginosa* in the inoculated WWTP effluent. For *E. coli,* the concentrations at the first timepoint were higher on the microplastic compared to the glass particles despite the particles being exposed to the same background *E. coli* concentrations; the same pattern was also recorded for *E. faecalis* and *P. aeruginosa* (Appendix C). The mean concentration of *E. coli* remained consistently higher on plastic compared to glass particles throughout the experiment (Fig. 3.2; ANOVA, $F_{1, 190} = 9.048$, $p < 0.01$); this was also the case for *E. faecalis* (ANOVA, $F_{1, 190} = 38.26$, $p < 0.001$) *and P. aeruginosa* (ANOVA, $F_{1, 190} = 11.59$, $p < 0.001$). Despite the manufacturer describing both the microplastic and glass particles as being 2 mm in diameter, the two particles were clearly different sizes (Appendix D). Surface roughness and potential colonisation area also varied with material; the glass particles had smoother homogenous surfaces (providing less colonisation area) compared to the rough microplastic surface.

E. coli, E. faecalis and *P. aeruginosa* concentrations decreased over time for all treatments, showing an exponential biphasic decay curve, with the most rapid decrease occurring within the first 10 days. During this period, *E. faecalis* concentrations decreased more rapidly on the microplastic compared to glass particles; concentrations had dropped below 4 $\log_{10}$ CFU/40 glass particles by day 3, whereas concentrations above 4 $\log_{10}$ CFU/40 microplastic particles were still recorded at day 9. After day 9 concentrations plateaued. In the majority of treatments, at the final timepoint (day 27), viable bacteria were still detected (the only exceptions being *E. faecalis* on glass particles in freshwater and *P. aeruginosa* on glass particles in seawater). *E. faecalis* colonising microplastic particles were the most persistent by the final timepoint, particularly in seawater, with concentrations of 2.54 ± 0.14 $\log_{10}$ CFU/40 particles still present.

Although freshwater and seawater had differing physicochemical properties (Table 3.1), there were no significant differences in biofilm concentrations between freshwater and seawater for *E. coli, E. faecalis* and *P. aeruginosa* (ANOVA, $F_{1, 574} = 2.76$, $p = 0.097$). However, for the final three sampling points (days 17, 22 and 27) concentrations of *E. coli* were significantly higher in freshwater on both microplastic and glass particles (*t* test; Plastic: $t(22) = 4.3787$, $p < 0.001$; Glass: $t(22) = 6.5733$, $p < 0.001$).

The exponential rate constants (λ) calculated from fitting the non-linear model to all treatments are shown in Table 3.2. Overall, there was no significant association between λ, water type, particle material, or bacterial species (Scheirer Ray Hare test, $H = 0.075$, $p = 0.785$). The only factor to have a significant effect on λ was bacterial species (Scheirer Ray Hare test; $H = 4.557$, $p < 0.05$), with *E. faecalis* having a higher value of λ than *E. coli* (Tukey HSD, $p < 0.01$); there were no other significant differences. Therefore, despite there being higher concentrations of *E. coli, E. faecalis* and *P. aeruginosa* detected on the surface of microplastic particles, there was no resulting influence of water type or material on the exponential rate constant influencing the die-off rates of these bacteria.

Table 3.2. Parameter values for bacterial die-off associated with non-linear models from Experiment 1. These models were fitted for the first 15 days of the experiment.

			Exponential Rate Constant l (Day^{-1})	
			Mean	SE
Freshwater	Plastic	*E. coli*	0.197	0.011
		E. faecalis	0.075	0.044
		P. aeruginosa	0.251	0.013
	Glass	*E. coli*	0.115	0.015
		E. faecalis	0.236	0.037
		P. aeruginosa	0.173	0.040
Seawater	Plastic	*E. coli*	0.001	<0.001
		E. faecalis	0.347	0.147
		P. aeruginosa	0.108	0.003
	Glass	*E. coli*	0.001	<0.001
		E. faecalis	0.500	0.131
		P. aeruginosa	0.162	0.032

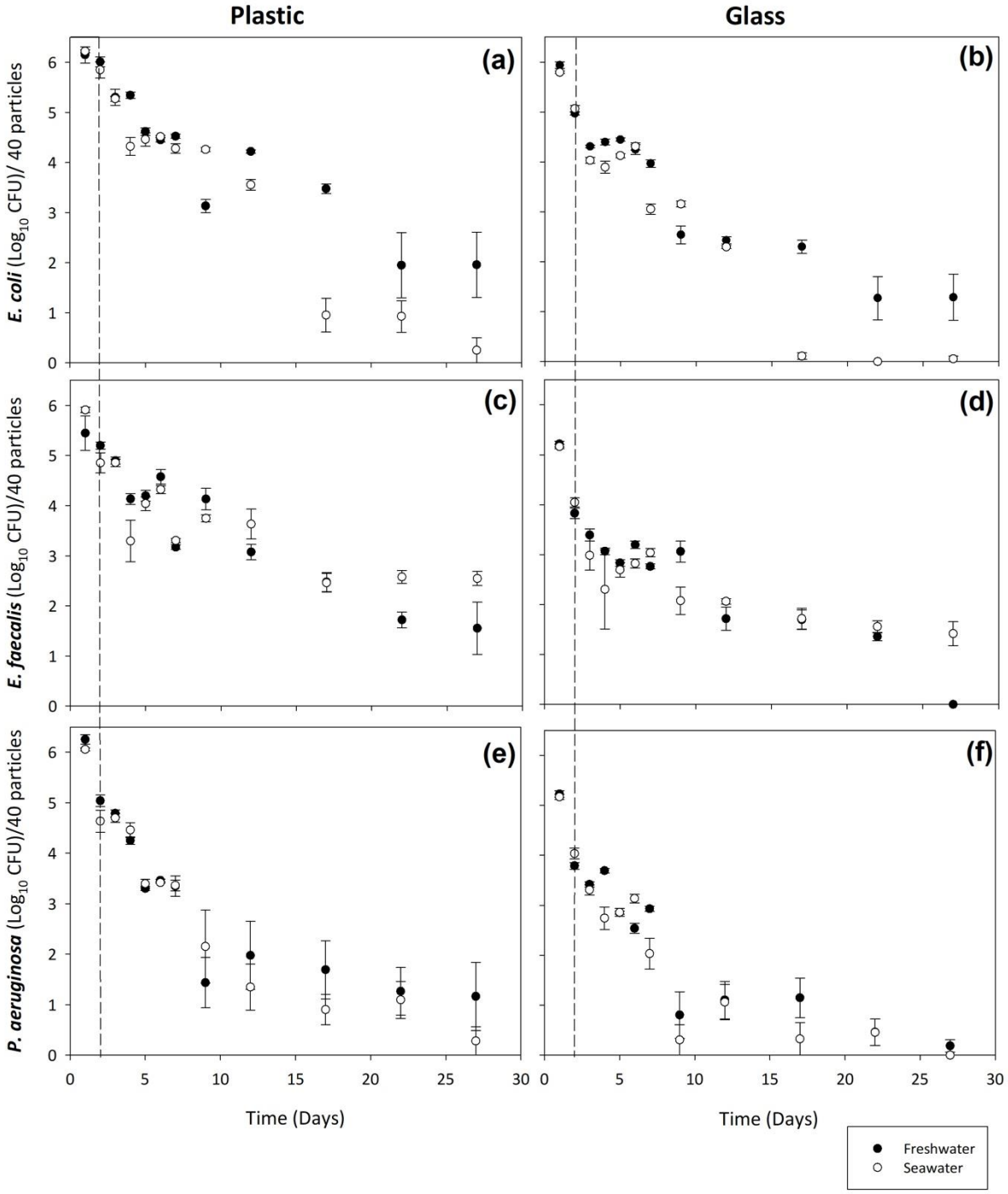

Figure 3.2. Concentration of *E. coli* (a, b), *E. faecalis* (c, d) and *P. aeruginosa* (e, f) on microplastic beads (a, c, e) and glass beads (b, d, f) in freshwater (filled triangles) and seawater (empty triangles). The mean was calculated from four replicates, ± the standard error. Cages containing the beads were moved from the inoculated WWTP effluent to freshwater or seawater tanks on day 2 of the experiment (indicated by the vertical dashed line).

Experiment 2

3.3.2 Physicochemical characteristics and bacterial die-off

At the time of sampling, background concentrations of *E. coli, E. faecalis* and *P. aeruginosa* were detected in all water samples subsequently used in the mesocosm tanks; although no *E. coli* or *E. faecalis* were detected in the sand samples (Table 3.3). *E. coli, E. faecalis* and *P. aeruginosa* successfully colonised the surfaces of the plastic and glass particles. The amount of biofilm increased with time (Fig. 3.3); however, there was a decrease after the particles reached the seawater. The amount of biofilm was higher on the microplastic compared to glass particles at all timepoints (Fig. 3.3; *t* test, $t(78) = -3.30$, $p < 0.01$) and, similar to Experiment 1, microplastics were consistently colonised by higher concentrations of *E. coli, E. faecalis* and *P. aeruginosa* than glass particles (Fig. 3.4; ANOVA, $F_{1, 238} = 20.03$, $p < 0.001$). However, the concentration of all three pathogens in the plastisphere decreased with time as the particles transitioned between the different environmental matrices (Fig. 3.4; ANOVA, $F_{9, 227} = 73.24$, $p < 0.001$). In contrast to Experiment 1, the die-off of *E. coli, E. faecalis* and *P. aeruginosa* in Experiment 2 showed a linear monophasic die-off pattern. Viable *E. coli, E. faecalis* and *P. aeruginosa* were still detected at the final sampling point (14 d) after one week on the sand surface; however, no viable *P. aeruginosa* were detected colonising the glass particles at the final timepoint (Fig. 3.4).

The greatest change in physicochemical characteristics occurred as particles moved between the freshwater and estuary mesocosms; salinity increased by 2.13 ‰ and electrical conductivity by 45.31 mS (Table 3.3). Concentrations of *E. coli* in the plastisphere decreased most rapidly upon their exposure to estuarine conditions, after which there was an initial cessation in the rate of die-off in the seawater. There was also a similar cessation in die-off rate of *E. faecalis* and *P. aeruginosa* colonising the surfaces of microplastic particles when entering the seawater. In contrast, the concentration of *P. aeruginosa* colonising glass particles increased by 1.30 $\log_{10}$ CFU/40 particles following the move into the seawater which corresponded with the background concentrations of *P. aeruginosa* in the sample water collected from the environment (Table 3.3).

Log linear regression models were applied to all replicates (r^2 ranged from 0.693 to 0.962) to determine modelled linear decline rate constants and *D*-values (Table 3.4). There was no significant association between *k*, material or bacterial species (Two-way ANOVA, $F_{2,18} = 0.183$, $p = 0.834$); and neither factor had a significant effect on the linear decline rate (Material: *t* test, $t(21) = -1.147$, $p =$

0.264; Bacterial species; ANOVA, $F_{2,21} = 1.848$, p = 0.182). Similar to Experiment 1, despite the material type explaining differences in *E. coli, E. faecalis* and *P. aeruginosa* concentrations (Fig. 3.4) and biofilm quantity (Fig. 3.3), there was no influence on the die-off rates. However, unlike Experiment 1, bacterial species also had no influence on die-off rate during Experiment 2. Additionally, material type and bacterial species had no significant effect on *D* values (Material: *t* test, t(21) = 0.951, p = 0.3519; bacterial species; ANOVA, $F_{2,21} = 1.961$, p = 0.166).

Table 3.3. Background bacteria and physicochemical properties of water types used in Experiment 2. The mean was calculated from 3 replicates, ± the standard error. (nd, not done)

	Bacteria concentration ($\log_{10}$ CFU/100 ml or $\log_{10}$ CFU/g dry sand)			Physicochemical characteristics		
Water Type	*E. coli*	Intestinal enterococci	*Pseudomonas aeruginosa*	Salinity (‰)	Electrical conductivity (mS)	Turbidity (NTU)
Effluent	4.62 ± 3.70	4.06 ± 2.67	2.66 ± 1.92	0	0.24 ± 0.01	0.86 ± 0.06
Freshwater	3.20 ± 1.85	1.00 ± 0.00	5.54 ± 4.79	0	0.36 ± 0.02	1.55 ± 0.22
Estuary	1.48 ± 1.08	1.47 ± 1.18	3.61 ± 2.50	2.13 ± 0.07	45.67 ± 0.67	6.04 ± 0.23
Seawater	< 1.00	< 1.00	4.24 ± 3.21	2.93 ± 0.07	50.83 ± 0.55	20.83 ± 4.13
Sand	0	0	3.74 ± 3.49	nd	nd	nd

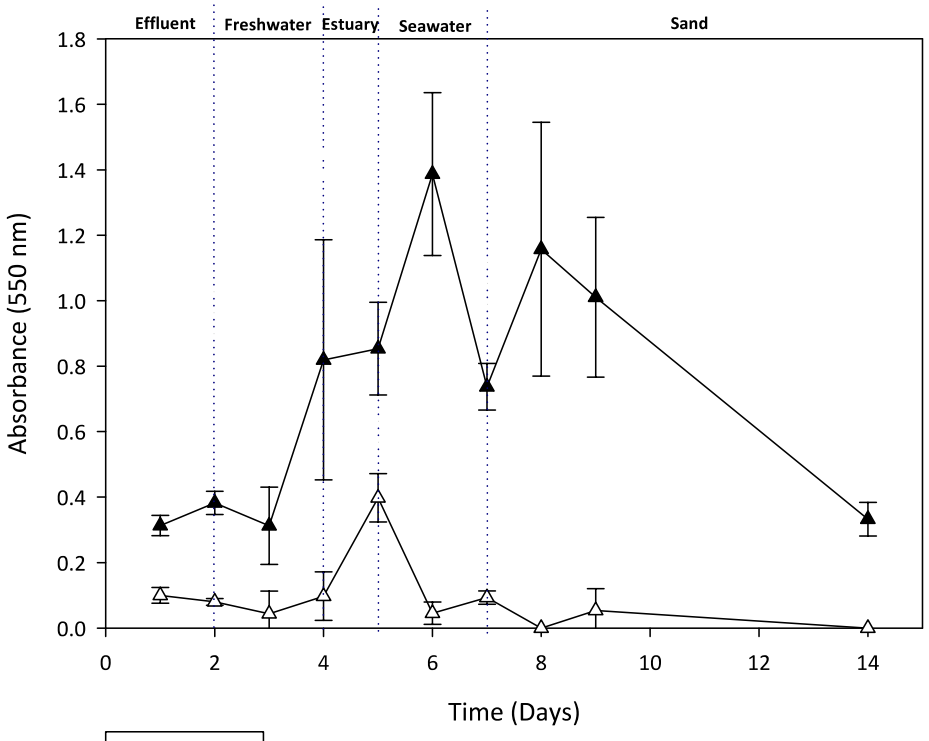

Figure 3.3. Crystal violet absorbance values (A550) for biofilm on microplastic beads (filled triangles) and glass beads (empty triangles). The mean was calculated from four replicates, ± the standard error.

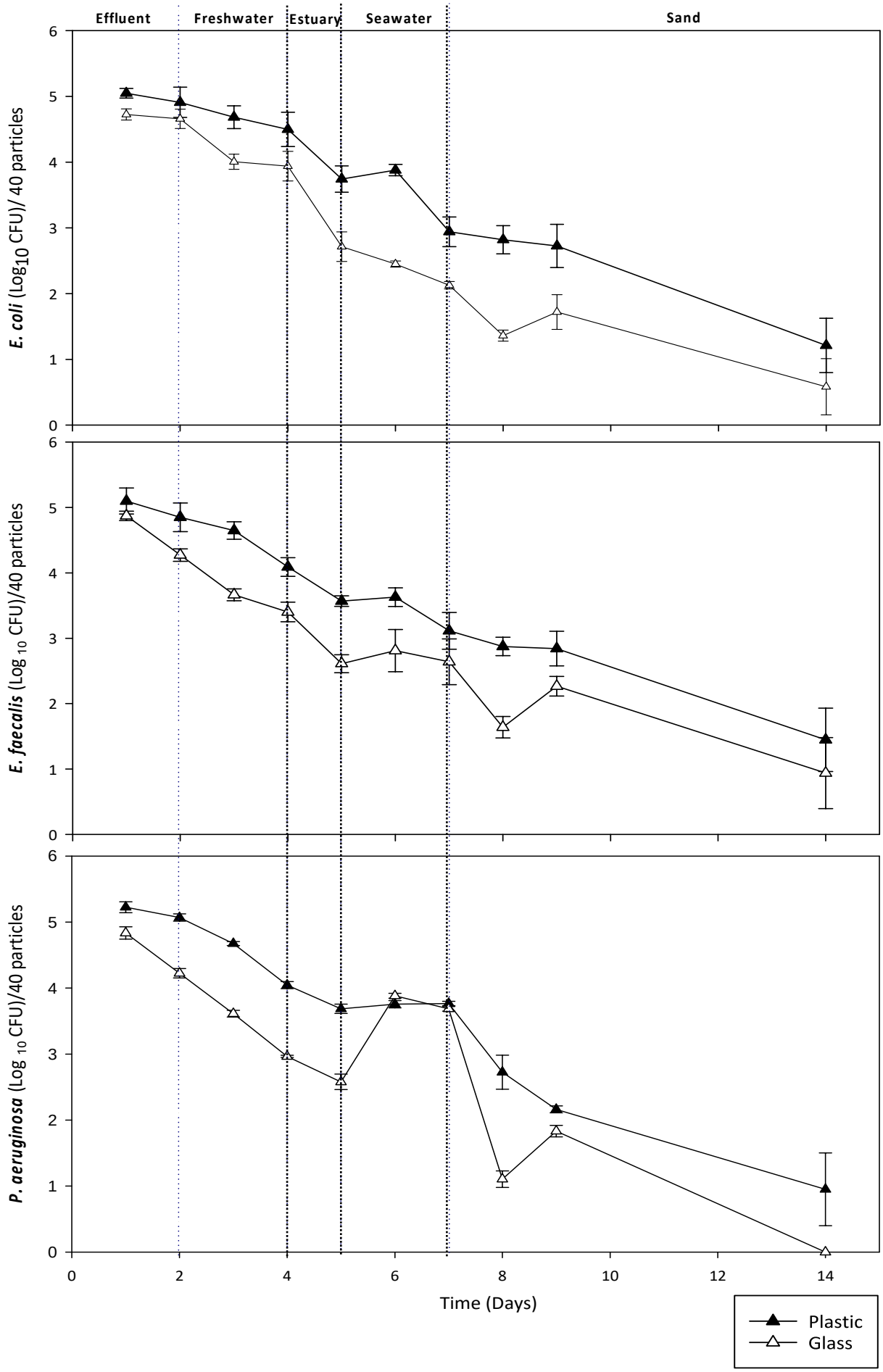

Figure 3.4. Concentration of (a) *E. coli*, (b) *E. faecalis*, and (c) *P. aeruginosa*, on microplastic beads (filled triangles) and glass beads (empty triangles). The mean was calculated from four replicates, ± the standard error. The dashed vertical lines represent the transition to the next treatment.

Table 3.4. Parameter values for bacterial die-off associated with linear models from Experiment 2.

		Modelled Linear Decline Rate (Day^{-1})*		*D*-Values (Days)		R^2	
		Mean	SE	Mean	SE	Mean	SE
Plastic	*E. coli*	0.731	0.074	3.265	0.380	0.873	0.026
	E. faecalis	0.669	0.070	3.549	0.347	0.867	0.021
	P. aeruginosa	0.771	0.070	3.052	0.242	0.895	0.026
Glass	*E. coli*	0.842	0.099	2.886	0.426	0.866	0.059
	E. faecalis	0.692	0.081	3.468	0.405	0.795	0.053
	P. aeruginosa	0.842	0.006	2.736	0.019	0.793	0.011

* Linear decline rate constant = (2.303 x Figure 5 slope gradient)

3.4. Discussion

3.4.1 Survival of FIOs and P. aeruginosa in different environmental matrices

E. coli, E. faecalis and *P. aeruginosa* successfully colonised microplastic and glass particles and were able to survive and persist as these particles remained in one environmental matrix or transitioned between different environmental matrices. The protective environment provided by a biofilm can facilitate the increased survival of surface-associated bacteria, which could be transported further than free-living bacteria, particularly during stressful conditions, e.g., during the transfer from freshwater to seawater (Goldstein *et al.,* 2014; Keswani *et al.,* 2016; De Carvalho, 2018). Our study has also provided new parameter values that characterise die-off of *E. coli*, *E. faecalis* and *P. aeruginosa* when associated with biofilm of microplastic and glass particles. These novel data could be used to help parameterise multi-pollutant models or risk assessment frameworks. Although these models already incorporate plastic and pathogen pollution (Kroeze *et al.,* 2016), they are only beginning to recognise the importance of associations and interactions between different pollutants (Strokal *et al.,* 2022). Viable FIOs and *P. aeruginosa* were still detected after 25 days in freshwater and seawater, and there is potential for microplastics to be transported over large distances during this time, moving into areas where they are more likely to come into contact with humans e.g., wild swimmers. Similarly, the ability for potential pathogens to persist on microplastics as they move downstream through different environmental matrices increases the risk

in places where human exposure is likely to be greater (e.g., bathing waters and beach environments).

3.4.2 Influence of time on the concentrations of FIOs and P. aeruginosa in the plastisphere

During both experiments, the concentrations of *E. coli, E. faecalis* and *P. aeruginosa* colonising both the microplastic and glass particles decreased with time. As the biofilm matured, the plastisphere communities would have changed through the process of succession (Wright *et al.,* 2020; Lear *et al.,* 2022), with potential pathogen populations experiencing increased levels of competition, e.g., for space, and nutrients (Kirstein *et al.,* 2019; Zhang *et al.,* 2022). Limiting such resources can result in higher levels of competition in the plastisphere compared to the surrounding water or sediment (Li *et al.,* 2021; Zhang *et al.,* 2022). During Experiment 2, nutrient levels would have been refreshed as the particles were moved into new environmental matrices; however, such transitions would have created increasing levels of abiotic stress, which was reflected by the decreasing concentrations of *E. coli, E. faecalis* and *P. aeruginosa* within the plastisphere. By the time the particles have reached areas with increased human exposure (e.g., bathing waters and beaches), there may be such a low concentration of potential pathogens still attached to microplastics that they present a minimal human health risk. However, although the relative risk of pathogens binding to microplastics has not yet been determined (Beloe et al., 2022), the fact that viable FIOs and *P. aeruginosa* could still be cultured from the surface of microplastics after more than three weeks in environmental conditions suggests that there is still a potential risk of pathogen transfer following human exposure.

Differences in physicochemical characteristics can influence plastisphere communities (Zhang *et al,* 2021), and the survival of free-living bacteria (Song *et al.,* 2020), and physicochemical gradients are likely to be a key driving factor behind the die-off in the plastisphere. Increases in turbidity can increase the survival of free-living bacteria due to the nutrient rich conditions and protection from light intensity (Saha *et al.,* 2019). In contrast, increases in salinity can cause rapid changes in osmotic pressure, which can lead to cell lysis (Auer *et al.,* 2017). When microplastic and glass particles remained in either the freshwater or the seawater mesocosms, there was an exponential biphasic decay curve with an initial rapid die-off of labile cells followed by the slower die-off of resistant cells. Similar biphasic decay patterns have been seen for FIOs in seawater and sand (Zhang *et al.,* 2015; Ahmed *et al.,* 2019), likely due to population heterogeneity, with subpopulations having different

adaptations to environmental conditions and different decay rates (Brouwer *et al.,* 2017). Comparable values for exponential rate constants (λ), which govern the decay of the die-off rate constant over time, were found for FIOs and *P. aeruginosa* in both freshwater and seawater in Experiment 1, possibly due to the plastisphere providing protection from non-optimal physicochemical characteristics. During Experiment 2, microplastic and glass particles transitioned through each environmental matrix for relatively short periods without enough time for the secondary lag phase of bacterial die-off to be reached (for example, the secondary lag phase in Experiment 1 was reached by about day 12). Therefore, when particles were moved between different environmental matrices there was a linear monophasic die-off pattern.

The complexity of bacterial survival dynamics in the plastisphere include factors other than changing physicochemical characteristics. For example, the influence of planktonic microbial communities, heavy metals, antibiotics, environmental contaminants and plastic additives can all influence plastisphere communities (Na *et al.,* 2018; Song *et al,* 2020; Sridharan *et al.,* 2022; Zhang *et al.,* 2022). The formation of the plastisphere itself can also influence the toxicity and biodegradation of plastics as well as their vertical transport; this could have subsequent implications on the survival and transport of any potential pathogens (Wang *et al.,* 2021; Sooriyakumar *et al.,* 2022). Additionally, although not observed during this study, the aggregation of microplastics has also recently been highlighted as an additional factor which could impact colonisation area and plastisphere communities (reviewed in Wang *et al.,* 2021). Antimicrobial resistance is increasingly being detected in potential pathogens within the plastisphere (e.g., *Vibrio* spp., *Legionella* and *Mycobacterium*; Zhao *et al.,* 2021; Junaid *et al.,* 2022; Metcalf *et al.,* 2022), which is likely to increase the survival of these pathogens and present a heightened risk to human health. Despite the level of protection afforded by living in a biofilm, exposure to stressful environmental conditions will still select against less resistant strains (Zhu et al., 2022). Bacteria often regulate their gene expression in response to environmental stress, e.g., pathogens can express virulence genes, (Everest, 2007), and resistance to environmental stress could select fitter subpopulations of pathogens with increased virulence; however, increased stress resistance comes at a cost in terms of fitness and so conversely, these populations could actually become less virulent (Pandey et al., 2022).

The production of the neurotransmitter, norepinephrine, in response to stress by a host can potentiate the virulence of bacteria, e.g., *P. aeruginosa* (Everest, 2007; Cambronel *et al.,* 2019). However, norepinephrine can also be produced by bacteria when they experience stress, e.g., *E. coli* (Shishov *et al.,* 2009), which may affect the composition and virulence of pathogens in the

plastisphere as microplastics move between environmental matrices and experience different levels of stress (e.g., the transfer from freshwater to marine water). The implications for this could be increased virulence of potential pathogens in the plastisphere and therefore a greater risk to human health as pathogen transmissibility and infectiousness can both be amplified with increasing virulence (Fleming-Davies *et al.,* 2018). Rising water temperatures can increase the virulence of plastisphere populations of *Vibrio parahaemolyticus* (Billaud *et al.,* 2022), which could increase the risk of pathogens on microplastics in the marine environment, particularly under projected climate change parameters.

During this study, we aimed to replicate the movement of particles moving downstream through an estuary system under moderate flow conditions, with a journey time of approximately seven days. However, transition times through a catchment continuum may vary substantially due to their different sizes, characteristics and hydrology (Windsor *et al.,* 2019). Under low flow conditions, microplastic particles have longer transition times and can also become trapped or entrained. Tidal currents can also influence the retention of plastics within an estuary; during spring tides microplastics are dispersed further downstream (Chen *et al.,* 2022), and often become more concentrated in estuarine sediments than in the water (Biltcliff-Ward *et al.,* 2022). During storm surges and high flow events, catchments are flushed through very quickly, which can result in high levels of sewage and agricultural run-off reaching recreational beaches (Messenger, 2022). With concentrations of waterborne pathogens being so high following these events, the relative risk posed by pathogens associated with the plastisphere will be negligible. However, following the peak of such events, microplastics colonised by pathogens provide a mechanism for longer term persistence in the environment, and could increase the likelihood of exposure risk.

3.4.3 Plastics vs. Glass

Microplastics were colonised by consistently higher concentrations of *E. coli, E. faecalis* and *P. aeruginosa* (and total biofilm) throughout both experiments. Differences in surface properties were probably a major factor for this, with the increased surface roughness of the microplastic particles promoting bacterial colonisation and cell attachment (Gong et al., 2019). The higher contact angles and more hydrophobic surfaces of the microplastic surfaces were also likely to facilitate microbial colonisation (Cai et al., 2019). Although the quantity of biofilm and the concentrations of bacteria were consistently higher on plastic particles, there were no differences in die-off rates in either experiment. This suggests that although plastics become more heavily colonised by bacteria, in the

environmental conditions tested here they are not able to persist for any longer than on the glass control material. However, the buoyant nature of microplastics including polyethylene (density 0.94 g/cm^3) enables them to be transported for longer and over greater distances than similar glass particles (density 2.6 g/cm^3), increasing dissemination and the likelihood of plastics coming into contact with humans. Although there are several factors influencing the transport and fate of materials in the environment, density is suggested as the most important factor to consider, with high density particles sinking and becoming incorporated into the sediment, limiting their ability to transport pathogens (Harris, 2020; Metcalf *et al.,* 2022a). There is also evidence of microplastics becoming trapped in coastal environments where the likelihood of human exposure is greater (Harris, 2020); Ritchie and Roser (2020) predicted 40 million tonnes of microplastics accumulating, being buried and resurfacing along shorelines globally. Natural materials, such as seaweed and feathers, can also harbour potential pathogens (Quilliam *et al.,* 2014; Song *et al.,* 2020; Metcalf *et al.,* 2022b); compared to plastics these materials may provide a more readily available source of nutrients for biofilm communities and potentially enable pathogens to survive for longer. However, such materials decompose more quickly than durable plastic polymers, whilst the longevity of plastic particles allows them to act as a substrate for pathogen colonisation on numerous occasions throughout their lifetime in the environment.

3.5 Conclusion

This work has demonstrated that viable FIOs and potential pathogens bound to the surface of microplastics were able to persist in freshwater and seawater, and during the transition between these environmental matrices. Large volumes of microplastics are released from WWTPs and subsequently transported into areas of high human exposure (e.g., bathing waters and beach environments), and the ability of microplastics to act as a substrate for biofilm could also increase the risk of pathogen transfer to such areas. Therefore, there is an urgent need for updated regulations to prevent discharge of microplastics from WWTPs. In terms of existing infrastructure this will be a significant challenge and the development of potential mechanisms for stripping microplastics out of wastewater will need to come at a stage before the final screening prior to discharge. Under EU law, sewage discharges are only legal in exceptional circumstances; despite this, in the UK, 400,000 such events were reported in 2021 (Marshall, 2022). Therefore, before there is an investment in engineering solutions to reduce microplastic discharge into the environment from WWTPs, there first needs to be much tighter enforcement of existing laws on uncontrolled sewage discharge.

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4. Evidence of interspecific plasmid uptake by pathogenic strains of *Klebsiella* isolated from microplastic pollution on public beaches

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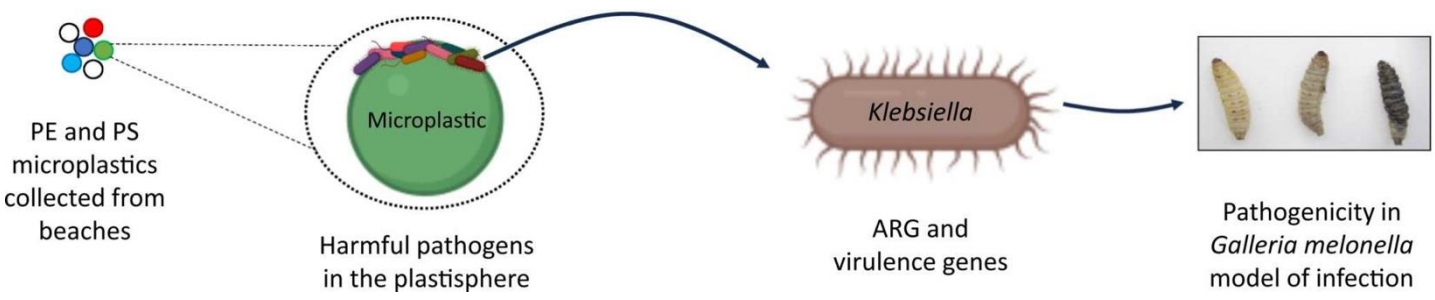
ABSTRACT

Microplastic beads are becoming a common feature on beaches, and there is increasing evidence that such microplastics can become colonised by potential human pathogens. However, whether the concentrations and pathogenicity of these pathogens poses a public health risks are still unclear. Therefore, the aim of this study was to determine realistic environmental concentrations of potential pathogens colonising microplastic beads, and quantify the expression of virulence and antimicrobial resistance genes (ARGs). Microplastic beads were collected from beaches and a culture-dependent approach was used to determine the concentrations of seven target bacteria (*Campylobacter* spp.; *E. coli;* intestinal enterococci; *Klebsiella* spp.; *Pseudomonas aeruginosa*; *Salmonella* spp.; *Vibrio* spp.). All seven target bacteria were detected without the need for a pre-enrichment step; urban sites had higher bacterial concentrations, whilst polymer type had no influence on bacterial concentrations. *Klebsiella* was the most abundant target bacteria and possessed virulence and ARGs, some of which were present on plasmids from other species, and showed pathogenicity in a *Galleria melonella* infection model. Our findings demonstrate how pathogen colonised microplastic beads can pose a heightened public health risk at the beach, and highlights the urgency for improved monitoring and enforcement of regulations on the release of microplastics into the environment.

Keywords: Public health; Plastic pollution; Human pathogens; Plastisphere; Antimicrobial Resistance; Virulence

Graphical Abstract:



4.1 Introduction

Microplastics (particles < 5 mm in diameter) are the most abundant type of plastic pollution in the marine environment (Xu *et al.,* 2019). Virgin pre-production plastic pellets (also known as microplastic beads or nurdles) are produced by the petrochemical industry for use as the raw material in the production of many thermally moulded plastic products. Once in the environment, microplastic beads can be disseminated over large distances and reach areas with high potential for human interaction, such as in rivers and on designated bathing water beaches (Karkanorachaki *et al.,* 2018; Karlsson *et al.,* 2018). The accumulation of microplastic beads in beach and coastal environments has been reported globally, with beads being distributed heterogeneously along the high tide mark (Rodrigues *et al.,* 2019; Ajith *et al.,* 2020). This is not only unsightly but may also provide a public health risk for recreational beach users (Keswani *et al.,* 2016; Metcalf *et al.,* 2022a).

Microplastics are rapidly colonised by microorganisms, which form 'plastisphere' communities (Zettler *et al.,* 2013) that are distinct from free-living communities and often contain or even enrich potential human pathogens (Metcalf *et al.,* 2022a; Ormsby *et al.,* 2023). Antimicrobial resistance genes (ARGs) are also frequently present in the plastisphere, where they can be up to 5000 times more abundant compared to the surrounding water (Zhang et al., 2020). Discharge from wastewater treatment plants (WWTPs) are known hotspots for microplastics and human pathogens (Conco et al., 2022; Raza et al., 2022), whilst hospital effluents provide an additional risk, with bacteria in these effluents carrying five to ten times more resistant genes per cell than from domestic sources (Quintela-Baluja et al., 2019). On their passage through WWTPs, microplastics can become colonised by pathogens, which can facilitate their persistence though the freshwater-estuary-coastal-beach sand continuum (Metcalf et al., 2023). Therefore, microplastics have the ability to facilitate the persistence and transport of pathogens in the environment, with beach and coastal environments being one of the main human exposure routes due to recreational activities occurring year-round. Beach plastics (e.g., microplastic beads, wet wipes and cotton bud sticks) are often colonised by faecal indicator organisms and potential pathogens, (e.g., *Escherichia coli*, intestinal enterococci, and *Vibrio* spp.) (Quilliam *et al.,* 2014; Rodrigues *et al.,* 2019; Metcalf *et al.,* 2022b). However, to quantify colonisation dynamics these previous studies have included a pre-enrichment step under optimal conditions, which provides no information on realistic environmental concentrations of each pathogen attached to the microplastic surface, and no assessment of the pathogenicity of these potential pathogens.

The public health risks of pathogens within the plastisphere are poorly resolved, due to limited evidence of pathogenicity and/or expression of virulent factors (Wright et al., 2020; Lacerda *et al.,* 2022). The presence of pathogenic-related taxa in the plastisphere does not necessarily correlate with pathogenicity and virulence (Beloe *et al.,* 2022; Bowley *et al.,* 2022); and there remains uncertainty about whether pathogens attached to microplastics pose a relevant risk to human health (Bhagwat et al., 2021; Oberbeckmann et al., 2021). To date, studies which have assigned genes encoding virulence mechanisms to specific pathogens in the plastisphere have been unable to confirm whether these genes are expressed *in situ* (Radisic *et al.,* 2020; Rasool *et al.,* 2021). Two metaproteomic studies have demonstrated that potential pathogens were less active compared to other plastisphere taxa, with no proteins involved in pathogenicity detected (Oberbeckmann *et al.,* 2021; Delacuvellerie *et al.,* 2022). More recently, plastisphere studies have used the insect virulence model *Galleria mellonella* as a proxy for the mammalian innate immune system (e.g., Ormsby *et al.,* 2023) to address the uncertainty of whether pathogens in the plastisphere are actually pathogenic (Beloe *et al.,* 2022).

The aims of this study were to determine the environmental concentrations of potential human pathogens attached to microplastic beads washed up onto coastal beaches, identify the presence of genes associated with virulence and antimicrobial resistance (AMR), and evaluate virulence using the *Galleria mellonella* infection model. Microplastic beads were collected from ten different beaches, and a culture-dependent approach was used to determine the concentrations of seven target bacteria (*Campylobacter* spp.; *E. coli;* intestinal enterococci; *Klebsiella* spp.; *Pseudomonas aeruginosa*; *Salmonella* spp.; *Vibrio* spp.). These target bacteria were selected due to their previous detection in the plastisphere (Metcalf *et al.,* 2022a), along with the lack of understanding on their realistic environmental concentrations present within the plastisphere; most bacteria in the plastisphere have only been detected using 16S rRNA sequencing which is unable to differentiate between live and dead bacteria. Additionally, these target bacteria are all potential human pathogens, known to cause human infections and mortality worldwide (Bartlett *et al.,* 2022). *Klebsiella* was the most abundant pathogen isolated, and therefore whole genome sequencing was used to determine presence of genes for virulence and AMR, in addition, pathogenicity of these environmental isolates was determined by using the *Galleria* assay.

4.2 Materials and Methods

4.2.1 Sampling location

Ten beaches in central Scotland, UK, were selected for sampling (Erskine, Largs, Irvine, Ayr, Turnberry, Montrose, Broughty Ferry, Portobello and Eyemouth), which included both urban and rural sites on the east and west coast of Scotland (Figure 4.1; Appendix F). Eight of these beaches are designated bathing water beaches, which are regulated under the EU Bathing Water Directive (BWD) 2006/7/EC. Most of Scotland's population live in urban centres in the central belt, including the capital city of Edinburgh (population: 550,000) and the coastal city of Dundee (population: 150,000). The city of Glasgow on the west coast is the largest urban centre in Scotland with a population of 1.7 million. There are several large plastic production and processing plants in central Scotland, which are commonly situated at estuary or coastal sites.

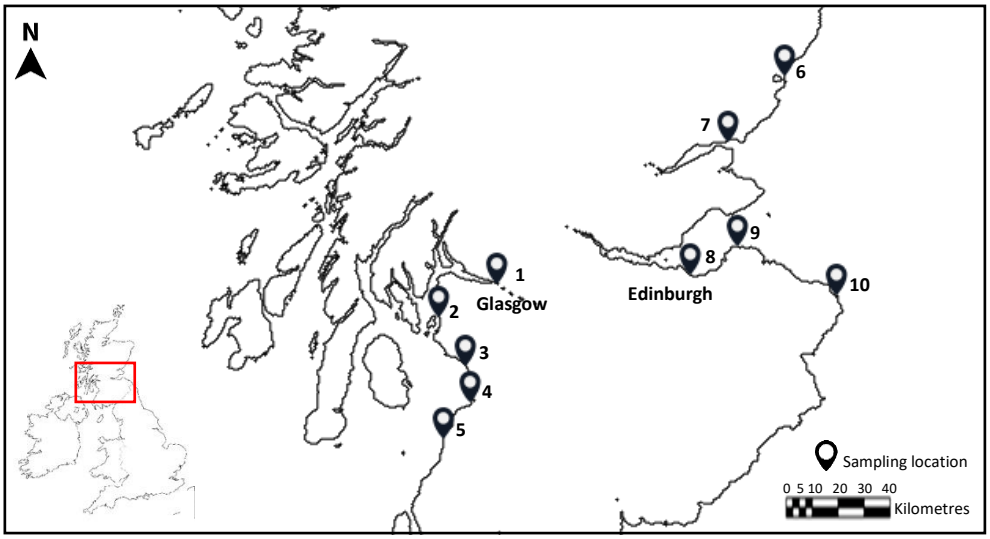


Figure 4.1. Map of sampling locations (black points) on the east and west coasts of Scotland numbered according to Appendix E.

4.2.2 Sample collection and processing

Microplastic beads (polyethylene (PE) and polystyrene (PS) beads) were collected on five days in November 2022 (Fig. 4.1; Appendix E; Appendix G) using sterile forceps and placed into sterile sample bags. Microplastic beads were identified as small round plastic particles. They were selected for this study due to their similar size and shape compared to the diversity of other microplastic particles found on beaches. Microplastic beads were the most abundant shape of microplastics found on all beaches sampled. PE beads were smooth, hard and a variety of colours, whilst PS beads were soft, rough and white. Beads were characterised (Zeiss Stemi 305 with an Axiocam 208; Zen software, Zeiss; 0.8× magnification; Appendix G) and measured using callipers (WIHA dialMax ESD, Germany). The beads collected ranged in size from 2- 5 mm diameter (mean = 4.1 ± 0.1 mm (*n* = 100)). Sand was also collected in sterile 50 ml Falcon tubes from the same location. Water temperature and salinity at each site were also measured with, a thermometer and a salinity refractometer (RGBS). All samples were stored at 4°C, and processed within 48 h. Microplastic beads were sorted by material (PE or PS), and PE beads further separated by colour; only white PE beads were analysed in this study as they were the most abundant colour collected during this and previous studies (Appendix H; Rapp *et al.,* 2020). Sites where fewer than 10 microplastic beads of each polymer were collected were excluded from further analysis due to too few replicates.

4.2.3 Chemical characterisation of microplastic beads

Fourier Transform Infrared Spectrometry (FTIR) was conducted on a Thermo Scientific Nicolet is50 FTIR (Thermo Scientific, USA) to identify polymer composition. Particles were washed with water to improve identification (Jung *et al.,* 2018). Spectra were obtained using attenuated total reflectance (ATR). A minimum of five replicate samples of both PE and PS beads from multiple sites were analysed. Polymer type was confirmed by comparing the spectra with reference plastic spectra (Jung *et al.,* 2018; Appendix K).

4.2.4 Recovery of potential pathogens using selective media

Microplastic beads were thoroughly rinsed with sterile phosphate buffered saline (PBS) to remove loosely adhering microorganisms, and transferred, using sterile forceps, to a 15 ml Falcon tube containing 5 ml sterile PBS. Bacteria were removed using methods previously described in Metcalf *et al.,* (2023). Briefly, samples were vortexed vigorously at 1500 rpm for 3 min and the wash

solution removed. Next, 5 ml of fresh PBS was added to the particles and vortexed again before the two wash solutions were combined. The wash solutions were directly filtered through 0.45 mm cellulose acetate membrane filters (Sartoris Stedim Biotech., Gottingen, Germany) and transferred onto the surface of selective media (*Campylobacter* blood free agar [CA] [plus CCDA supplement] for *Campylobacter*; membrane lactose glucuronide agar [MLGA] for *E. coli*; Slantez and Bartley agar [SB] for Intestinal enterococci; *Klebsiella* ChromoSelect agar [KCA] for *Klebsiella* spp.; *Pseudomonas* base agar [PA] [plus CN supplement] for *P. aeruginosa*; bismuth sulfite agar [BSA] for *Salmonella* spp.; and thiosulfate citrate bile salts sucrose agar [TCBS] for *Vibrio* spp. [Oxoid, UK]). All plates were inverted and incubated; CA plates were incubated at 37°C for 48 h under microaerophilic conditions (Campy GEN Sachet; Oxoid, UK); MLGA plates incubated at 37°C for 24 h; SB plates incubated at 44°C for 44 h, KCA plates incubated at 37°C for 24 h; PA plates incubated at 35°C for 48 h; BSA plates incubated at 37°C for 48 h; and TCBS incubated at 37°C for 24 h. After incubation, positive colony forming units (CFU) were enumerated. A culture-dependent approach was used to quantify the number of viable potential pathogens present within the plastisphere, improving our understanding on the risk this may pose to human health. Positive colonies were removed from each plate using a sterile loop and overnight cultures grown in Luria-Bertani (LB) broth (Fisher Bio-reagents, UK) in an orbital shaking incubator (120 rpm, IncuShake MIDI, SciQui, UK) at 37°C, before glycerol stocks of all isolates (final concentration 40% glycerol) were frozen at -20°C.

To recover bacteria associated with sand samples, 20 g sand was added to 20 ml sterile PBS and vortexed (1500 rpm, 10 min); the samples were left to settle and the supernatant filtered through 0.45 mm cellulose acetate membranes (Sartoris Stedim Biotech., Gottingen, Germany), before being aseptically transferred onto the surface of selective media (as above). CFU were enumerated to quantify the concentration of *Campylobacter, E. coli,* Intestinal enterococci, *Klebsiella*, *P. aeruginosa*, *Salmonella* spp. and *Vibrio* spp., and glycerol stocks made as described above.

4.2.5 PCR for Klebsiella identification

Klebsiella from Broughty Ferry (Site 7; Figure 4.1) were the most commonly isolated bacteria and were detected on both PE and PS beads at this site. Subsequently, colonies presumptively identified as *Klebsiella* were confirmed by PCR analysis (following the methods of Silva *et al*. 2019). Glycerol stocks of isolated *Klebsiella* were grown overnight in LB (37°C, 120 rpm, 24 h), and DNA

extracted using a Genomic DNA purification kit (Monarch, UK), using 1 ml of culture from each sample. Primers targeting *acrAB* were used to identify *K. pneumoniae* (Alsanie *et al.,* 2020). Amplification reactions consisted of 12.5 ml master mix (New England Biolabs, UK), 2 ml primer stock (10 mmol/L) and 1 ml of each DNA sample in a final reaction volume of 25 ml. PCR amplification was carried out in a thermal cycler (Techne TC-412; Keison Products, UK) using the following cycle: 3 min initial denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension of 72°C for 7 min. All PCR products were run through a 1.5% agarose gel using GelRedâ staining (Biotium, USA) and visualised with UV light; positive products had an amplicon size of 312 bp. DNA sequencing was carried out for all isolates for confirmation of species and to determine the presence of virulence and resistance genes.

4.2.6 Isolate sequencing and bioinformatic analysis

DNA was extracted from *Klebsiella* isolates using a Genomic DNA purification kit (Monarch, UK), and quantified, and the purity assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, UK); integrity was assessed by running through a 1.5% agarose gel. Ligation sequencing libraries were prepared using the NEBNext ONT Companion Module (New England Biolabs, UK) and the Native Barcoding Kit 24 (SQK-NBD112.24; Oxford Nanopore Technologies, UK), following the manufacturers protocols. The library was sequenced for 48 h using an Oxford Nanopore MinION (Mk1C) flowcell (FLO-MIN106; Oxford Nanopore Technologies, UK). Guppy (v6.3.8) was used to perform the basecalling in Fast mode with reads displaying Q>8 and >1000 bp length used for further analysis.

Genomes were assembled into contigs using Geneious software (Auckland, New Zealand); briefly, reads were trimmed using BBDuk, normalised using BBNorm and assembled using Flye (Kolmogorov *et al.,* 2019). Annotation of the assembled genomes was carried out in K Base (Department of Energy Systems Biology Knowledgebase, USA) and the Center for Genomic Epidemiology (DTU National Food Institute, Denmark), to assess completeness and contamination of the genome (CheckM, v 1.0.18; Parks *et al.,* 2015), determine species ID (GTDBtk, v 1.7.0; GTDB R06-RS202; Chaumeil *et al.,* 2019), build a phylogenetic tree (SpeciesTree, v 2.2.0; Sutormin, 2023), predict pathogenicity (PathogenFinder, v 1.1; Cosentino *et al.,* 2013), and determine the presence of virulence genes (VirulenceFinder, v 2.0; Camacho *et al.,* 2009; Joensen *et al.,* 2014; Malberg Tetzschner *et al.,* 2020), resistance genes (ResFinder, v 4.1; Camacho *et al.,* 2009; Bortolaia *et al.,* 2020; Zankari *et al.,* 2020), plasmids (PlasmidFinder, v 2.0.1, Enterobacteriales database; Camacho *et*

al., 2009; Carattoli *et al.,* 2014) and viruses (VirSorter, v 1.0.5; Roux *et al.,* 2015). Taxonomy was confirmed for the combined assemblies, which included the replicates from each substrate. Once the replicates had been confirmed to be the same species, the replicate with the most complete genome was then selected for further analysis (Appendix L).

4.2.7 Galleria mellonella *infection model*

G. mellonella larvae (Livefood, UK) were kept in darkness at 15°C and used within one week of purchase. Healthy larvae showing no signs of melanisation and measuring between 2.0 – 2.5 cm in length were used for all experiments using methods adapted from Ormsby *et al.,* (2023) (Appendix I). Glycerol stocks of *Klebsiella* from Site 7 were grown overnight in LB (37°C, 120 rpm, 24 h); these same cultures were used for sequencing (described above). To ensure that bacterial cells were in their exponential growth phase when injected into *Galleria,* 500 ml overnight cultures were added to LB (5 ml) and grown at 37°C, with shaking (120 rpm) to an OD_{600} of 0.2. Cells were then centrifuged (3000 rpm, 5 min) and resuspended in PBS. Serial dilutions in PBS were carried out, plated on KCA selective media and incubated (as above) for retrospective enumeration. To determine virulence of each of the selected *Klebsiella* strains following their isolation from the microplastic beads and sand, groups of 10 larvae were injected with 10 ml of bacteria (approximately 10^4 to 10^6 CFU) using a 100 ml Hamilton syringe (Bonaduz, Switzerland) with a 0.6 x 30 mm needle. Bacteria were injected into the hemocoel via the last right pro-limb, with all experiments conducted in biological triplicate. Needles were sterilised between samples by flushing with 70% ethanol followed by PBS. As a positive control, a reference clinical pathogenic strain of *K. pneumoniae* (strain ATCC 13883) was also included. A buffer negative control of 10 ml PBS was used to account for mortality caused by physical injury or infection caused by a contaminant. After injection, larvae were incubated at 37°C and inspected 24, 48 and 72 h post-injection; larvae were considered dead when they did not respond to touch stimuli.

4.2.8 Statistical analysis

Statistical analyses were conducted using R Studio version 3.3.2 (R Core Team, 2016). Analysis of variance (ANOVA) with Tukey's post-hoc test were used to compare the bacterial concentrations between material, species and site. All data were tested for distribution and homogeneity of variance (Shapiro-Wilk and Levene's) before parametric tests were used. Where assumptions were not met, data were either log transformed, or a non-parametric Scheirer Ray Hare

test used for a two-way factorial design (Scheirer et al., 1976), followed by Tukey's post-hoc test. Data is reported as mean ± SE. *P* values < 0.05 are considered significant.

4.3 Results

4.3.1 Recovery of microplastics beads from coastal beaches

Microplastic beads were verified as either PE or PS (Table 4.1; Appendix J; Appendix K). PE was present at all sites, whilst PS was collected from nine beaches (Appendix E). All PS beads were white, and the majority of PE beads were white (Appendix H). The abundance and type of microplastic beads varied within and between sites, but most were found accumulated at the hightide strandline and between rocks. Beaches were mainly sandy, with salinity ranging from 2 to 36 practical salinity units (PSU) and water temperatures ranging from 8.0 to 10.7°C. Although sampling was carried out during weekdays in the winter, there were beach users present at all sites (Appendix E).

Table 4.1. Concentrations of *Campylobacter*, *E. coli*, Intestinal enterococci, *Klebsiella*, *P. aeruginosa, Salmonella* and *Vibrio* spp. recovered from polyethylene (PE) and polystyrene (PS) beads (CFU/100 beads) and sand (CFU/ 100 g^{-1} dry weight sand) collected from the ten sampled sites on the Scottish coast. For PE and PS, the mean was calculated from 4 pseudo replicates. For sand, the mean was calculated from 3 replicates, ± the standard error.

Material	Bacteria	Site									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
PE (CFU/100 beads, n = 4)	***Campylobacter***	0	BLD	0	0	0	-	0	0	0	-
	E. coli	13	0	223	0	0	-	25	128	68	-
	Intestinal enterococci	0	0	BLD	0	BLD	-	0	25	BLD	-
	Klebsiella	40	20	225	220	200	-	1280	33	BLD	-
	P. aeruginosa	0	0	0	0	0	-	150	0	0	-
	Salmonella	20	BLD	68	65	300	-	140	48	13	-
	Vibrio spp.	53	59	BLD	13	BLD	-	260	200	30	-
PS (CFU/100 beads, n = 4)	***Campylobacter***	0	-	-	-	-	13	BLD	-	-	0
	E. coli	26	-	-	-	-	10	BLD	-	-	18
	Intestinal enterococci	10	-	-	-	-	15	BLD	-	-	BLD
	Klebsiella	515	-	-	-	-	50	190	-	-	343
	P. aeruginosa	0	-	-	-	-	103	BLD	-	-	0
	Salmonella	120	-	-	-	-	110	25	-	-	80
	Vibrio spp.	481	-	-	-	-	1065	185	-	-	321
Sand (CFU/ 100 g^{-1} dry weight sand, n = 3)	***Campylobacter***	0	401 ± 46	192 ± 17	264 ± 30	0	228 ± 35	209 ± 52	0	0	0
	E. coli	2059 ± 375	262 ± 60	192 ± 122	11902 ± 10931	1660 ± 1495	5447 ± 440	6226 ± 2768	606 ± 158	250 ± 95	71 ± 18
	Intestinal enterococci	3605 ± 700	262 ± 160	891 ± 424	16226 ± 6200	0	2267 ± 878	7774 ± 2818	1140 ± 139	268 ± 164	71 ± 18
	Klebsiella	5381 ± 1286	750 ± 76	367 ± 80	5995 ± 3034	11180 ± 11014	8943 ± 904	3513 ± 490	2601 ± 479	947 ± 228	335 ± 157
	P. aeruginosa	19241 ± 842	227 ± 97	0	35 ± 18	74 ± 74	70 ± 46	0	53 ± 31	0	0
	Salmonella	16888 ± 2162	1954 ± 915	314 ± 109	3024 ± 1529	5036 ± 4667	8328 ± 211	10331 ± 4304	2351 ± 375	4003 ± 331	1852 ± 433
	Vibrio spp.	29992 ± 4037	2682 ± 745	692 ± 75	5638 ± 1734	1024 ± 678	1860 ± 442	1788 ± 29	2206 ± 785	3316 ± 267	1125 ± 189

* BLD = Below limit of detection (<10 CFU)

- indicates where no samples were collected from a particular site.

4.3.2 Bacterial concentrations on microplastic beads and in beach sand

All seven target bacteria were detected on microplastic beads. There was a significant interaction between bacterial concentration, polymer, species, and site (Table 4.1; four-way ANOVA, $F_{6,252} = 7.653$, $p < 0.001$). *Klebsiella* was the most abundant of the seven target bacteria, with concentrations of up to 1280 CFU/100 PE beads. *P. aeruginosa* was the least common species, only being detected at two sites (6, 7). Two sites (1, 7) had both PE and PS beads, although the polymer type had no significant influence on bacterial concentrations (ANOVA, site 1: $F_{6,42} = 1.264$, $p = 0.30$, site 7: $F_{6,42} = 1.979$, $p = 0.09$). Three sites (1, 7, 8) were located near large urban centres and potential hospital effluents; these urban sites had significantly higher bacterial concentrations attached to both PE (Scheirer Ray Hare, $H = 4.91$, $p < 0.05$) and PS (ANOVA, $F_{1,98} = 4.301$, $p < 0.05$).

As with the microplastic beads, all seven bacteria were detected in the sand, with there being a significant interaction between bacterial concentrations, species and site (Three-way ANOVA, $F_{54,140} = 3.644$, $p < 0.001$). *Klebsiella* was also the most abundant bacteria detected in beach sand, with concentrations ranging from 335 to 11,180 CFU 100 g^{-1} (dry weight). *Campylobacter* and *P. aeruginosa* were the least abundant bacteria in the sand; however, there were high concentrations of *P. aeruginosa* (19,241 CFU 100g^{-1} dry weight) at site 1, the site near the urban centre of Glasgow (where there was also signs of sewage discharge).

4.3.3 Virulence factors and antimicrobial resistance genes from Klebsiella isolates

Taxonomic classification of the sequenced *Klebsiella* isolates using the Genome Taxonomy Database, identified the isolates from PS and sand as *K. pneumoniae* (average nucleotide identity to the closest reference isolate ATCC 13883 of 98.82% for PS and 98.94% for sand). Whereas *K. variicola* (average nucleotide identity to the closest strain DSM 15968 of 98.91%) was identified as the strain isolated from PE (Appendix L; Appendix M); this species is an emerging opportunistic human pathogen, which is closely related to, and lies within, the *K. pneumoniae* complex (Rodriguez-Medina *et al.,* 2019). According to the Pathogen Finder programme which predicts the pathogenicity of an isolate by identifying proteins with a known involvement in pathogenicity (Cosentino *et al.,* 2013), all isolates had a high probability of being a human pathogen (PE = 81%, PS = 89%, Sand = 88%, *K. pneumoniae* = 90%; Appendix L), with several virulence and resistance genes being encoded (Fig. 4.2).

All isolates possessed between five and six common virulence mechanisms identified using the package VirulenceFinder (Fig. 4.2; Fig. 4.3; Appendix N). These included the gene *fimH* which was present in all isolates and encodes for type 1 fimbriae, a major adhesive factor; interestingly, type 3 fimbriae were only present in the PS isolate. Siderophores, used to acquire iron, were present in all isolates; however, the siderophore yersniabactin was only present in the *K. pneumoniae* clinical isolate. The gene *clpK1*, which was only present in the isolates colonising the PE and PS, encodes for heat shock protein.

ARGs belonging to a variety of classes were also identified within the *Klebsiella* genomes (Fig. 4.2; Appendix O). The genes *fosA, OqxA* and *OqxB* were present in all isolates and confer resistance to clinically relevant antibiotics such as fosfomyscin, chloramphenicol and trimethoprim. Resistance to the common antibiotics amoxicillin and ampicillin was also conferred by different genes in the *Klebsiella* isolated from PE (*blaLen12*), sand (*blaSHV-33, blaSHV-187*) and the *K. pneumoniae* clinical isolate (*blaSHV-40, blaSHV-56, blaSHV-79, blaSHV-85, blaSHV-89*). The *Klebsiella* strain isolated from PS was the only isolate to possess the *blaSHV-187* gene, which encodes for beta-lactam resistance. Arsenic resistance genes were also present in all isolates.

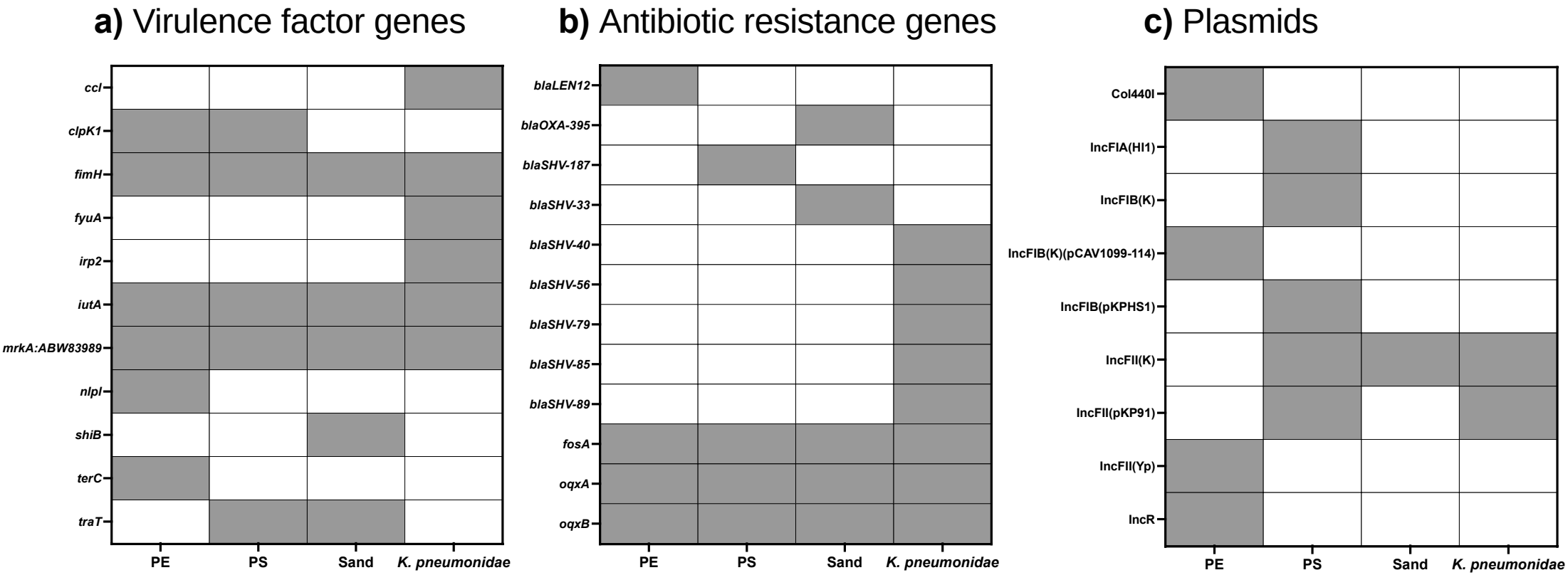


Figure. 4.2 Presence (grey) and absence (white) of (a) virulence factor genes, (b) antibiotic resistance genes and (c) plasmids, present in strains of *Klebsiella* isolated from PE, PS, and sand. For comparison, the profile of a clinical strain of *K. pneumoniae* has also been included.

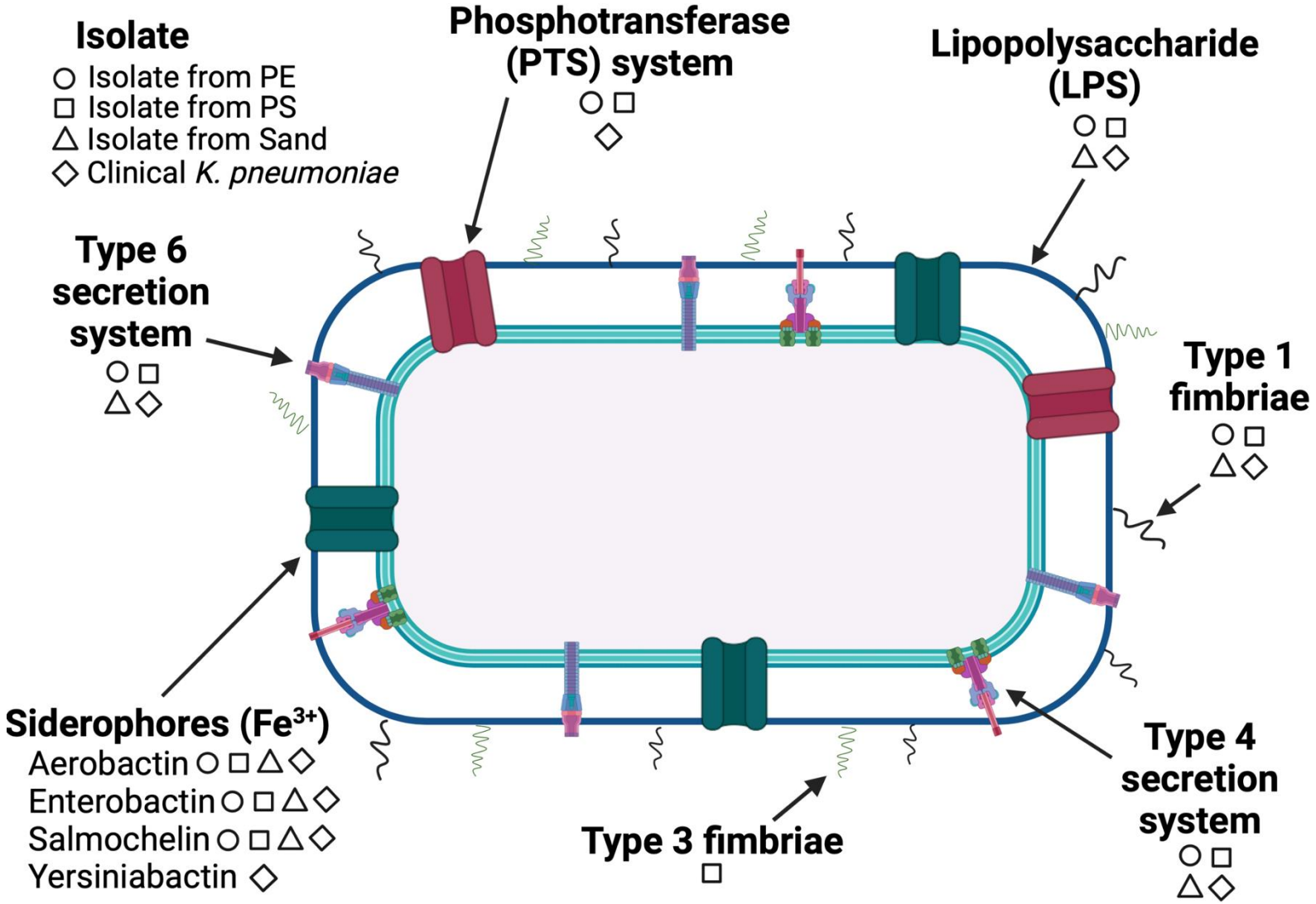


Figure. 4.3 Occurrence of *Klebsiella* virulence factors in strains of *Klebsiella* isolated from PE (circle), PS (square), sand (triangle), and for comparison, from a clinical strain of *K. pneumoniae* (diamond). Figure created with BioRender.com.

Several plasmids were also detected (Fig. 4.2; Appendix P; Appendix Q); IncFIB(K) was the most common plasmid, detected in the *Klebsiella* isolates from PS, sand and the *K. pneumoniae* clinical isolate, a *K. pneumoniae* plasmid which contained virulence (*clpK1, mrkA:ABW83989, traT, ccl*) and resistance (*blaSHV-40, blaSHV-56, blaSHV-79, blaSHV-85, blaSHV-89*) genes. The isolates from the microplastic beads contained more plasmids (the isolates from both PE and PS had five plasmids) than the isolates from sand (one plasmid) and the *K. pneumoniae* clinical isolate (two plasmids). The *K. pneumoniae* clinical isolate, and the *Klebsiella* isolates recovered from the sand all contained *K. pneumoniae* specific plasmids. However, the *Klebsiella* isolates recovered from PE contained IncFIB(K)(pCAV1099-114) which is a plasmid from *K. oxytoca* and IncFII(Yp) a plasmid from *Yersinia pestis;* whilst the *Klebsiella* isolates from PS contained INCFII(pKP91) a plasmid from *K. variicola* and INCFIA(HI1) a plasmid from *Salmonella* Typhi; suggesting increased horizontal gene transfer occurring within the plastisphere. The IncFIB(K)(pCAV1099-114) plasmid found in the *Klebsiella* isolated from the PE beads also contained the virulence genes (*clpK1* and *terC*), signifying the plastisphere as a site of interspecies transfer of virulence factors.

4.3.4 Expression of virulence in a Galleria mellonella *model*

Strains of *Klebsiella* recovered from both PE and PS microplastic beads, and from beach sand, were all still pathogenic as demonstrated in a *Galleria mellonella* model of infection (Fig. 4). The highest mortality of *Galleria* larvae occurred within the first 24 h, during which the percentage survival decreased by 83 – 93% following injection with 10^6 bacteria and 50 – 67% when *Galleria* were injected with 10^4 bacteria. Although there were differences in percentage survival between the two concentrations, *Klebsiella* isolates were still virulent even at the lower concentration. At 72 h after inoculation, the percentage survival of *Galleria* was significantly higher for all isolates compared to the PBS control at both concentrations (Fig.4.4; ANOVA, 10^6: $F_{5,12} = 8.382$, $p < 0.01$, 10^4: $F_{5,12} = 8.382$, $p < 0.01$). However, there was no significant difference between the different *Klebsiella* isolates in terms of larval mortality, indicating that isolates of *Klebsiella* found colonising plastic microplastic beads were at least as pathogenic as the clinical *K. pneumoniae* positive control and the isolates from the sand. Although there were differences in the types of virulence factor genes found in the different environmental isolates of *Klebsiella* (Fig. 4.2) the substrate they were isolated from did not significantly affect virulence gene expression in the *Galleria* model.

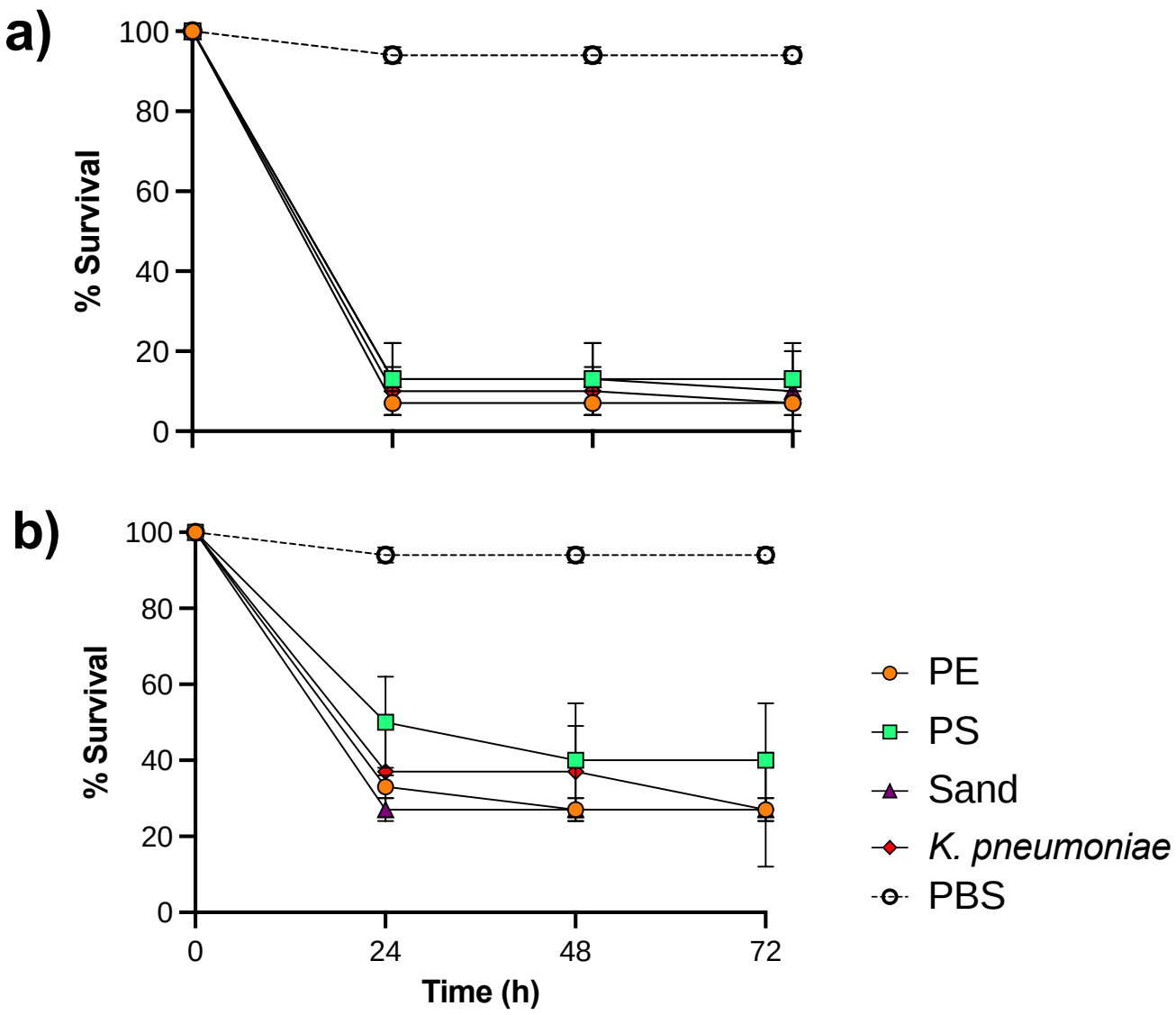


Figure. 4.4 *Galleria melonella* survival following challenge with isolates of *Klebsiella* recovered from PE, PS, and sand, at concentrations of (a) 10^6 CFU/10 µl, or (b) 10^4 CFU/10 µl, injected into each larva. Data points (*n* = ten *G. melonella* larvae) represent the mean of three independent biological replicates ± SE. * indicates a significant difference between at each time point.

4.4 Discussion

Microplastic pollution washed up on beaches is commonly colonised by a variety of potential human pathogens, particularly at more urban sites. In this study, we have demonstrated that concentrations of several human pathogens colonising microplastic beads on coastal beaches were at detectable levels without the need for a pre-enrichment step. Strains of *Klebsiella* colonising microplastics on the beach possessed ARGs and virulence genes, and expressed pathogenicity when introduced into a *Galleria* model. We have also demonstrated that *Klebsiella* isolates from the microplastic beads possessed plasmids containing virulence genes from other species of bacteria, suggesting the occurrence of interspecific plasmid uptake in the plastisphere.

4.4.1 Colonisation of microplastic beads by potential human pathogens

Although previous studies have identified potential pathogens on microplastic beads (Rodrigues *et al.,* 2019; Murphy *et al.,* 2020; Metcalf *et al.,* 2022a), this study is the first to quantify realistic concentrations of potential pathogens in the plastisphere of environmental microplastics. Species of *Klebsiella*, which are often found in the plastisphere, can cause opportunistic and nosocomial infections; however, there is also evidence that *Klebsiella pneumoniae* is capable of biodegrading PE (Awasthi *et al.,* 2017). Yang *et al.,* (2023) previously showed *Klebsiella* to be more abundant in PS compared to PE plastispheres, although in our study there was no difference between the two materials. *Vibrio* spp. was one of the most abundant species detected on microplastic beads in this study; similarly, Delacuvellerie *et al.,* (2022) found *Vibrio* to be the most abundant human pathogenic bacteria (up to 88% of total reads) on macroplastics collected from a beach in Corsica. *P. aeruginosa* are common biofilm formers, and both *P. aeruginosa* and *E. coli* can persist, or even become enriched, in the plastisphere (Lear *et al.,* 2022; Kelly *et al.,* 2022; Metcalf *et al.,* 2023); however, *P. aeruginosa* was only detected on microplastic beads at two sites during this study. *E. coli* also had low concentrations and was only detected on microplastic beads at seven sites despite being present in the sand at all of the sites.

Microplastic beads at beaches near urban sites had higher concentrations of potential pathogens in the plastisphere. These urban sites are all near to large WWTPs (which also receive hospital effluent) and it is likely that this is a major source for plastics to become colonised by

pathogens. The potential for pathogens to persist in the plastisphere, and the subsequent transport and dissemination of microplastics through the landscape increases the potential for human exposure routes and environment transfer pathways (Metcalf *et al.,* 2023). This study was carried out in the winter when concentrations of both *E. coli* and *Pseudomonas* in stormwater are known to be lower (Selvakumar *et al.,* 2006). Therefore, human exposure risk is likely to vary temporally, with concentrations being higher in the summer when environmental conditions are more optimal, coinciding with an increased number of beach users over the summer months.

The concentrations of human pathogens detected in the plastisphere were below the infectious doses of most of our target bacteria (Kothary and Babu, 2001). However, different serotypes and strains have different infectious doses (Schmid-Hempel and Frank, 2007). For example, the infectious dose of certain enterohemorrhagic strains of *E. coli* is approximately 10 cells, while other *E. coli* strains require a large infectious dose of $>10^5$ cells (Kothary and Babu, 2001; Schmid-Hempel and Frank, 2007). We did not identify the serotypes and strains of all isolates so cannot exclude the possibility that particular strains with lower infectious doses were present. Immunocompromised individuals would also be more susceptible to infection, being vulnerable to a lower infectious dose (Dropulic and Lederman, 2016). The infectious dose is also lower if gastric acid production in the gut is reduced following recent consumption of food with a buffeting effect (e.g., chocolate) or the use of acid-reducing medications (Martinsen *et al.,* 2019; Mennah-Govela *et al.,* 2020). Importantly, more than one pathogenic species was present at each site, potentially increasing the likelihood of infection as multi-species biofilms can have higher pathogenic potential (Seth *et al.,* 2012).

4.4.2 Klebsiella *isolates and virulence genes*

There are several pathogenic species of *Klebsiella,* including *K. variicola*, and *K. pneumoniae* which accounts for 3 – 8 % of all nosocomial bacterial infections (Ashurst and Dawson, 2023). *Klebsiella* can become abundant in the plastisphere in WWTPs, with ten times greater concentrations on microplastics in effluent compared to microplastics in sewage (Kelly et al., 2021). All sequenced *Klebsiella* isolates in this study possessed the main factors essential for a high level of, e.g., lipopolysaccharide, fimbriae and siderophores (Parrott et al., 2021; Riwu et al., 2022), and demonstrated pathogenicity in a *Galleria* model of infection. Lipopolysaccharides prevent phagocytosis and inhibit

complement-mediated lysis, enabling *Klebsiella* to establish infection (Paczosa et al., 2016). Type 1 fimbriae were present in all isolates, an adhesion factor that contributes towards biofilm formation and stability in *Klebsiella* (Riwu et al., 2022). Type 3 fimbriae, which provides additional adhesins, were also present in the isolate from the PS microbeads. This enables *Klebsiella* to successfully bind to PS and become key members of the plastisphere community. Siderophores enable *Klebsiella* to acquire iron from the host during infection; the various siderophores each have different affinities for iron and can be inhibited by different host molecules (Paczosa et al., 2016). Therefore, it is advantageous for *Klebsiella* to produce a variety of different siderophores; the clinical *K. pneumoniae* isolate from this study had the most siderophores present meaning it may be more virulent than the environmental isolates. Although similar virulence mechanisms were also detected in sand isolates, microplastic beads are pollutants which further increase the presence of harmful potential pathogens in beach environments and also have the potential to transfer them between different environments.

Virulence genes have previously been detected in *Vibrio* spp. and *E. coli* isolated from the plastisphere, although a greater amount of virulence genes were detected in the surrounding water compared to the plastisphere (Silva et al., 2019). Bacterial communities in seawater are dynamic and constantly changing due to tidal flows and currents (Yu et al., 2023), which means planktonic bacterial human pathogens may only remain in bathing waters for short periods of time. In addition, the decay of bacteria originating from wastewater is more rapid in seawater compared to in beach sand (Zhang et al., 2015). Therefore, colonising microplastic beads may increase the persistence of virulent human pathogenic bacteria in beach environments, and thus increase the likelihood of transfer to humans. Importantly, virulence factor expression in biofilm on plastics increases at higher temperatures, e.g., *Vibrio parahaemolyticus* (Billaud *et al.,* 2022); therefore, with projected warmer climates as a result of climate change the potential risk of pathogen-colonised microplastic beads in the beach environment will increase.

4.4.3 Klebsiella *isolates were pathogenic in a* Galleria *model of infection*

Few studies have confirmed the pathogenicity of pathogens in the plastisphere beyond demonstrating the presence of virulence genes. Our study has successfully used a *Galleria* model of infection to show that *Klebsiella* isolated from the plastisphere of beach microplastics remains

pathogenic. Different pathogens within the plastisphere are likely to express different virulence mechanisms which results in differing die-off characteristics, e.g., in contrast to *Klebsiella,* clinical strains of *E. coli* recovered from the plastisphere showed a linear increase in mortality of *Galleria* (Ormsby et al., 2023). The virulence of plastisphere communities can also differ with polymer type, with communities on LDPE being less virulent than other polymers (Lear *et al.,* 2022), although no such differences were seen in virulence between the isolates of *Klebsiella* colonising PE and PS. Previously, the limitations of plastisphere studies have been highlighted in terms of their public health risk (Beloe et al., 2022); however, our data supports the assertion that human pathogens in the plastisphere do retain their virulence.

Although *K. pneumoniae* has previously been identified in the plastisphere (Kelly *et al.,* 2021; Rasool *et al.,* 2021), this is the first study to identify *K. variicola*, the isolate from the PE sample; this emerging species is often misidentified as *K. pneumoniae* and although it possesses different virulence mechanisms (Fouts *et al.,* 2008; Rodriguez-Medina *et al.,* 2019), it can be just as virulent (Long *et al.* 2017). In our study, the *K. variicola* strain possessed *nlpl* (which encodes for a lipoprotein processor) and *terC* (which encodes for tellurium ion resistance) which were not present in any of the other *Klebsiella* isolates. Despite these differing virulence genes, when introduced into the *Galleria model* there was no difference in mortality rate between the two *Klebsiella* species. Both *K. pneumoniae* and *K. variicola* had high mortality rates (up to 93 %), although at lower concentrations of inoculation there may have been differences in the rate of mortality (Maatallah et al., 2014).

4.4.4 Klebsiella *ARGs and mobile genetic elements*

Increasing numbers of *Klebsiella* strains have become resistant to antibiotics (Bengoechea *et al.,* 2019), and our study detected the presence of several ARGs in all *Klebsiella* isolates; therefore, treatment of an infection with one of these strains is likely to be more difficult. Previous studies have shown plastisphere pathogens to be most resistant to amoxicillin and ampicillin (Metcalf *et al.,* 2022b; Liu *et al.,* 2021), and several genes resistant to these antibiotics were also detected in our study. The presence of pollutants and horizontal gene transfer can selectively enrich AMR in the plastisphere (Liu *et al.,* 2021). Although we only examined ARGs in *Klebsiella* isolates, there is increasing evidence of AMR in other potential pathogens detected on the microplastic beads (e.g., *E. coli, P. aeruginosa* and *Vibrio*

spp.; Metcalf *et al.,* 2022b; Shi *et al.,* 2021); *Pseudomonas* has been previously reported as the dominant host for ARGs, carrying 50 % of ARGs on microplastics (Wu *et al.,* 2019; Sun *et al.,* 2021). Silva *et al.,* (2023) also showed higher concentrations of AMR bacteria colonising microplastics compared to sand, suggesting that the hydrophobic plastisphere enhances AMR bacteria colonisation. Additionally, *K. pneumoniae* isolates display higher antibiotic resistance rates compared to *K. variicola* (Garza-Ramos *et al.,* 2016); this is further supported by this study where *K. pneumoniae* isolates from the clinical control and sand contained a higher number of ARGs compared to the *K. variicola* isolate from PE.

The transfer of virulence and ARGs is usually mediated by mobile genetic elements such as plasmids, insertion sequences and bacteriophages; plasmids remain as extrachromosomal circularised DNA or linearised DNA and can be transferred intra- and interspecifically between bacteria within the plastisphere before being integrated into the bacterial genome (Brito, 2021; Xu *et al.,* 2022). Plasmid transfer increases in frequency in bacteria associated with microplastics compared to free-living bacteria (Arias-Andreas *et al.,* 2018), whilst a higher proportion of plasmid associated ARGs are expressed in the plastisphere compared to in water (Wu *et al.,* 2022). Yuan *et al.,* (2022) also showed evidence of increased horizontal ARG transfer on aged plastics compared to pristine microplastics, suggesting that aged microplastic beads found in beach environments will possess increased levels of ARGs. Several plasmids were detected in the *Klebsiella* isolates, including ones which contained ARGs and virulence genes. Importantly, unlike the clinical *K. pneumoniae* and sand isolates, the isolates recovered from the microplastic beads contained plasmids from other species (e.g., *Salmonella* Typhi and *Y. pestis*). Interspecific plasmid transfer is known to have lower conjugation and success rates (Jonsdottir *et al.,* 2023); the protective environment provided by the plastisphere may increase the likelihood of successful interspecific plasmid transfer. One of the plasmids in the isolate of *Klebsiella* recovered from PE (IncFIB(K)(pCAV1099-114)) came from *K. oxytoca* and contained the virulence genes (*clpK1* and *terC*); this *terC* gene encodes for tellurium resistance which was not present in any other isolates. Tellurium resistance and other metal homeostasis genes have previously been detected in greater abundances on microplastics compared to the surrounding water (Rahman *et al.,* 2021); this is likely due to the fact that microplastics contain higher metal ion concentrations compared to the surrounding environment (Holmes *et al.,* 2014). Taken together, we speculate that mobile genetic elements may increase virulence and AMR of plastic associated *Klebsiella* as a result of horizontal gene transfer, further highlighting the importance of plasmids as a source of ARGs in the plastisphere.

4.5. Conclusion

Klebsiella isolates colonising microplastic beads possessed ARG and virulence genes, some of which were present on plasmids originating from other species, and showed pathogenicity in a *G. melonella* infection model. This demonstrates a heightened public health risk at the beach, which will vary both spatially and temporally. Therefore, there is a pressing need to improve public awareness and prevent the release of microplastics as they pass through the plastics supply chain and through WWTPs. In the UK, illegal sewage discharges continue to occur and be at the forefront of the media and public attention (Laville, 2023). Improved monitoring and enforcement of regulations is required to prevent the continual release of microplastic beads with the potential to be colonised by harmful pathogens.

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5. Sewage-associated plastic waste washed up on beaches can act as a reservoir for faecal bacteria, potential human pathogens, and genes for antimicrobial resistance

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ABSTRACT

Sewage-associated plastic wastes, such as wet wipes and cotton bud sticks, commonly wash up on beaches; however, it is unclear whether this represents a public health risk. In this study, sewage-associated plastic waste, and naturally occurring substrates (seaweed and sand), were collected from ten beaches along the Firth of Forth estuary (Scotland, UK) and analysed using selective media for the faecal indicator organisms (FIOs) *E. coli* and intestinal enterococci (IE), and potential human pathogens (*Vibrio* spp.). Minimum inhibitory concentration (MIC) analysis was used to determine antibiotic resistance in selected strains. FIOs and *Vibrio* were more often associated with wet wipes and cotton bud sticks than with seaweed, and there was evidence of resistance to several antibiotics. This work demonstrates that plastics associated with sewage pollution can facilitate the survival and dissemination of FIOs and *Vibrio* and thus, could present an as yet unquantified potential risk to human health at the beach.

Keywords: Beach pollution; Environmental pathogens; Plastic pollution; Plastisphere; Sewage discharge; Wet wipes

5.1 Introduction

Wastewater treatment plants (WWTPs) are important sources of plastic waste (Gatidou *et al.,* 2019), with an increasing number of reports (and media interest) of sewage-associated plastic waste being discharged directly into the aquatic environment (Mourgkogiannis *et al.,* 2018; Okoffo *et al.*, 2019; Picken and Ellison, 2021). Such items include disposable wet wipes (baby wipes or moist towelettes), cotton bud sticks and sanitary products (e.g., sanitary pads, tampon applicators), which are composed in part, or fully, of plastic polymers, including polyethylene terephthalate (PET), polypropylene (PP) and polyethylene (PE) (Briain *et al.*, 2020). Once in rivers or coastal environments, the organic fraction of sewage effluent is eventually broken down and metabolised by microorganisms. However, sewage-associated plastic waste can wash up and accumulate on beaches, including popular public bathing beaches (Walker *et al.,* 2006; Araújo and Costa, 2007; Storrier *et al.*, 2007; Rapp *et al.*, 2020), with inevitable negative consequences for beach aesthetics, tourism, and local coastal economies (Nelms *et al.*, 2017).

There is currently significant media and public interest in sewage discharge, which corresponds with the increase in the release of untreated sewage being reported globally (e.g., Sydney, Australia; Edinburgh, UK; Los Angeles, USA) (Calderwood, 2021; Picken and Ellison, 2021; Papenfuss, 2022). Such discharge influences environmental and bathing water quality, causes public outrage, and leads to increased reports of adverse health effects (e.g., gastroenteritis, ear, nose and eye infections) (Slack *et al.,* 2021). Although there are strict environmental regulations in place, e.g., in the UK a new Environment Bill was approved in November 2021, these are often breached by water companies who are releasing untreated sewage into the environment in volumes that exceed their permitted discharge limits (Environment Agency, 2021; Environmental Audit Committee, 2022; Stallard, 2022). Blocked sewerage systems reduce the efficiency of WWTPs and can lead to increases in the number of sewage spill events (Briain *et al.,* 2020; Environmental Audit Committee, 2022), with wet wipes responsible for 90% of these blockages (Brown, 2021). With many commercially available brands of wet wipes being labelled as 'flushable' and 14% of people in the UK admitting to incorrectly flushing wet wipes down the toilet (Marine Conservation Society, 2021), it is not surprising that there are increased sewage blockages and subsequent sewage spills. Media attention has focused on the accumulation of wet wipes on beaches (Grierson, 2021; Picken, 2021), with the *Great British Beach Clean* reporting an average 25 wet

wipes per 100 m on Scottish beaches (Marine Conservation Society, 2021). In addition to signifying recent sewage discharge, sewage-associated plastic waste washing up on beaches can be also an indicator of 'legacy sewage discharge' that has accumulated in the environment from previous discharge events.

Plastics in the environment become rapidly colonised by microbial biofilm (Zettler *et al*., 2013), and such 'plastisphere' communities are highly variable, diverse and importantly can harbour human pathogens (Galafassi *et al.*, 2021; Wang *et al.*, 2021; Zhang *et al.*, 2021). The protective environment provided by the plastisphere is hypothesised to enhance the survival, persistence, and dissemination of human pathogens in the environment (Keswani et al., 20216). Natural organic materials, such as sand and seaweed, also have the potential to transport microbes, including pathogenic species, in naturally occurring biofilm (Quilliam *et al.,* 2014; Pham *et al.,* 2021). However, there is emerging evidence that suggests that some pathogens can become 'enriched' on plastics compared to natural materials (Pham *et al.,* 2021; Zhao *et al.*, 2021; Metcalf *et al*., 2022); although conflicting studies have found potential pathogens in higher abundance on other materials (Beloe *et al.,* 2022; Metcalf *et al.,* 2022). Pathogens detected on PET, PP and PE in the environment include *Vibrio* spp. (Jiang et al., 2018; Laverty et al., 2020; Zhang et al., 2021), and the faecal indicator organisms (FIOs) *E. coli* and intestinal enterococci (IE) (Rodrigues et al., 2019; Kelly et al., 2021), which are the key monitoring parameters used for regulating and classifying microbial bathing water quality, e.g., in the EU Bathing Water Directive (Quilliam et al., 2019). Pathogens colonising plastics are often isolated from around sewage discharge sites or other places of anthropogenic activity (Basili *et al.*, 2020; Pazos *et al.,* 2020), and recovery of potential human pathogenic bacteria, such as *Legionella* and *Mycobacterium*, have been reported on plastics inoculated with raw sewage (Wang *et al.*, 2020; Zhao *et al.*, 2021). WWTPs are one of the most important hotspots for human pathogenic bacteria, antimicrobial resistance genes (ARGs) and metal resistance genes (MRGs) (Li *et al.,* 2015; Guo *et al.,* 2017; Martinez-Campos *et al.,* 2021). Sewage-associated plastic waste is likely to be colonised by these bacteria and their associated ARGs and MRGs during their passage through WWTPs (Martinez-Campos *et al.,* 2021); additionally, they can become colonised during the period they remain in the environment. Recreation at bathing waters and on beaches provide opportunities for human exposure to such colonised plastic waste, with the potential for pathogen transfer and subsequent risk to public health.

Due to the high abundance of sewage-associated plastic waste washing up on beaches there is an urgent need to fully understand the risk of this material for facilitating the persistence and transport of human pathogens, particularly in areas with poor wastewater management and high sewage discharges. Therefore, the aim of this study was to quantify the abundance of sewage-associated plastic waste on public beaches in the Firth of Forth estuary (Scotland, UK) and determine whether this waste was colonised by FIOs and *Vibrio* spp., and whether any of these bacteria were expressing ARGs.

5.2. Materials and Methods

5.2.1 Sampling location

The Firth of Forth is a tidal estuary (tidal range >5 m) in the east of Scotland (UK), with several nearby urban centres, including the capital city of Edinburgh (population: 518,000). WWTPs are located throughout the catchment, serving approximately 1.6 million people, with 33 plants discharging directly into the estuary, for example, Alloa WWTP, which is just upstream of the study sites, was reported to discharge over 3 million m^3 of untreated sewage between 2016 and 2020 (Picken and Ellison, 2021). Additionally, Seafield WWTP in Leith is Scotland's largest WWTP, processing 300 million litres of wastewater every day and serving almost 1 million people (Scottish Water, 2021). There are numerous beaches along the shores of the Forth Estuary, including popular public beaches and EU designated bathing water beaches (Fig. 5.1).

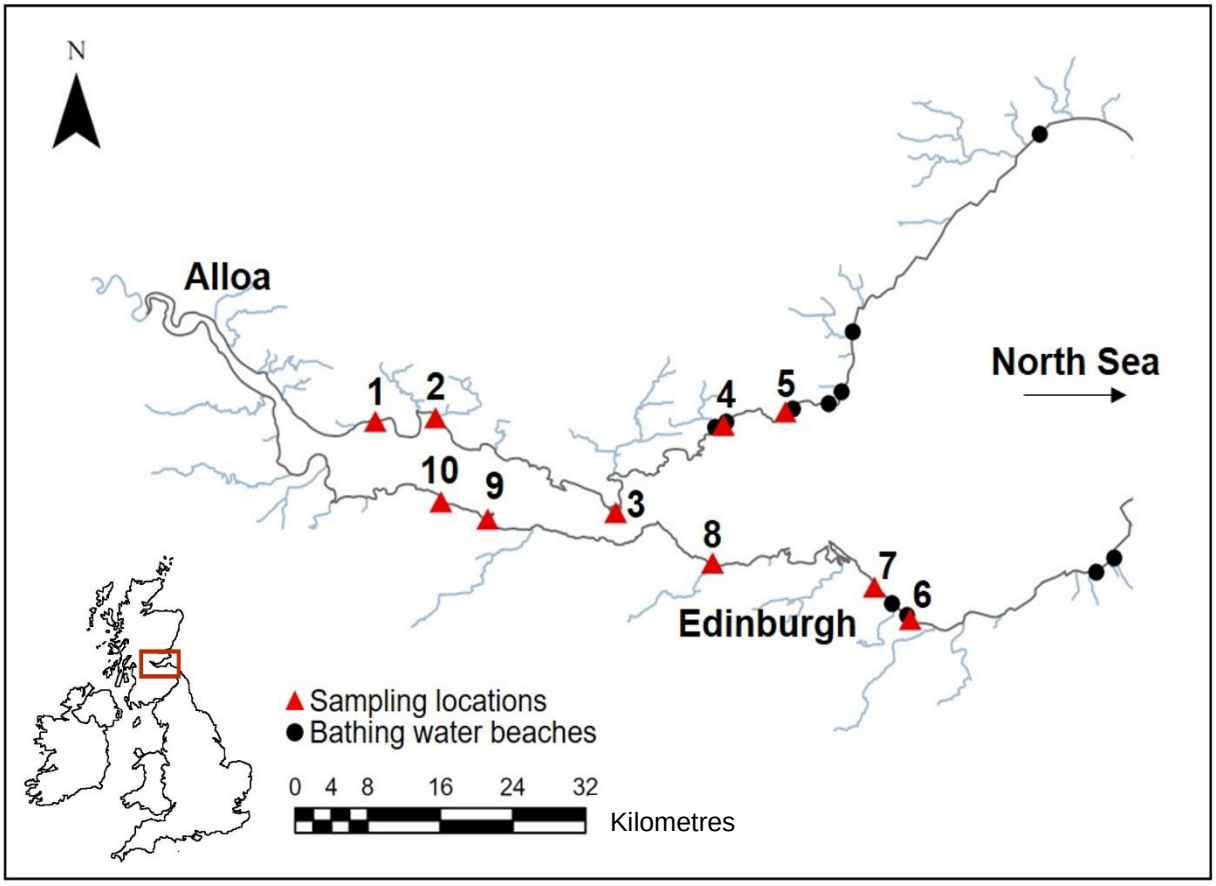

Figure 5.1. Sampling sites and EU designated bathing water beaches along the Firth of Forth estuary. Sampling locations (red triangles) are numbered according to Table 5.1. Bathing water beaches are indicated by black dots.

5.2.2 Sample collection and processing

Sewage-associated plastic waste (wipes, cotton bud sticks and sanitary products) was collected from ten beaches (Culross, Torryburn, North Queensferry, Aberdour, Burntisland, Portobello, Leith, Crammond, Blackness and Bo'ness) on two consecutive days in December 2021 (Fig. 5.1; Appendix R). At each site a transect (100 m x 10 m) was set out along the hightide strandline, and all sewage-associated plastic waste within the transect collected and placed into sterile ziplocked bags. Due to the high volume of wet wipes at Site 8, a 10 m x 10 m transect was used instead. Naturally occurring substrate, i.e., brown seaweed (*Ascophyllum nodosum*) and sand, were collected at intervals along the transect (0, 50 and 100 m). Estuary water samples (200 ml) at each beach were collected in triplicate in sterile plastic bottles, and temperature and salinity of the water measured *in situ* at each site. All

samples were stored at 4°C and processed within 48 h. Plastic sewage waste samples were sorted by type (wipes, sanitary products and cotton bud sticks) and weighed. Three representative samples of seaweed, sand, and wipes from each site were placed into a drying oven (Swallow Oven, UK) at 75°C for 24 h, to determine their dry weights.

Replicate composite samples of wipes (30 g in 100 ml), cotton bud sticks (four sticks in 20 ml) and seaweed (3 g in 20 ml) from each site were pre-enriched in non-selective media Luria-Bertani (LB) broth (Fisher Bio-reagents, UK) or *Vibrio* selective Bile Peptone broth (1% peptone, 0.5% Taurocholic acid, 1% NaCl, pH 9.0 [Faruque *et al.*, 2006]) at 37°C in a shaking incubator (100 rev min^{-1}; Gallenkamp Orbital incubator) for 18 h. For those sites where less than 30 g of wipes had been collected (i.e., S2-4), 3 g of wipes were placed in 10 ml broth. No cotton bud sticks were present at two sites (S1 and S9) and only two cotton bud sticks were collected from S8, resulting in a composite sample of <4 sticks for this site. Following pre-enrichment, serial dilutions were carried out in phosphate buffered saline (PBS) and dilutions (10^{-4} – 10^{-7}) plated onto selective media (membrane lactose glucuronide agar (MLGA) for *E. coli*, thiosulphate citrate bile salts agar (TCBS) for *Vibrio*, and Slantez and Bartley agar (SB) for intestinal enterococci (IE) (Oxoid, UK)). All plates were inverted and incubated; MLGA and TCBS plates were incubated at 37°C for 24 h and SB plates incubated at 37°C for 4 h followed by 44°C for 44 h. The presence or absence of target bacteria on all sewage-associated plastic waste samples was determined by enumerating colony forming units (CFUs).

Samples of estuary water from every site (100 ml, n = 3) were directly filtered through 0.45 µm cellulose acetate membrane filters (Sartorius Stedim Biotech., Gottingen, Germany) and aseptically transferred onto the surface of selective media. To remove bacteria associated with the sand samples, 20 ml of sterile PBS was added to 20 g of sand for each replicate sample and shaken for 10 min (100 rev min^{-1}), and vortexed for a further 10 min (1,500 rpm). The sand was allowed to settle for a few seconds and then the supernatant was filtered through 0.45 µm cellulose acetate membrane filters and placed on selective media. Following incubation, CFUs were enumerated to quantity the concentration of each target microorganism (100 ml^{-1} water or 100 g^{-1} dry weight sand).

5.2.3 Multiplex PCR for Vibrio *spp. identification*

Eight representative colonies (encompassing a range of different colony morphologies and colour) of *Vibrio* spp. from the seaweed (S1, S6), wet wipe (S1, S7), and cotton bud stick (S2, S7) samples were isolated from agar plates and grown overnight in LB broth. DNA extraction was carried out using the QIAamp DNA mini kit (Qiagen, USA), using 250 µl of culture from each sample. Primers from Nhung *et al.,* (2007) targeting *dnaJ were used to identify five Vibrio species, i.e., V. alginolyticus, V. cholerae, V. mimicus, V. parahaemolyticus, and V. vulnificus.* Amplification reactions consisted of 5 µl Multiplex master mix (New England Biolabs, UK), 3.75 µl of the multiplex primer stock (10 µmol/L) and 5 µl of each DNA sample in a final reaction volume of 25 µl. PCR amplification was carried out in a thermal cycler (Techne TC-412; Keison Products, UK) using the following cycle: 3-min initial denaturation at 94°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 7 mins. All PCR products were subjected to electrophoresis down a 1.5% agarose gel using GelRed® staining (Biotium, USA) and visualised with UV light. Different amplicon sizes were used to differentiate between distinct *Vibrio* species: *V. alginolyticus (144bp), V. cholerae (375bp), V. mimicus (177bp), V. parahaemolyticus (96bp), and V. vulnificus (412bp).*

5.2.4 Minimum Inhibitory Concentration (MIC)

Representative individual colonies of *E. coli*, IE and *Vibrio* spp. isolated from seaweed, wet wipe, and cotton bud stick samples, were picked off agar plates, grown further overnight in LB broth, and subjected to MIC analysis to determine antibiotic resistance. Resistance or sensitivity to five antibiotics, listed as critically or highly important antimicrobials for human medicine (WHO, 2019), was examined. These included amoxicillin, ampicillin, streptomycin, cephalexin, and tetracycline (at ten concentrations between, 256 mg/L and 0.5 mg/L) (Melford, UK; Duchefa Biochemie, Netherlands). Two-fold serial dilutions of each antibiotic were made in LB broth in 96-well plates. Bacterial cultures were adjusted to an OD_{600nm} of 0.1, and 10 µl added to each well of a 96-well plate. Samples were incubated overnight at 37°C. The MIC was recorded visually as the lowest concentration of the antimicrobial agent that inhibited bacterial growth (CLSI, 2009; Chen *et al.,* 2021; Paudyal *et al.,* 2021).

5.2.5 Statistical Analysis

Graphs were prepared and statistical analysis conducted using R Studio version 3.3.2 (R Core Team, 2016). Analysis of variance (ANOVA) was used to compare the bacterial concentrations between sites for water and sand. All data were tested for distribution and homogeneity of variances (Shapiro-Wilk and Levene's) before parametric tests were used. Where assumptions were not met, data was square root transformed. Data is reported as mean ± standard error. P values < 0.05 are considered significant.

5.3. Results

5.3.1 Quantity of plastic sewage waste

Sewage-associated plastic waste was present on all of the beaches sampled in the Forth Estuary, with higher concentrations found on beaches on the south side of the estuary (Table 5.1). Wet wipes were the most abundant item found, with >70 kg (dry weight) present at Crammond beach within the 100 m transect (extrapolated from a 10 m transect). Beaches further downstream (S3-S7), including the three EU designated bathing waters, had lower quantities of wet wipes, but higher numbers of cotton bud sticks (Table 5.1). Sanitary products were present on eight of the ten beaches (Appendix R). The abundance and composition of sewage-associated plastic waste varied both within and between beaches, but the majority was found accumulated at the hightide strandline (Table 5.1). Water temperatures ranged from between 3.3 and 6.6°C and salinity between 0.8 and 3.0% salt. At the point of sampling, over two cold weekdays in December, people (mainly dog walkers and families) were recorded on eight of the beaches (Appendix R).

Table 5.1. Quantity of sewage-associated plastic waste collected at each beach.

	Wipes		Cotton Bud Sticks	
Site	**Wet weight (g per 100 m)**	**Dry weight (g per 100 m)**	**Weight (g per 100 m)**	**Number**
S1 - Culross	915	385	-	-
S2 - Torryburn	1,000	196	2.82	11
S3 - North Queensferry	10.20	5.64	8.37	34
S4 - Aberdour	25.46	15.85	13	52
S5 - Burntisland	13.13	7.45	6.12	24
S6 - Portobello	23.80	10.73	37.89	126
S7 - Leith	162	106	91.25	303
S8 - Crammond	90,003	72,929	1.09	2
S9 - Blackness	3,000	1,697	-	-
S10 - Bo'ness	938	538	26.76	38

5.3.2 Bacterial colonisation

At the time of sampling, FIOs and *Vibrio* spp. were detected in the water at all sites, with there being differences in concentration between sites (Fig. 5.2; ANOVA, Water: $F_{9,60} = 3.734$, $p < 0.001$, Sand: $F_{9,60} = 5.040$, $p < 0.001$). On the day of sampling, the concentration of *E. coli* in the water at all sites was indicative of good water quality under the EU Bathing Water Directive classifications (i.e., ≤500 CFU/100 ml), whilst only eight sites were indicative of good water quality (i.e., ≤200 CFU/100 ml) for IE concentrations. Sand samples had high levels of both FIOs and *Vibrio* spp., with the mean concentrations for each site ranging from 54 – 3,815 CFU 100 g^{-1} (dry weight).

Wipes had the highest levels of colonisation, with *E. coli* and IE being present on 100% of samples (Fig. 5.3). Colonisation by *Vibrio* spp. was consistently lower than FIO colonisation across all materials. Colonisation of seaweed by FIOs and *Vibrio* spp. was consistently lower than for wet wipes and cotton bud sticks. Four *Vibrio* species (*V. alginolyticus, V. parahaemolyticus, V. cholerae* and *V.* vulnificus) were detected on the seaweed, wet wipe, and stick samples (Appendix S) by PCR, with *V. alginolyticus* present on all materials, and *V. vulnificus* only present on cotton bud sticks. Importantly, this method of species identification did not infer whether these were pathogenic strains of *Vibrio*.

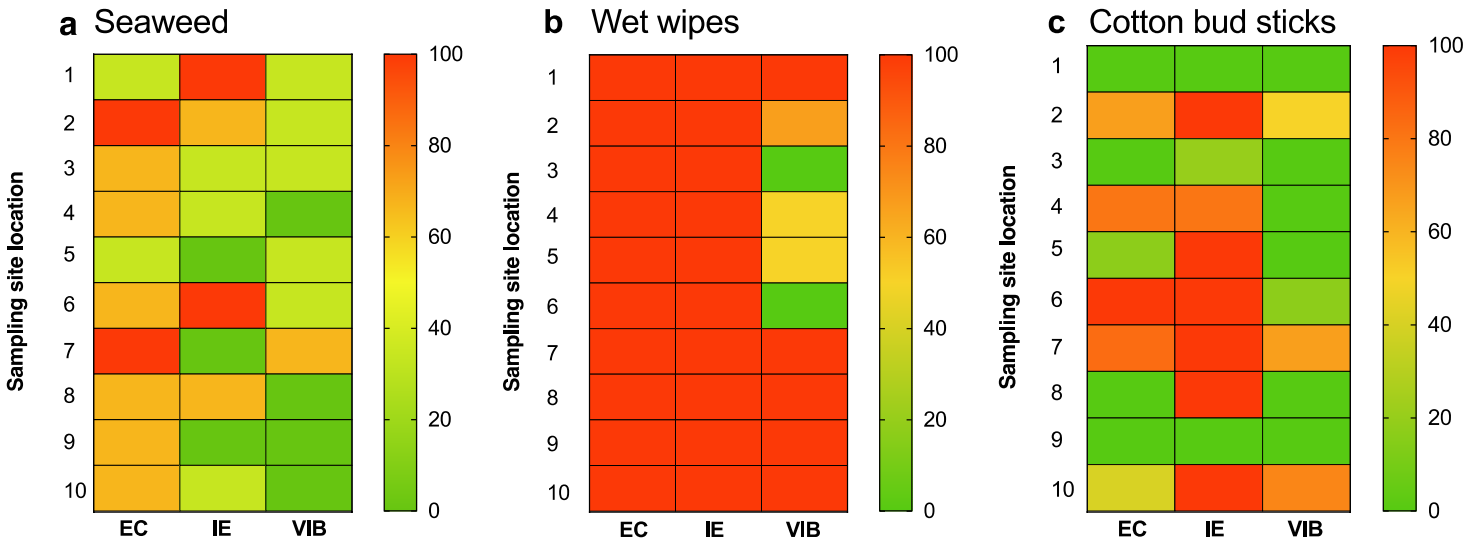

Figure 5.3. FIOs and *Vibrio* spp. recovered from seaweed, wet wipes and cotton bud sticks in the Forth estuary. Using selective media, FIO and *Vibrio* spp. were identified on (a) seaweed, (b) wet wipes and (c) cotton bud sticks from the ten sampled sites along the Firth of Forth. The colour scale indicates the percentage of material that was colonised by each species.

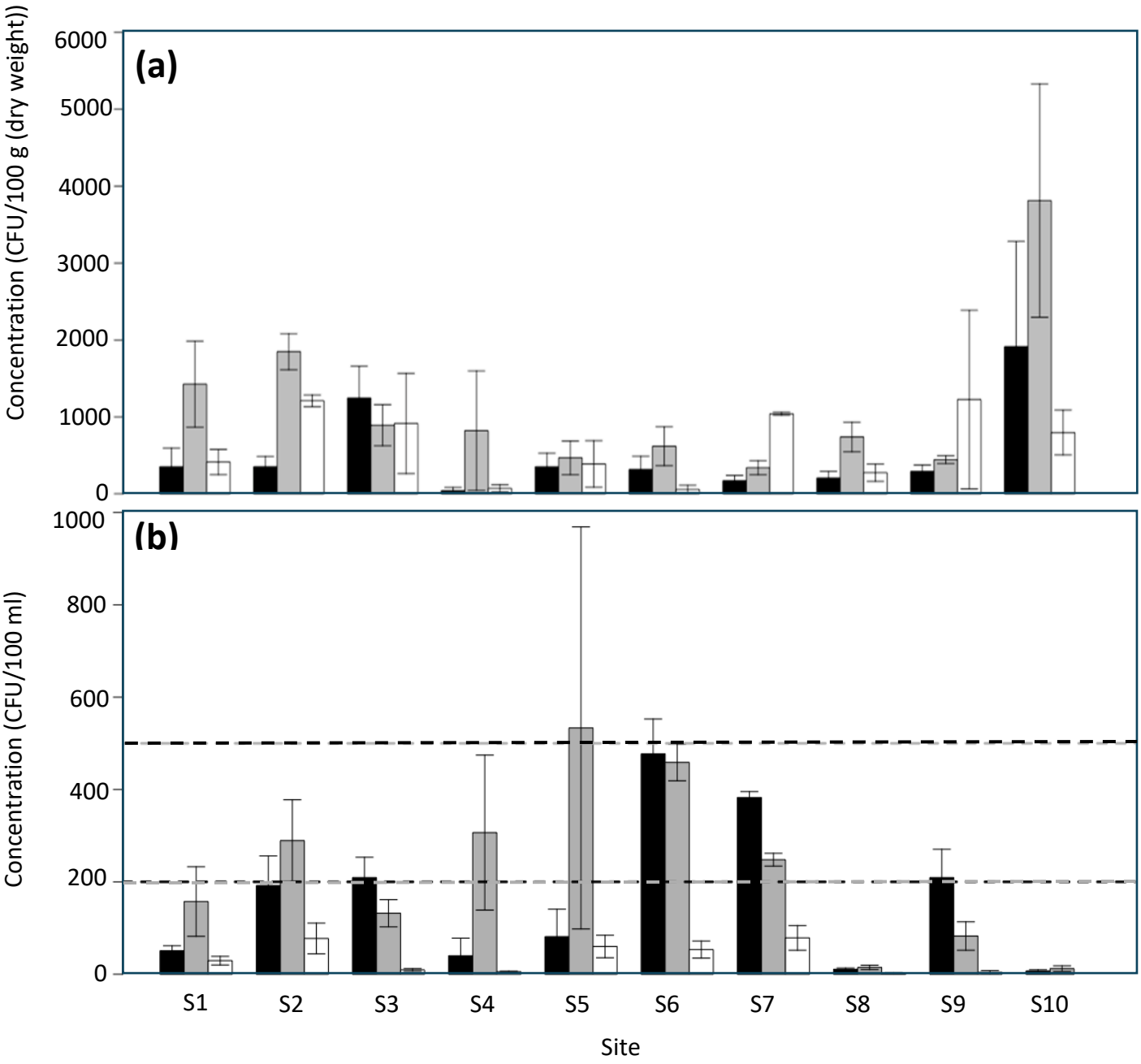

Figure 5.2. Concentration of *E. coli* (black bars), Intestinal enterococci (grey bars) and *Vibrio* spp. (white bars) in sand (a) and water (b). The mean was calculated from 3 replicates, ± the standard error. The dashed horizontal lines represent the 'Good' water quality classification thresholds for the EU Bathing Water Directive classifications for *E. coli* (black) and IE (grey), based on a 95th percentile evaluation that would be calculated over the entire bathing water season.

5.3.3 Antibiotic Resistance

MIC to five antibiotics were determined for isolates of FIOs and *Vibrio* spp. colonising the surfaces of seaweed, wet wipes, and cotton bud sticks (Fig. 5.4). The most common forms of resistance were to amoxicillin, ampicillin and cephalexin, with isolates most susceptible to tetracycline. *E. coli* and isolates of *Vibrio* spp. had the highest resistance, although there was little difference in resistance profiles of bacteria isolated from the different materials. However, IE isolated from the wet wipes and cotton bud sticks appeared to be more resistant to the antibiotics tested than those isolated from seaweed. Two thirds of all isolates were resistant to multiple antibiotics, at the concentrations examined (Fig. 5.

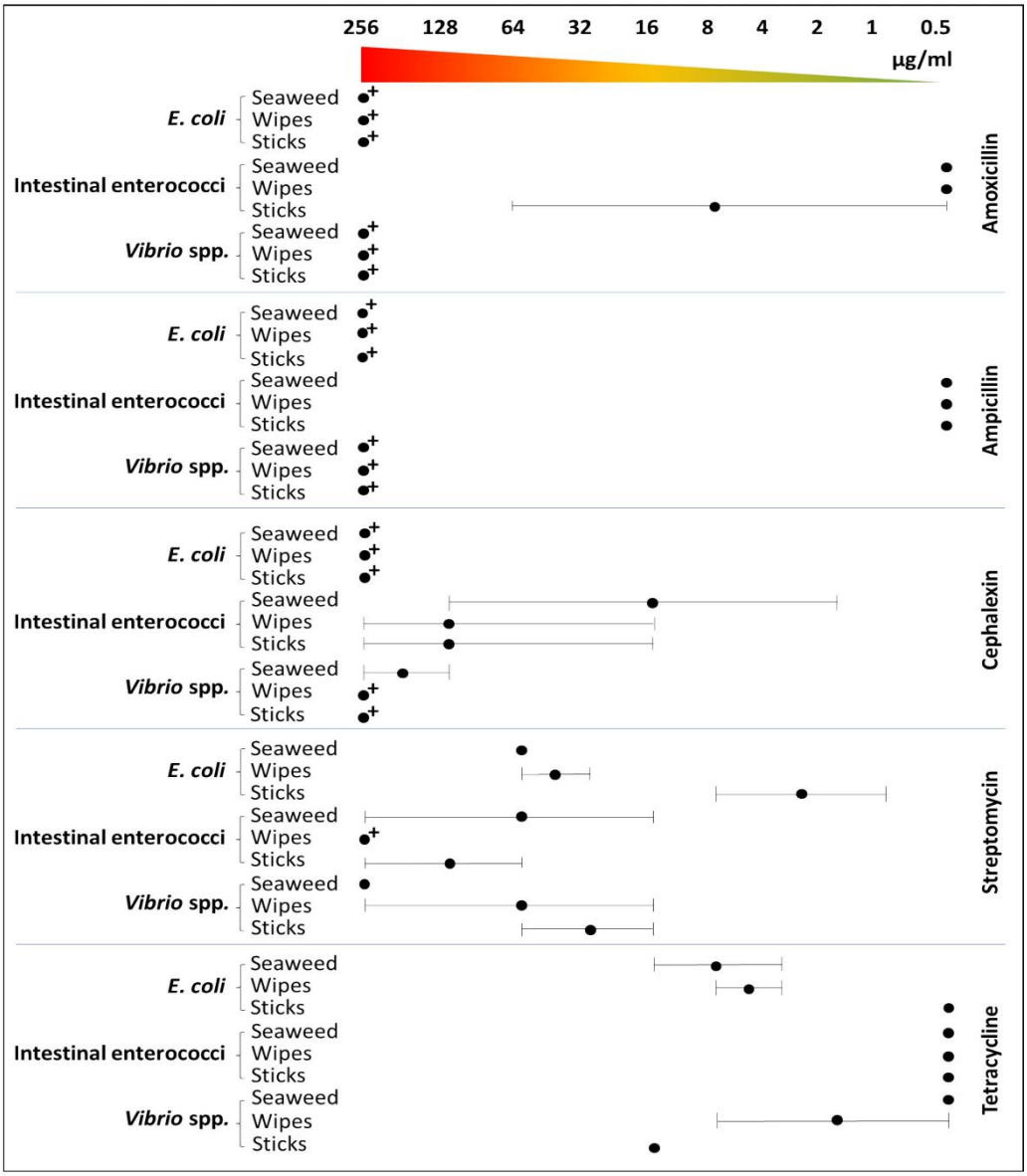

Figure 5.4. Minimum Inhibitory Concentration (MIC) of *E.* coli, Intestinal enterococci and *Vibrio* spp. recovered from beaches in the Forth Estuary. Strains able to grow at the highest concentration (**+**) indicates that their MIC would probably be higher than this. There were three replicates per treatment, error bars indicate the range.

5.4. Discussion

Seven million wet wipes, 2.5 million tampons and 1.5 million sanitary pads are incorrectly flushed down the toilet every day in the UK, resulting in sewage blockages and the release of untreated sewage into the aquatic environment (Environmental Audit Committee, 2022). This results in large quantities of sewage-associated plastic waste accumulating on beaches, and in this study, all beaches sampled were polluted, with wet wipes being the most abundant item found. This waste is not only unsightly but can also present an increased public health risk. FIOs and *Vibrio* spp. were more often associated with sewage-associated plastic waste than seaweed, and there was also evidence of resistance to several antibiotics. This highlights the potential impact this waste could have on human health at beaches through facilitating the survival and dissemination of FIOs and potential human pathogens.

5.4.1 Sewage-associated plastic waste washed-up on beaches

Most of the anthropogenic litter observed on the sampled beaches was sewage-associated plastic waste, with perhaps surprisingly no evidence of plastic waste associated with the COVID-19 pandemic, e.g., face masks. It has been previously reported that only a small proportion of anthropogenic litter collected on British beaches was sewage waste (5%) (Nelms *et al.*, 2017), which suggests that in recent years the quantity of sewage-associated plastic waste on beaches in the UK has grown, e.g., the quantity of wet wipes washing up on UK beaches is estimated to have increased by 400% in the last decade (Marine Conservation Society, 2019). This is likely due to a rise in the number of sewage spills, which have increased by 40% in Scotland in the last five years (Slack *et al.*, 2021; Environmental Audit Committee, 2022). Thirty-three WWTPs directly release treated wastewater effluent into the Forth Estuary; this includes Alloa WWTP, which according to recent media reports is one of the top 10 locations in Scotland for the volume of sewage discharge (Picken and Ellison, 2021). Once discharged, sewage waste travels downstream, accumulates in the estuary, and is washed-up on the beaches due to local wind and tidal patterns, which was evident by the abundance and composition of collected sewage-associated plastic waste varying with location, both within and between beaches. This is likely to be a result of coastal currents and bidirectional tidal flows within the Forth Estuary, which control the transport and accumulation of plastic waste (Lam *et al.,* 2020; Pinheiro *et al.,* 2021); the estuary has a large tidal range of up to 5.8 m and a tidal flux up to 30,000 m^3/s (Elliott and Neill,

2007). Estuaries often entrap and accumulate plastic waste, where it either remains suspended or sinks to the estuary bed where it can become incorporated into sediments (Lam *et al.,* 2020; Pinheiro *et al.,* 2021; Schernewski *et al.,* 2021). Plastics can remain 'trapped' within the estuary over a range of timescales before being washed up onto beaches; therefore, the accumulation of sewage-associated plastic waste on the beaches in this study could be an indication of 'legacy' sewage discharge over long timescales. The beach location and geomorphology, as well as the western prevailing winds and weather conditions will all have influenced where the waste was deposited and the rate of its accumulation on the shores of the estuary (Ghaffari *et al.*, 2019; Pinheiro *et al.*, 2019; Jong *et al.*, 2022; Nelms *et al.,* (2017).

5.4.2 Sewage-associated plastic waste on beaches are colonised by FIOs and Vibrio

Sewage-associated plastic waste on the beaches of the Firth of Forth estuary were colonised by both FIOs and species of *Vibrio*. Three of the beaches sampled are EU designated bathing water beaches (Aberdour, Burntisland and Portobello), where human exposure to sewage waste will be higher during the bathing water season, or for beach users outside of the bathing water season, such as wild swimmers, or during recreational water sports. FIOs are likely to have colonised sewage-associated plastic waste as it passed through WWTPs (Guo *et al.*, 2017; Martínez-Campos *et al.*, 2021), but opportunites for additional colonisation could occur as plastics move through faecally-contaminated water (contaminated from either sewage discharge or agricultural run-off), or through direct contact on beaches (e.g., by dog or bird faeces). It is unclear how long different bacterial species can survive and persist within the plastisphere of sewage-associated plastic waste, but survival could be facilitated by the protective environment provided by the biofilm (Keswani *et al.,* 2016).

Both FIOs and *Vibrio* spp. were more often associated with sewage-associated plastic waste than with seaweed, and potentially pathogenic species of *Vibrio* (e.g., *V. parahaemolyticus*) were isolated from the plastisphere on most of the beaches sampled. Although, multiplex PCR can differentiate between species of *Vibrio,* it is unable to determine virulence (and therefore risk) and testing for virulence genes would be required to determine actual pathogenesis (Wright *et al.*, 2020). Bacteria are ubiquitous on beaches, colonising both sand and seaweed, which can provide a more

readily available source of nutrients compared to plastics (Quilliam *et al.*, 2014; Song *et al.*, 2020), and FIOs and potential pathogens can become enriched on the surfaces of both plastics and natural materials, such as leaves, feathers and sand (Metcalf et al., 2022). However, the distinct properties of plastics (e.g., buoyancy and durability) could allow bacteria colonising these surfaces to persist for longer and be more widely transported within the environment.

5.4.3 Antibiotic Resistance

Bacteria isolated from plastic samples in this study showed resistance to several antibiotics. Horizontal gene transfer and contaminants on plastic surfaces are thought to increase the development of antimicrobial resistance (Teuten *et al.,* 2007; Arias-Andres *et al.,* 2018; Vos *et al., 2020*). Reports have shown that plastisphere pathogens can be most resistant to amoxicillin and ampicillin and least resistant to tetracycline (Moore *et al.*, 2020; Liu *et al.*, 2021), and here we have identified similar patterns of resistance to these antibiotics. Such resistance causes concern for human health; ampicillin is one of the most widely used antibiotics for the treatment of IE infections (Gavalda *et al.*, 2007). Antimicrobial resistance is now considered an important global threat to human public health, and it is predicted that antimicrobial resistance will be a major cause of mortality by 2050 (Dadgostar, 2019). With sewage-associated plastic waste acting as a reservoir of FIOs and ARGs, the opportunity for increased environmental dissemination and subsequent pathways for human exposure will be increased at beaches and in coastal waters.

5.5. Conclusion

The release of untreated sewage into aquatic environments is commonly reported in the media, by NGOs and in the literature (e.g., McCoy et al., 2020; Slack et al., 2021). Improved infrastructure and associated funding provision, together with increased monitoring and management, is required to prevent (or at least reduce) the release of untreated sewage into the aquatic environment. Sewage blockages are partly responsible for an increase in the number of sewage spills; they are primarily caused by plastic waste, including wet wipes, and annually cost water companies £100 million to clear (Environmental Audit Committee, 2022). Although businesses and private households are encouraged not to dispose of items that block sewers, millions of items are incorrectly flushed down toilets every

day in the UK (Environmental Audit Committee, 2022). The demand for wet wipes has increased as a result of the COVID-19 pandemic (Hu et al., 2022), therefore, now more than ever, there is an urgent need for greater investment in public awareness programmes to eliminate wet wipes entering sewerage systems. FIOs and species of *Vibrio* were more often associated with wet wipes on the beaches of the Forth Estuary than with seaweed, and there was evidence of resistance to several antibiotics. This work has demonstrated that plastics associated with sewage pollution can facilitate the survival and dissemination of harmful microorganisms and as such could present an increased potential risk to human health at the beach.

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Data Availability

Data is available at University of Stirling DataSTORRE (http://hdl.handle.net/11667/196).

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6. Persistence of ‘wet wipes’ in beach sand: an unrecognised reservoir for localised *E. coli* contamination

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Abstract:

The flushing of wet wipes down toilets leads to blockages of sewerage systems. This, together with unregulated sewage discharge, often results in increasing numbers of wet wipes washing up onto beaches. However, it is unclear how long wet wipes can persist on the beach and whether they pose a prolonged public health risk if contaminated by *E. coli.* In this mesocosm study, three types of wet wipes (plastic containing, and home and commercially compostable) colonised with *E. coli* were buried in beach sand and their degradation, tensile strength, and concentration of *E. coli* was quantified over 15 weeks. Wet wipes containing plastic remained largely intact for 15 weeks, whilst both compostable wet wipes fragmented and degraded. Importantly, *E. coli* persisted on all three wet wipe types, representing localised reservoirs of *E. coli* in the sand, which could present a human health risk at the beach.

Keywords: Public health; Environmental pathogens; Sewage discharge; Plastic pollution; Sand microbiology

6.1 Introduction

Reports of illegal sewage discharges are becoming more common, although governments and water companies are failing to make the appropriate changes to prevent future sewage spills or discharge events. For example, in the UK, despite the implementation of fines and stricter environmental regulations (e.g., UK Environment Bill 2021), illegal sewage discharges continue even during periods of drought (Stallard *et al.,* 2023). Furthermore, the disposal of wet wipes in toilets causes sewerage blockages, that also leads to an increase in sewage spill events (Water UK, 2023). Collectively, this results in an increasing number of wet wipes washing up onto our beaches, with a recent survey reporting 63 wet wipes for every 100 m of beach in Scotland, an increase of 150% compared with 2021 (Marine Conservation Society, 2022). This has negative consequences for beach aesthetics and tourism and has attracted significant media attention (Heany, 2023; Taggart, 2023), but may also pose a sanitary risk to human health.

Wet wipes are multipurpose non-woven textiles, used for personal hygiene and disinfection, and are composed of several different polymer fibres and chemical additives (e.g., lotions, antimicrobial agents, and preservatives) (Das and Pourdeyhimi, 2014). These polymer fibres include synthetic plastics (e.g., polyethylene and polyester) and cellulose from either a natural source (e.g., wood pulp, cotton, and bamboo) or chemical regeneration (e.g., viscose) (Pantoja Munoz *et al.,* 2018; Allison *et al.,* 2023). In 2021, 90% of wet wipes contained plastics (Zhang *et al.,* 2021), which can accumulate and persist in the environment (O Briain *et al.,* 2020; Rapp *et al.,* 2020). However, as public awareness improves, compostable non-plastic wet wipe alternatives have become increasingly available (although many brands of compostable wipes still contain plastics (O Briain *et al.,* 2020)). To be considered compostable, wet wipes must fulfil certain criteria (e.g., BS EN 13432 compostability standard); a ‘commercially compostable’ wipe must decompose in an industrial composting facility (at 58°C) within 180 days, whilst a ‘home compostable’ wipe must decompose at ambient temperature (20-30°C) within 365 days (British Plastics Federation, 2023). However, under natural environmental conditions, there is evidence of compostable products, including wet wipes, failing to fully degrade within these timeframes (Manfra *et al.,* 2021; Allison *et al.,* 2023).

The presence of plastic-associated sewage waste is becoming more prevalent at beach environments (Metcalf et al., 2022), yet the persistence and/or degradation of wet wipes in beach sand is yet to be quantified. Wastewater treatment plants (WWTPs) are known hotpots of human bacterial, viral, and fungal pathogens, which can harbour antimicrobial resistance genes (ARGs) and mobile genetic elements (MGEs) (Conco *et al.,* 2022). MGEs including plasmids and bacteriophages can facilitate the transfer of antimicrobial resistance and virulence genes, which can increase in frequency in microplastic associated bacteria compared to free-living bacteria (Aris-Andres *et al.,* 2018; Metcalf et al., 2024). Having a water content of more than 90%, wet wipes are highly susceptible to microbial colonisation (Salama *et al.,* 2021), and due to their larger area for colonisation compared to microplastics could become enriched with harmful bacteria on their transition through WWTPs (Pham *et al.,* 2021). Once discharged or spilled from the WWTP, pathogens colonising microplastics can survive the transition through the freshwater-marine continuum to beach environments (Metcalf *et al.,* 2023); and it has previously been shown that potential bacterial pathogens are more often assocaited witg wet wipes compared to seaweed (Metcalf *et al.,* 2022) however, it is unclear whether these potential pathogens could continue to survive on wet wipes when buried in beach sand. We are also yet to understand how different wet wipe types degrade in beach sand and how this will influence pathogen survival and dispersal within the environment. As beach environments are a main route for human exposure to potential pathogens colonising wet wipes, it is timely that we increase our understanding of the public health risks associated with this. Therefore, this study aimed to determine the physical degradation of wet wipes (plastic-containing and compostable), and the persistence of *E. coli* on their surfaces when buried in beach sand. To address this, we have used a culture-dependent approach to determine how long *E. coli* can remain viable on wet wipe surfaces in beach sand environments, and quantified tensile strength measurements of the wet wipes as a proxy for degradation.

6.2 Materials and Methods

6.2.1 Mesocosm set-up

Wastewater effluent, seawater, and sand were collected from sites within the river Forth catchment (wastewater effluent, Dunblane [56.184°N, -3.963°W]; seawater and sand, Kirkaldy [56.117°N, -3.146°W]) in Scotland, UK between 10th-17th May 2023. Samples were stored at 4°C and used within 24 h. Salinity and pH were measured with a salinity refractometer (RGBS) and a HI 2209 pH meter (Hanna Instruments, UK), respectively (Appendix T). To determine background *E. coli*

concentrations, water samples (100 ml, *n* = 4) were vacuum filtered through 0.45 µm cellulose acetate membranes (Merck, Germany). To extract background *E. coli* in the sand samples, 20 g sand was added to 20 ml sterile phosphate buffered saline (PBS) and vortexed (1500 rpm, 10 min); the samples were left to settle before 1 ml of the supernatant was vacuum membrane filtered as above. Membranes were aseptically transferred onto the surface of selective media (membrane lactose glucuronide agar [MLGA]; Oxoid, UK). Colony forming units (CFU) were enumerated after incubation at 37°C for 24 h (Appendix T). To determine the sand dry weight, four replicate sand samples were placed into a drying oven (Swallow Oven, UK) at 75°C for 24 h.

Three commercial wet wipe brands were selected with differing categories of advertised compostability (A: non-compostable, containing plastic; B: home compostable; C: commercially compostable). All wet wipes were cut into replicate 7 x 7 cm squares and passed through a series of treatments to simulate their journey from the bathroom to the beach (Fig. 6.1), i.e., flushed down the toilet, discharged from the WWTP into seawater, and finally washed up onto beach sand. Initially, wet wipes were placed into a bucket containing 10 L of tap water containing 250 g sterile human faeces supplemented with an additional inoculum of *E. coli* (1×10^3 CFU/ml). Wet wipes were stirred continuously for 2 min to simulate being flushing down the toilet, before being moved into glass beakers containing 2 L of fresh effluent discharged from a WWTP with an additional inoculum of *E. coli* (5×10^3 CFU/ml). All beakers were subsequently incubated on an orbital shaking incubator for 48 h (15°C, 80 rpm). Wet wipes were then transferred into glass beakers containing 2 L seawater and incubated for a further 24 h (15°C, 80 rpm), before being transferred into environmental beach sand mesocosms (18th May 2023; Fig. 6.1). The sand mesocosms were constructed from drainpipes (11 cm circumference; Tool Station, Bridgwater, UK) cut into 30 cm long sections and filled with sand to 10 cm deep. Frost hessian fabric (EU Fabrics, Birmingham, UK) was used to cover the bottom end of the tube to prevent loss of sand but allow drainage. Each wet wipe was folded in half (to form a triangle), and added to each pipe and covered with a further 5 cm of beach sand. I-Button temperature logger chips (iButtonLink, WI, 176 USA, *n* = 4) were placed into mesocosms to monitor the temperature throughout the study. Rainfall and temperature data for the duration of the study were obtained from the closest Met Office weather observatory at Grangemouth, Scotland (56.017°N, -3.700°W; Met Office, 2023).

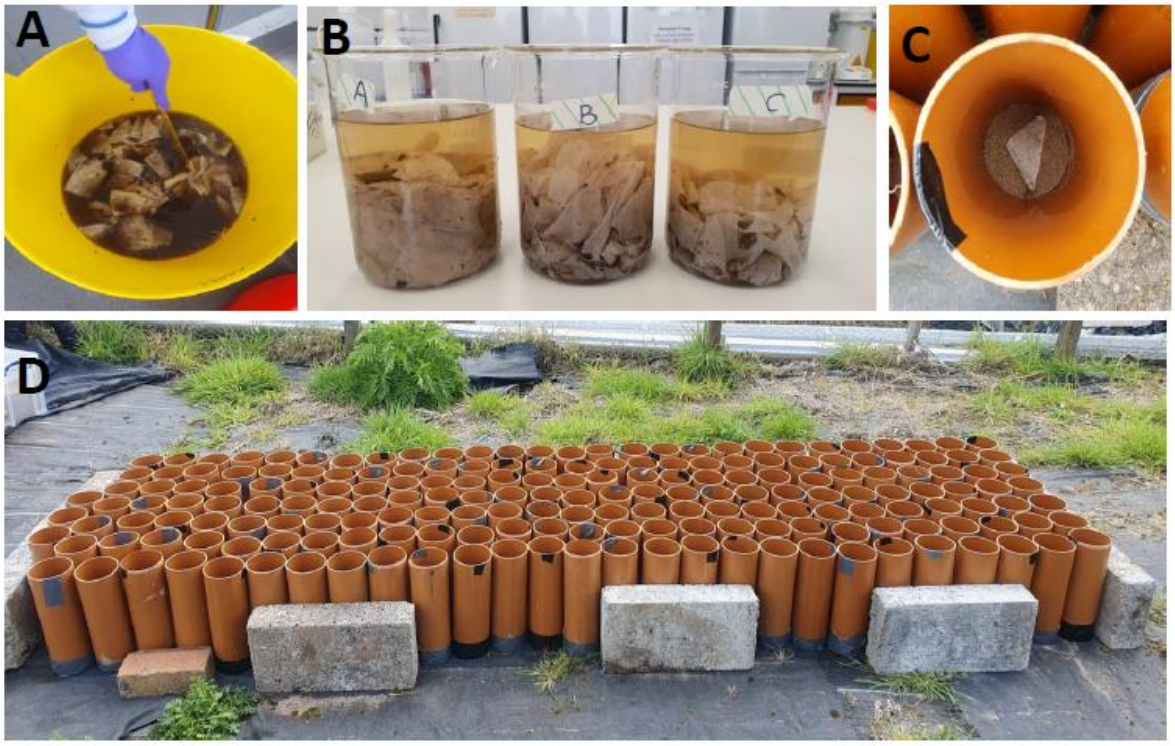

Figure 6.1. Mesocosm set-up. Wipes were passed through a simulated toilet flush (A), incubated in effluent followed by seawater (B), before being placed into the sand mesocosm tubes (C, D).

6.2.2 Sample processing

At weekly timepoints (for 15 weeks), four of each wet wipe type were randomly selected, and removed from the mesocosm using sterile forceps. The loosely adhering sand was shaken off and each wet wipe transferred into a sterile glass vial containing 20 ml PBS. Vials were then vortexed at 1500 rpm for 5 min and *E. coli* enumerated as described above and previously (Metcalf *et al.,* 2022). The wet wipes were removed from the glass vials and left to dry overnight. The following day, a 50 N digital force meter (Vogueing Tool, Hubei, China) was used to quantify tensile strength by measuring the force required to break each wipe.

6.2.3 Statistical analysis

Statistical analyses were conducted using R Studio version 3.3.2 (R Core Team, 2016). Analysis of variance (ANOVA) with Tukey's post-hoc test was used to compare the tensile strength and *E. coli* concentrations between the three different types of wet wipes. All data were tested for distribution and homogeneity of variance (Shapiro-Wilk and Levene's) before parametric tests were used. Where

assumptions were not met, non-parametric Kruskal-Wallis tests were used. Data is reported as mean ± SE. *P* values ≤ 0.05 are considered significant.

6.3. Results

6.3.1 Wet wipe degradation

Although all wet wipes became darker over time, the plastic-containing wet wipes (A), remained intact compared to both of the compostable wipes (B and C) which fragmented and degraded during the 15-week course of the experiment (Fig. 6.2); wipe C had completely degraded by week 10 while wipe B had degraded by week 14. Plastic-containing wipes started with a lower tensile strength than the compostable wipes (Fig. 6.3; ANOVA, $F_{2, 9} = 26.32$, $p < 0.001$). However, unlike the compostable wet wipes whose tensile strength decreased with time as the wipes degraded (Fig. 6.3; ANOVA; Wipe B: $F_{13, 38} = 58.64$, $p < 0.001$; Wipe C: $F_{7, 24} = 39.52$, $p < 0.001$), the tensile strength of the wet wipes containing plastic remained constant throughout the experiment (ANOVA, $F_{14, 44} = 1.206$, $p = 0.305$). The increase in fragmentation of wipes B and C after week five (Fig. 6.2), may be linked to the increased rainfall from week 5 (Appendix U). The first four weeks of the study were warm and dry, with only four days of recorded rainfall in this period (7.5 mm in total). In comparison, the subsequent four weeks of the study (weeks 5-8) were wet; a total of 140 mm rain fell within this period (appendix U).

6.3.2 Survival of E. coli on the surfaces of wet wipes buried in beach sand

Background *E. coli* concentrations for water used in the simulated flush were highest in the effluent (10^4 CFU/100 ml; Appendix T), and wet wipes became colonised by *E. coli* during the inoculated simulated toilet flush and transfer to WWTP effluent, which persisted during the incubation in seawater. Concentrations of *E. coli* in the sand mesocosms decreased with time for all three wet wipe types (Fig. 6.4; ANOVA, Kruskal-Wallis; Wipe A: $F_{15, 48} = 17.41$, $p < 0.001$; Wipe B $H(13) = 47.47$, $p < 0.001$; Wipe C: $F_{7, 24} = 17.36$, $p < 0.001$). Wipe A showed an exponential biphasic decay curve, with the most rapid decrease occurring within the first three weeks. Between weeks 3 and 7, *E. coli* concentrations increased; this corresponded with an increase in temperature and rainfall in the mesocosms at weeks 4 and 5 (Appendix U). *E. coli* was able to withstand high temperatures in the mesocosms (max temperature 48.5°C recorded during weeks 4 and 5). This was followed by a rapid decrease in *E. coli* concentration for wipe A between weeks 7 and 8, when there was increased rainfall saturating the sand (66 mm of rain fell

within this week; Appendix U). After week 7 there was a cessation in die-off rate for wipe A as *E. coli* concentrations plateaued. Wipe C showed a similar biphasic decay curve, with concentrations plateauing between weeks 3 and 8 after the initial rapid die-off. In contrast, *E. coli* concentrations followed a steady decline over time for wipe B with concentrations decreasing by 5 log CFU/wipe between weeks 0 and 13. After week 14, *E. coli* still remained on wipe B, but results were excluded from analysis due to an absence of replicates (Mean CFU per wipe: Week 14 = 1.90 x 10^2, n = 2; Week 15 = 3.20 x 10^2, n = 1). At later timepoints, wipe B and C had a significantly lower colonisation area compared to wipe A (Fig. 6.2).

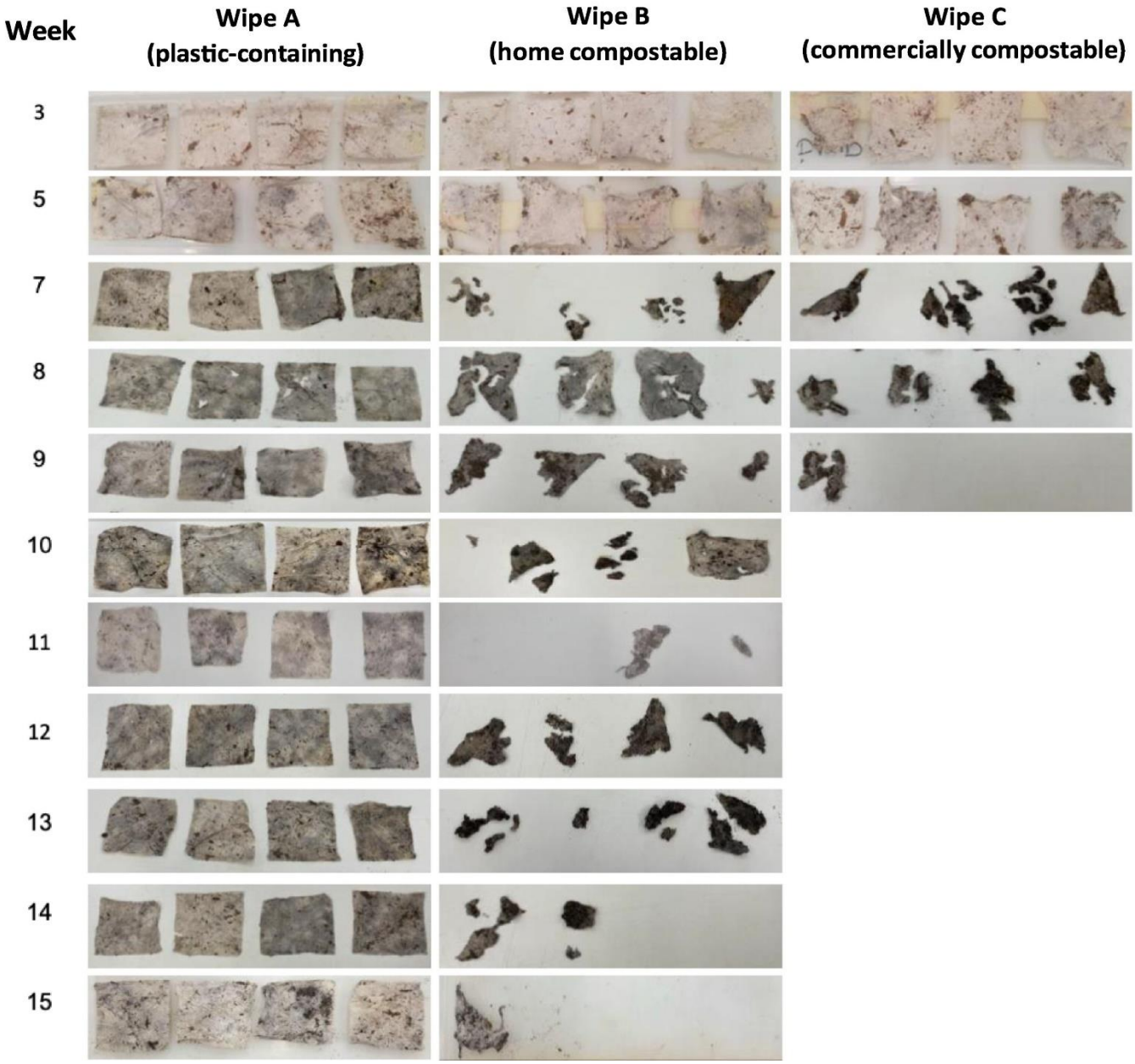

Figure 6.2. Visual assessment of wet wipe degradation.

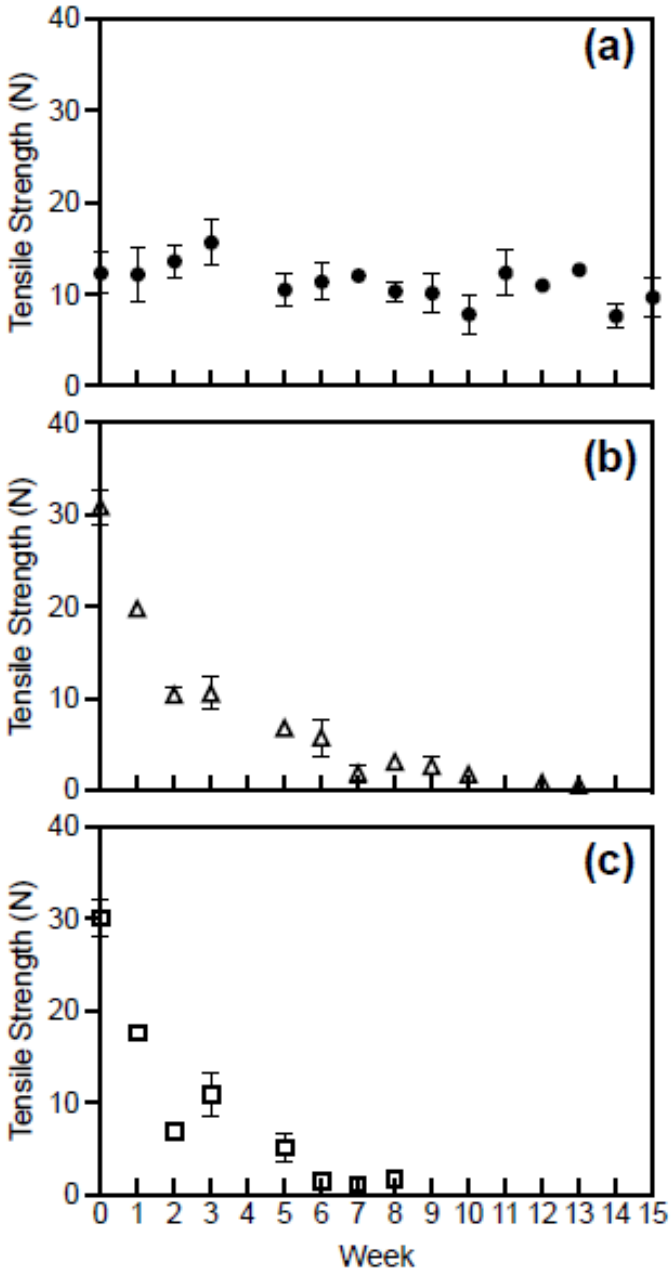

Figure 6.3. Tensile strength of wet wipes throughout the study: (a) plasticcontaining wipes (black circles); (b) home compostable wipes (white triangles); and (c) commercially compostable wipes (white squares). The mean was calculated from four replicates, ± standard error. The final measurements for home compostable (b) and commercially compostable (c) wipes were at weeks 13 and 8 respectively.

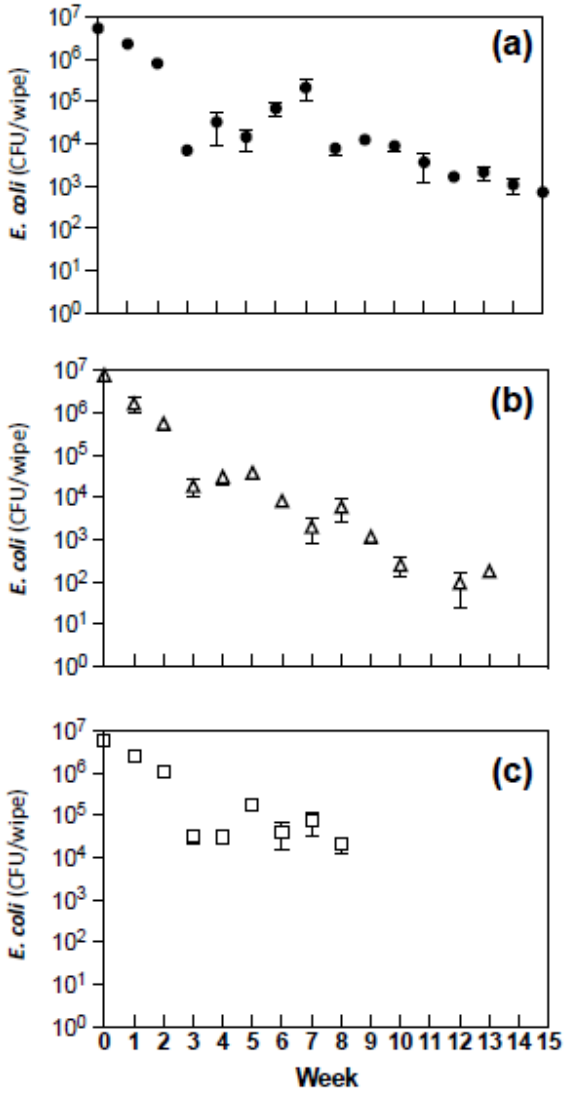

Figure 6.4. Concentration of E. coli on the wet wipes throughout the study: (a) plastic-containing wipes (black circles); (b) home compostable wipes (white triangles); and (c) commercially compostable wipes (white squares). The mean was calculated from four replicates, ± standard error. The final measurements for home compostable (b) and commercially compostable (c) wipes were at weeks 13 and 8 respectively.

6.4 Discussion

In this study, we have demonstrated that wet wipes containing plastic remained largely intact when buried in beach sand during a 15-week time course, whilst both types of compostable wet wipes fragmented and degraded. E. coli can persist on all three types of wet wipe when buried in beach sand, with a concentration of 10^3 per wipe remaining after 15 weeks on those wet wipes containing plastic. This as yet unquantified reservoir for potential pathogenic bacteria at the beach could pose a significant public health risk, and highlights the need for (i) increased public awareness of incorrect flushing of all wet wipe types, (ii) improved management of wastewater discharge and spills, including more effective regulation, and (iii) a greater impetus for policy change concerning wet wipes, particularly those that contain plastic.

The wet wipes containing plastic showed little degradation during the study, suggesting they can persist for long periods in beach sand environments. Previous studies had demonstrated that *E. coli* can persist on the surfaces of plastics in the environment for at least 28 days (Metcalf *et al.,* 2023; Ormsby *et al.,* 2023), but here we have shown that *E. coli* can continue to persist and survive for at least four months. However, despite the plastisphere and wet wipe surfaces providing a protective environment for *E. coli* (Li *et al.,* 2024), concentrations decreased with time. This is likely due to the non-optimal survival and growth conditions within the mesocosm; the optimal temperature for *E. coli* growth is 37°C under aerobic conditions (Jang *et al.,* 2017). Several environmental factors may also have influenced the survival and die-off rate of *E. coli*, including temperature, pH, rainfall, solar radiation, and moisture (Williams *et al.,* 2005; Peteresen *et al.,* 2020). The moisture content of the sand will have changed due to fluctuating air temperatures and rainfall: low moisture content is associated with decreased cell survival and growth limiting conditions (Underthun *et al.,* 2018), whereas increases in sand moisture following rain can resuscitate dormant cells (Beversdorf *et al.,* 2007). In this study, the rainfall from week 4 would have increased the sand moisture and likely resuscitated any desiccated or water-stressed cells, which would have been further promoted by the corresponding increase in temperature. However, as the sand became saturated, *E. coli* cells would have been washed off the wet wipe and leached through the column and out of the bottom of the mesocosm.

Wet wipes are responsible for 75% of sewerage blockages which, in the UK costs around £100 million a year to clean up (Water UK, 2023). Blocked sewerage systems lead to an increase in spill events where raw sewage can be released directly into the environment, contaminating bathing waters and causing serious illness (Slack *et al.,* 2022). In 2021, around 90% of wet wipes contained plastics (Zhang *et al.,* 2021); however, more recently alternatives are becoming more widely available, and several retailers have already banned the sale of wet wipes containing plastics (DEFRA, 2023). Additionally, following much debate in parliament, the English government has proposed a ban on wet wipes containing plastic by 2024 (DEFRA, 2023); however, there appears to be little evidence of a wider global ban on wet wipes containing plastics.

As public awareness increases, alternative plastic-free wet wipes are increasingly becoming available. In the last decade the market size of compostable wet wipes has tripled (> US$3 billion in 2022; Allison *et al.,* 2023). Commercially compostable wet wipes buried in sand completely degraded

within 10 weeks, and home compostable wet wipes had almost completely degraded by 15 weeks. The degradation processes of wet wipes in aquatic environments are already well understood (reviewed in Allison *et al.,* 2023); however, the degradation process when buried in beach sand will be different, with factors, such as temperature, sand moisture, and the autochthonous microbial communities influencing the rate of degradation. Abiotic hydrolysis, a degradation process where molecular chains (e.g., polyesters, cellulose) are broken down when water reacts with the material's surface, is likely to increase following rainfall (Speight, 2017). The differing conditions (e.g., particle type and size, salinity, moisture, temperature) in beach sand compared to commercial composting facilities may have facilitated more rapid degradation. For example, salinity would have been higher in the beach sand, which can influence microbial decomposition rates (Morrissey *et al.,* 2014). The moisture content may also have been higher than in a commercial composting facility due to the high rainfall (i.e., at week 8 the sand was completely saturated), increasing microbial activity and the degradation rate. Under appropriate composting conditions (e.g., in an industrial composting facility), the physical fragmentation of cellulose fibres in wet wipes can be rapid; however, molecular degradation in the environment is a much slower process due to physicochemical manufacturing properties and non-optimal breakdown conditions (Allison *et al.,* 2023). This results in both cellulose and plastic microfibres persisting in the environment (O Briain *et al.,* 2020), where they can enter the food chain with the potential to transport harmful contaminants (Kwak *et al.,* 2022). For example, microfibres from wet wipes in the River Thames, London have been linked to decreasing populations of Asian clams (McCoy *et al.,* 2020).

Despite wet wipes being treated with a number of chemical additives (e.g., malic acid, sodium hydroxide) and antimicrobial agents (e.g., sodium benzoate, benzalkonium chloride) (Salama *et al.,* 2021), *E. coli* was still able to survive and persist on all three wet wipe types. However, such chemical additives and antimicrobial agents are likely to be significantly diluted or washed off during toilet flushing and transfer through the WWTP, facilitating the persistence of *E. coli* on wet wipes as they enter the environment. In a recent survey, 88% of people said they were aware wet wipes harmed the environment, but 22% still admitted to flushing them down the toilet anyway (Water UK, 2023), resulting in 2.9 billion wet wipes entering WWTPs in the UK every year (DEFRA, 2023). Although compostable wet wipes do degrade with time, they can still persist in beach environments for up to 14 weeks. Therefore, compostable wet wipes could still pose a public health risk after washing up on beaches by acting as a localised source of *E. coli* contamination. As these wet wipes fragment and

breakdown, potential pathogens associated with them could be released into the sand where they could continue to persist long after the wet wipe has degraded (Weiskerger *et al.,* 2019).

6.5 Conclusion

We have demonstrated that wet wipes can persist in beach sand, which could pose a heightened human health risk at the beach, depending on the wet wipe material. Wet wipes continue to be popular consumer products, with 1.36 million tons being produced in 2020 (Hadley *et al,* 2023). But there continues to be frequent confusion among consumers regarding appropriate wet wipe disposal, resulting in wet wipes being incorrectly flushed down toilets and causing sewerage blockages and spills. Therefore, there is a pressing need to increase public education and awareness to prevent the incorrect disposal of wet wipes down the toilet, together with improved wastewater management and environmental regulations. Collectively, this will help to ensure that wet wipes are prevented from entering the environment, which would reduce their occurrence at beaches and the introduction of potentially harmful pathogens into the beach environment.

6. Acknowledgements

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7. Screening environmental isolates of human pathogenic *Candida* spp. colonising plastic pollution.

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Author contribution:

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Abstract:

Plastic pollution can provide an important substrate for the survival and dissemination of a range of human pathogens, and could increase potential transfer routes to humans. Recently, five species of the pathogenic yeast *Candida* have been classified as priority fungal pathogens by the WHO, yet viable pathogenic species of *Candida* have never before been isolated from environmental plastic pollution. Therefore, we tested whether plastic pollutants in freshwater, estuarine, and marine environments were colonised by *Candida*. We successfully isolated five species (*C. glabrata, C. tropicalis, C. krusei, C. sojae, C. pseudolambica*), which includes two that are on the WHO fungal priority pathogens list. All environmental isolates were resistance to at least one antifungal drug, thermotolerant to human body temperature, and in many cases more pathogenic than comparable clinical isolates. The incidence of candidiasis, particularly by drug resistant strains, is globally increasing, and it is therefore critical that we increase our focus on the environmental persistence of these pathogens, and the role of environmental plastic pollution as novel transfer routes for enhanced human exposure.

Keywords: Antifungal resistance; Environmental pollution; Fungal pathogens; Plastisphere; Pathogenic yeast.

7.1 Introduction

Plastic pollution in the environment is of major global concern, with an estimated 390 million metric tonnes of plastic being produced annually, with production predicted to double in the next 20 years (Lebreton and Andrady 2019; Plastics Europe 2022). Only a small proportion of the total plastic produced globally is recycled, which results in large quantities of plastic waste ending up in the environment (Geyer et al. 2017; Kibria et al. 2023). Plastics are effectively recalcitrant to degradation and as such plastic pollutants can negatively impact both terrestrial and aquatic ecosystems (Li et al. 2016; Kibria et al. 2023). Importantly, once in the environment plastic surfaces become rapidly colonised by microbial biofilm comprised of complex microbial communities (Zettler *et al.,* 2013). Such 'plastisphere' communities can contain (or even enrich) human pathogens, which can then be disseminated within different environmental matrices (Metcalf *et al.,* 2022). The majority of plastisphere research has focussed on prokaryotic communities, including human bacterial pathogens (Jiang *et al.,* 2018; Wright *et al.,* 2021; Metcalf *et al.,* 2022); however, eukaryotic microorganisms, including human fungal pathogens can also associate with the plastisphere (Yang *et al.,* 2020; Gkoutselis *et al.,* 2021; Ormsby *et al.,* 2023).

Approximately 150 million severe cases and 1.7 million deaths per year are caused by fungal pathogens (Kainz *et al*., 2020). One of the most important fungal infections is invasive candidiasis, caused by several species of the yeast *Candida*, with an estimated 700,000 cases annually (Bongomin *et al.,* 2017). Candidiasis also includes less severe cutaneous and mucosal infections, such as thrush. Infections due to pathogenic *Candida* species are increasing globally, due in part to the emergence of more virulent strains of *Candida* (Siscar-Lewin *et al.,* 2022), which is further compounded by the simultaneous emergence of pathogenic strains of drug-resistant *Candida* (Friedman and Schwartz, 2019; Fisher *et al.,* 2022; Parslow and Thornton, 2022). This has led the WHO to add five species of *Candida* (*C. auris, C. albicans, C. glabrata, C. parapsilosis* and *C. tropicalis*) to the fungal pathogen priority list (World Health Organization, 2023).

Understanding the source of infection and interaction of fungal pathogens within the environment is important for the effective management of fungal infections (Miranda *et al*., 2009), particularly as *Candida* can persist on plastic surfaces in healthcare settings (Estivill *et al.,* 2011; Welsh *et al.,* 2017). Most *Candida* species are non-pathogenic environmental microbes, persisting in sources such as food, water or plants (Angebault et al. 2013; Miranda et al. 2009). However, several species are potentially pathogenic, taking advantage of the opportunity to colonise humans and cause disease (Ekdahl *et al.,* 2023). *Candida* can survive the transition through wastewater treatment plants (WWTPs) (Assress *et al.,* 2021) and persist in various environmental matrices (e.g., coastal wetlands (*C.* auris), freshwater (*C.*

krusei, C. tropicalis), seawater (*C. albicans, C. tropicalis*), and soil (*C. albicans, C. tropicalis*) (Arora *et al.,* 2021; Medeiros *et al.,* 2008; Fotedar *et al.,* 2022; Wójcik et al. 2013, Sautour *et al.,* 2021). During their transition through WWTPs and the environment, pathogenic species of *Candida* could encounter and colonise environmental plastic pollution. Previous studies with *C. auris* and *C. parapsilosis* have demonstrated that these species are capable of adhering to plastics under environmental mesocosm conditions (Oliveira *et al.,* 2022; Dire *et al.,* 2023; Akinbobola *et al*., 2024); whilst ITS sequencing has shown that *Candida* spp. are present in the plastisphere (Wallbank *et al.,* 2022). Therefore, plastic pollutants could constitute an important vehicle for the dissemination of human pathogenic *Candida* in the environment with significant implications for the epidemiology and environmental management of *Candida* infections; however, viable pathogenic *Candida* are yet to be isolated from environmental plastic pollution. In this study, we have screened different types of plastic pollution in marine, estuarine, and freshwater environments for pathogenic species of *C*andida, and subsequently assessed these isolates for their thermotolerance, anti-fungal drug resistance, and their pathogenicity in a *Galleria* model of infection, in order to determine the co-pollutant risk of plastic pollutants to human health.

7.2 Materials and methods

7.2.1 Sample collection and processing

Plastic pollution was collected from six sites on three sampling days during spring 2023 (27th March, 2nd May, 26th May; Fig. 7.1; Appendix V). These sites included marine, estuarine, and freshwater beaches on east (Firth) and west (Clyde) catchments in Scotland's central belt. Two of the beaches sampled are designated bathing water beaches, regulated under the EU Bathing Water Directive (BWD) 2006/7/EC. Scotland's central belt contains several large urban centres (e.g., Glasgow, Edinburgh) and has the highest population density in Scotland (population 4.2 million). At each site plastic was collected using sterile forceps and placed into sterile ziplock bags. All samples were stored at 4°C and processed within 24 h. Plastic samples were sorted by type (cotton bud sticks, hard plastic, plastic bags and wrappers, polystyrene, wet wipes; Appendix W). Hard plastics included a variety of hard plastic fragments, including tampon applicators and plastic fragments. Cotton bud sticks and polystyrene were classed as separate categories as they could easily be distinguished from the other types of hard plastic.

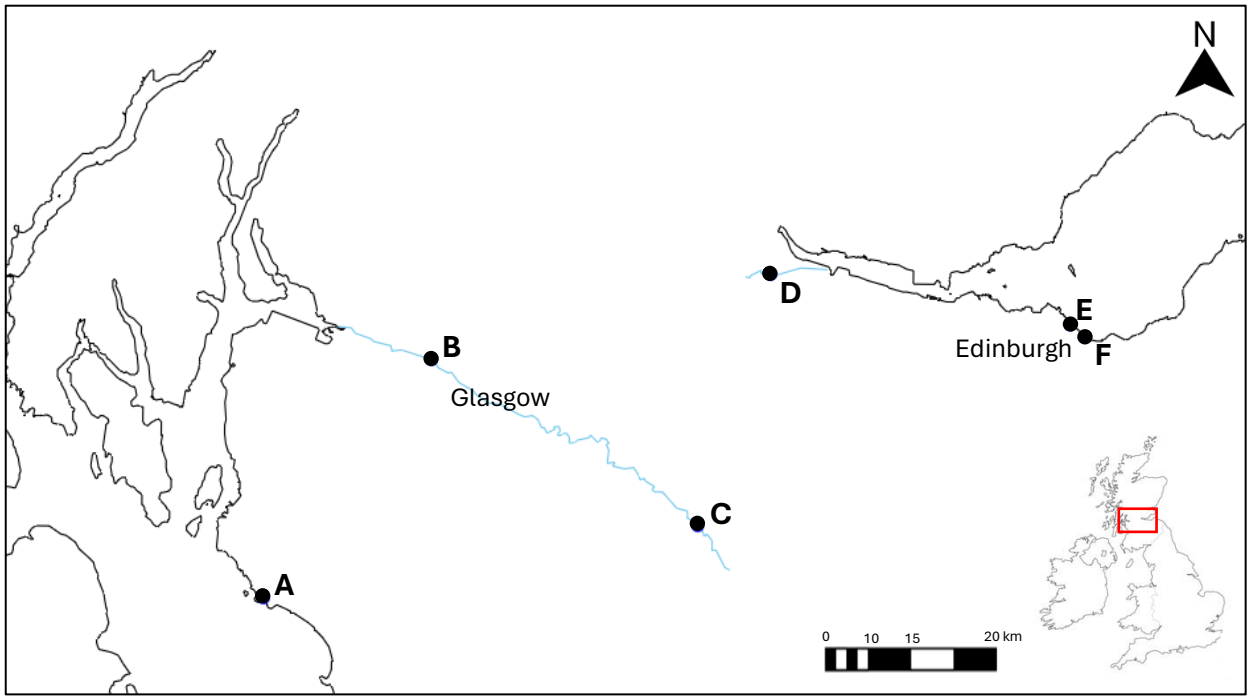

Figure 7.1. Sampling sites in central Scotland. Sampling locations (black circles) are labelled according to Appendix V.

7.2.2 Recovery of Candida spp. using selective media

Replicate composite samples of each plastic type from each site were pre-enriched in 100 ml yeast extract peptone dextrose (YPD) broth supplemented with antibiotics (gentamicin 50 mg/L, chloramphenicol 50 mg/L; Sigma-Aldrich, USA) and incubated at 30°C for 48 h in a shaking incubator (120 rpm; Incu-Shake MIDI Benchtop Shaking Incubator, SciQuip, UK). After incubation 100 ml of the overnight culture and a 1:1000 diluent of the same culture were separately spread onto sabouraud glucose agar with 50 mg/L chloramphenicol (SGA; Sigma-Aldrich, USA) supplemented with gentamicin 50 mg/L, and fluconazole at three different concentrations (0, 16, 64 mg/L). Plates were inverted and incubated for 48 h (30°C). Colonies were then selected and streaked onto CHROMagar™ *Candida* Plus agar (CHROMagar™, France) and incubated (30°C, 48 h). *Candida* species were presumptively identified based on colony colour and morphology on CHROMagar™. Twenty-seven colonies were isolated, and glycerol stocks (final concentration 40% glycerol) prepared and frozen at -20°C.

7.2.3 Characterisation of Candida isolates from environmental plastic pollution

7.2.3.1 PCR and sequencing for Candida identification

Glycerol stocks of 27 selected isolates were grown overnight in 5 ml YPD (120 rpm, 37°C, 24 h). Colony PCR was carried out with primers from Carvalho *et al.* (2007) targeting the ITS region to identify the five common pathogenic species of *Candida*, i.e., *C. albicans, C. glabrata, C. krusei, C. parapsilosis*, and *C. tropicalis*. Amplification reactions consisted of 12.5 µL multiplex master mix (New England Biolabs, UK), 1µL each of the forward and reverse primers (10 µmol/L), 2 µL of each DNA sample and 8.5 µL of sterile water in a final reaction volume of 25 µL. PCR amplification was carried out in a thermal cycler (Veriti 96-well thermal cycler, Applied Biosystems, USA) using the following cycle: 10 min initial denaturation at 94°C, followed by 40 cycles of 94°C for 15 s, 55°C for 30 s and 65°C for 45 s, with a final extension of 65°C for 10 min. All PCR products were run down a 2% agarose gel using GelRed® staining (Biotium, USA) and visualised under UV. Different amplicon sizes were used to differentiate between distinct *Candida* species: *C. albicans* (446 bp)*, C. glabrata* (839 bp)*, C. krusei* (169 bp)*, C. parapsilosis* (370 bp) and *C. tropicalis* (507 bp). Three reference clinical pathogenic strains of *C. albicans* (strain SC 5314), *C. glabrata* (strain ATCC 2001) and *C. tropicalis* (strain CAY676), were included as positive controls.

To further confirm the identity of each isolate, the ITS1 region was amplified and sequenced. Firstly, DNA was extracted using DNeasy Tissue kit (Qiagen, Germany) and eluted in 200 µL Buffer AE, and primers from Trost *et al.* (2004) used to amplify the ITS1 region (Forward primer: 5'-GTCAAACTTGGTCATTTA-3'; Reverse primer: 5'-TTCTTTTCCTCCGCTTATTG-3'). Amplification reactions consisted of 12.5 µL taq PCR master mix (Qiagen, Germany), 2 µL primer stock (10 µmol/L) and 5 µL of each DNA sample in a final reaction volume of 25 µL. PCR amplification comprised an initial denaturation step at 94°C for 3 min followed by 34 cycles of amplification (94°C for 30 s, 50°C for 30 s and 72°C for 1 min) followed by a final extension at 72°C for 10 min. PCR products were purified using a QIAquick PCR Purification kit (Qiagen, Germany), and eluted in 50 µL of elution buffer. All purified PCR products underwent Sanger sequencing using Applied Biosystems 3730 DNA analysers (DNA Sequencing and Services, Dundee, UK). Species ID was confirmed using NCBIs Basic Local Alignment Search Tool (BLAST; NCBI, USA).

7.2.3.2 Pathogenicity

To determine virulence, each isolate was introduced into a *Galleria mellonella* model of infection (Romera *et al.,* 2020). Healthy larvae of *G. mellonella* (Livefood, UK) between 2.0 to 2.5 cm in length were selected, kept in darkness at 15°C and used within one week of purchase. Glycerol stocks of each *Candida* isolate were cultured on SGA agar (37°C, 24 h), and distinct colonies selected and grown in 5 ml YPD (37°C, 24 h, 120 rpm). To ensure that cells were in their exponential growth phase when injected into *Galleria*, 1 ml overnight cultures were added to 5 ml YPD and grown to an OD_{570} of 0.7. Cells were then centrifuged (4000 rpm, 4 min) before being washed and resuspended in PBS. Groups of 10 larvae were injected with 10 µL of *Candida* cells (10^5 CFU/larvae) into the hemocoel via the last right pro-limb using a 100 µL Hamilton syringe (Bonaduz, Switzerland) with a 0.6 x 30 mm needle. All experiments were conducted in biological triplicate. Needles were flushed with ethanol followed by PBS to sterilise them between samples. An inoculation of 10 µL PBS was used as a control to account for mortality caused by physical injury or infection by contamination. Following injection, larvae were incubated at 37°C, and survival evaluated every 24 h for a total of 120 h. Larvae were considered dead when they did not respond to a touch stimulus.

7.2.3.3 Thermotolerance

To determine the thermotolerance profile, each isolate underwent an initial incubation at 18°C (simulating an environmental temperature) or 38°C (simulating human body temperature) for 24 h, before being moved to one of three different temperatures (18, 28 or 38°C) where their growth was measured. Colonies were selected from SGA plates and grown overnight in 5 ml YPD (37°C, 24 h, 120 rpm), and cells centrifuged (4000 rpm, 8 min), washed and resuspended in phosphate buffered saline (PBS). Cell concentrations were adjusted by dilution in sterile distilled water to give a final concentration of approximately 10^5 CFU/ml (PBS serial dilutions were plated on SGA agar and incubated for retrospective enumeration). Cells (20 ml) of each isolate were added to 180 ml YPD broth in 96-well plates (n = 3), and plates incubated at either 18, 28 or 38°C for 24 h. I-Button temperature logger chips (iButtonLink, WI, 176 USA) were placed into each incubator to monitor the temperature throughout incubation. Absorbance at 570 nm was measured before and after incubation in a spectrophotometer (Infinite M200 plate reader; Tecan, Switzerland) to determine the growth of the isolates.

7.2.3.4 Antifungal drug resistance

Each Candida isolate were subjected to minimum inhibitory concentration (MIC) analysis to determine antifungal resistance following the European Committee on Antimicrobial susceptibility testing (EUCAST) antifungal MIC method for yeasts (Arendrup *et al.,* 2020). Resistance to four antifungals at ten concentrations was examined: amphotericin B (0.008 – 4 mg/L), caspofungin (0.008 – 4 mg/L), fluconazole (0.125 – 64 mg/L), voriconazole (0.008 – 4 mg/L) (Thermoscientific, USA). Briefly, 96-well plates were filled with 100 µL double strength RPMI 1640 (2% glucose, Sigma-Aldrich, US) containing the different concentrations of antifungal drugs. Distinct colonies were selected from SGA plates and grown overnight in 5 ml YPD (37°C, 24 h, 120 rpm). Cells were recovered by centrifugation (4000 rpm, 8 min), washed and resuspended in PBS, and the concentration adjusted to approximately 10^5 cells/ml. Adjusted cultures (100 µL per well) of each isolate were added to 96-well plates and incubated without agitation at 35°C for 24 h. Control wells contained sterile drug-free medium, with 100 µL of the same adjusted cultures. Absorbance at 530 nm was measured in a spectrophotometer (Infinite M200 plate reader; Tecan, Switzerland) to determine growth of the isolates. The MIC of amphotericin B was determined as the lowest concentration giving rise to an inhibition of ≥90% of growth compared to the drug-free control. The MIC of caspofungin, fluconazole and voriconazole was determined as the lowest concentration giving inhibition of ≥50% growth compared to the drug-free control.

7.2.4 Statistical analysis

Statistical analysis was conducted in R Studio version 3.3.2 (R Core Team, 2016). Students t-tests and analysis of variance (ANOVA) were used to test for differences in pathogenicity, thermotolerance, and antifungal resistance. All data were tested for distribution and homogeneity of variance (Shapiro-Wilk and Levene's) before parametric tests were used. Where assumptions were not met, data were either log transformed, or non-parametric Mann-Whitney U or Kruskal Wallis tests used. Tukey's and Fisher's LSD post-hoc tests were used to compare groups. Data is reported as mean ± SE, and *P* values ≤0.05 are considered significant.

7.3 Results

7.3.1 Recovery of Candida *from environmental plastic pollution*

Five species of *Candida* (*C. glabrata, C. krusei, C. pseudolambica, C. sojae* and *C. tropicalis*) were isolated from the surfaces of plastic pollutants (Table 7.1), with three of these species (*C. glabrata, C. krusei* and *C. tropicalis*) listed on the WHO 'Fungal Priority Pathogens' list (World Health Organization, 2022). *C. glabrata* was the most commonly detected species, with 16 isolates from five different sites, which included all three environments, i.e., marine, estuarine, and freshwater. In contrast, all seven *C. tropicalis* isolates were only detected on plastics at one of the marine sites. *C. krusei* was only detected on plastic in freshwater and estuarine sites in the Clyde catchment; both these isolates had high similarity with the same accession number despite being from geographically different sites (OW988737).

7.3.2 Pathogenicity

All isolates were pathogenic towards *Galleria* larvae, although this varied between the different isolates (Fig. 7.2). At 120 h after pathogen challenge, the percentage survival of *Galleria* was significantly higher for environmental isolates compared to the PBS control (Fig. 7.2; Mann-Whitney U, $W = 64$, $p < 0.01$). However, in general, clinical strains of *Candida* demonstrated a higher level of virulence compared to the environmental isolates (Kruskal-Wallis, $H(1) = 15.05$, $p < 0.001$), killing between 80 and 93% of the *Galleria* after 120 h, with the highest mortality occurring within the first 24 h, during which the survival decreased by 37-60%. Isolate 21 (*C. tropicalis*) showed a similarly rapid die-off within the first 24 h, with a 60% reduction in larval survival. In terms of larval mortality, there was no significant difference between this environmental isolate of *C. tropicalis* and the clinical strain of *C. tropicalis* (*t*-test, $t(34) = -0.44$, $p = 0.66$), indicating that the environmental isolate was at least as pathogenic as the clinical control. Site where *Candida* had been isolated from had no significant influence on the percentage survival of challenged larvae at 120 h (ANOVA, $F_{5, 75} = 0.562$, $p = 0.729$); whilst species did have an effect on percentage survival at 120 h (ANOVA, $F_{4, 76} = 3.223$, $p < 0.05$), although the only significant difference was between *C. sojae* and *C. tropicalis* (Fisher's LSD; difference in means = 42.4%).

Table 7.1. *Candida* isolates from environmental plastic pollution.

Isolate number	Site	Site type	Species	% Identity	GenBank Accession Number of closest match	Plastic type
1	A	Marine	*C. glabrata*	100	OW988792	Soft plastic
2	B	Estuarine	*C. glabrata*	99.11	ON016558	Soft plastic
3	B	Estuarine	*C. glabrata*	95.41	ON391970	Polystyrene
4	B	Estuarine	*C. glabrata*	99.33	LC317501	Hard plastic
5	B	Estuarine	*C. glabrata*	95.20	MG560156	Hard plastic
6	B	Estuarine	*C. glabrata*	97.10	KX008750	Soft plastic
7	C	Freshwater	*C. glabrata*	98.80	OP876825	Soft plastic
8	C	Freshwater	*C. glabrata*	99.90	OP850582	Soft plastic
9	C	Freshwater	*C. glabrata*	99.10	OP850582	Hard plastic
10	C	Freshwater	*C. glabrata*	99.80	OP850582	Hard plastic
11	C	Freshwater	*C. glabrata*	99.00	NR_130691	Soft plastic
12	C	Freshwater	*C. glabrata*	95.60	KU987871	Soft plastic
13	C	Freshwater	*C. glabrata*	99.60	MF033154	Hard plastic
14	D	Freshwater	*C. glabrata*	95.22	MF187236	Soft plastic
15	E	Marine	*C. glabrata*	94.58	MZ255116	Soft plastic
16	E	Marine	*C. glabrata*	99.20	OW988778	Soft plastic
17	B	Estuarine	*C. krusei*	99.80	OW988737	Hard plastic
18	C	Freshwater	*C. krusei*	98.30	OW988737	Soft plastic

19	C	Freshwater	*C. pseudolambica*	95.80	MW895903	Soft plastic
20	B	Estuarine	*C. sojae*	100	NR_137087	Hard plastic
21	E	Marine	*C. tropicalis*	100	MK748468	Wet wipes
22	E	Marine	*C. tropicalis*	99.80	JKY102470	Wet wipes
23	E	Marine	*C. tropicalis*	97.20	OP627182	Hard plastic
24	E	Marine	*C. tropicalis*	99.50	MZ648456	Hard plastic
25	E	Marine	*C. tropicalis*	98.80	MH628218	Hard plastic
26	F	Marine	*C. tropicalis*	99.60	OW986301	Hard plastic
27	F	Marine	*C. tropicalis*	99.44	OW986301	Hard plastic

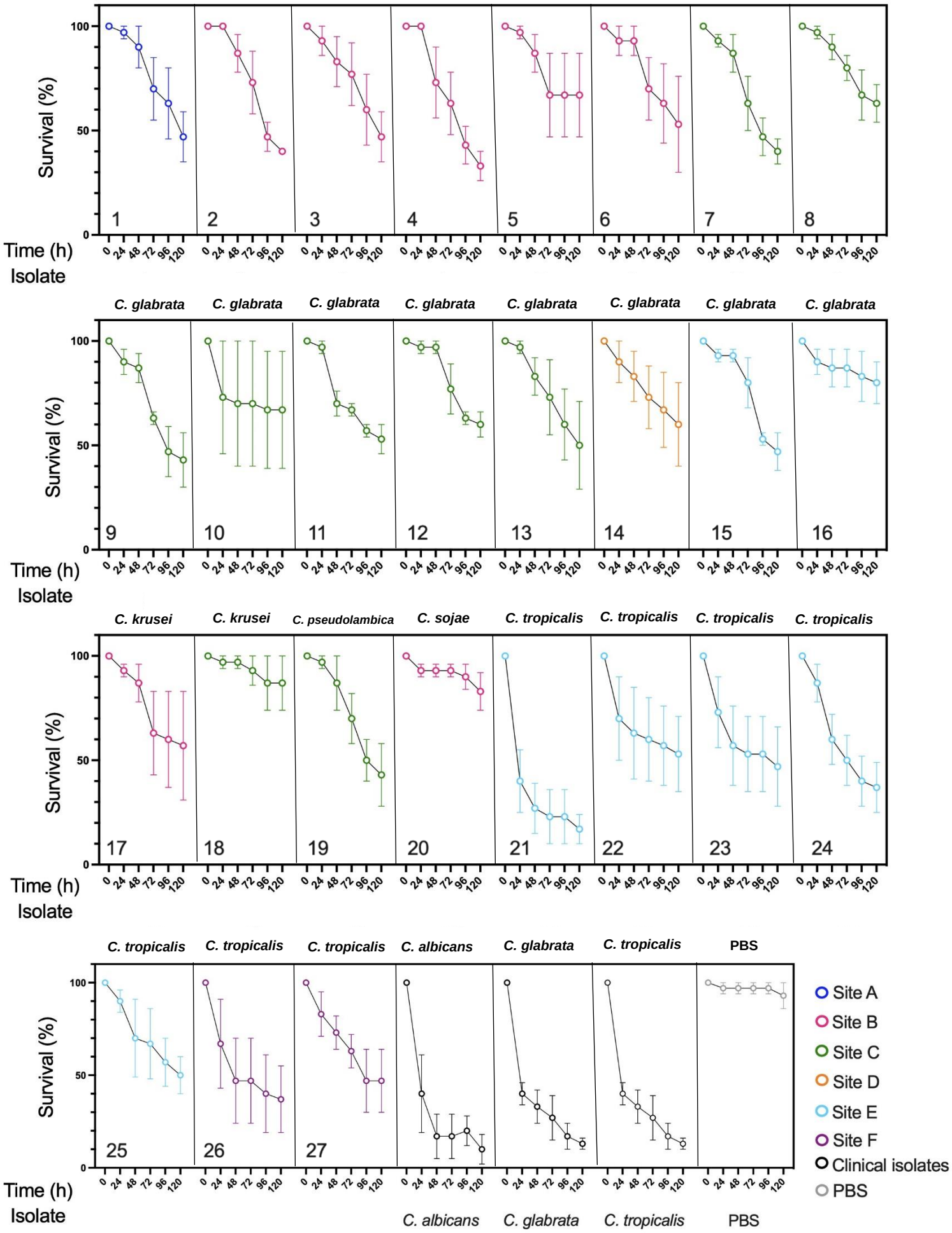

Figure 7.2. Virulence of *Candida* isolates from environmental plastic pollution at different sites (represented by the different colours) in a *Galleria mellonella* infection model. Clinical isolates (black) and a PBS control (grey) are also included. Data points (*n* = ten *G. mellonella* larvae) represent the mean of three independent biological replicates ± SE.

7.3.3 Thermotolerance

Growth of environmental isolates of *Candida* was significantly affected by temperature (ANOVA, $F_{2, 177} = 516.9$, $p < 0.001$), with growth at 28 and 38°C significantly higher than at 18°C (Fig. 7.3). Environmental isolates had the lowest levels of growth at 18°C when they had been initially incubated at 38°C (Fig. 7.3D), whereas acclimatisation at 18°C allowed these isolates to grow better (Fig. 7.3A). In general, there was a significant effect on the rate of growth between *Candida* species (ANOVA, $F_{5, 174} = 2.401$, $p < 0.05$); however, the only significant difference in growth was between *C. glabrata* and *C. pseudolambica* (Fisher's LSD; difference in means = 0.48). Two isolates (e.g., 19, *C. pseudolambica*; and 20, *C. sojae*) had low levels of growth at 38°C (regardless of the pre-incubation temperature suggesting that these species have a lower optimal growth temperature than the other *Candida* species, which are known to be human pathogens. In contrast, growth of *C. krusei* (i.e., isolates 17 and 18) at 38°C following an initial incubation at 18°C had the highest levels of growth, suggesting that these environmental isolates could successfully acclimate to the temperature of the human body. The nature of the site where *Candida* were isolated from had no significant influence on their growth profile or thermotolerance (ANOVA, $F_{6, 173} = 0.109$, $p = 0.995$).

7.3.4 Antifungal resistance

All environmental isolates of *Candida* showed some level of resistance to at least one of the four antifungals tested (amphotericin B, caspofungin, fluconazole, voriconazole), with a single isolate of *C. glabrata* having high levels of resistance to all four antifungals (Fig. 7.4). With the exception of *C. pseudolambica* (isolate 19), all of the environmental *Candida* isolates had comparable, or an increased, resistance to amphotericin B ($\geq$ 0.5 mg/L) compared to the clinical strains (Fig 7.4A). Most environmental isolates (and the clinical controls) were susceptible to caspofungin, being unable to survive concentrations higher than 0.031 mg/L; environmental isolates resistant to caspofungin included *C. glabrata* and *C. krusei* (Fig 7.4B). Clinical isolates of *C. glabrata* and *C. tropicalis* were able to grow at the highest concentration of fluconazole tested (64 mg/L), and resistance among the environmental isolates was also fairly high (Fig 7.4C). However, environmental isolates of *C. tropicalis* expressed a range of resistance profiles to fluconazole, i.e., three of the seven isolates were resistant to concentrations of 64 mg/L, whilst the other four isolates were only resistant to concentrations between 0.8 and 2.3 mg/L (Fig 7.4C). A similar pattern was observed for voriconazole, where six of the seven *C. tropicalis* isolates were highly susceptible (Fig 7.4D). Isolates of *C. tropicalis* (21 and 22) which showed high levels of

resistance to fluconazole were both isolated from wet wipes, whilst the other strains of *C. tropicalis* were isolated from hard plastic (Table 7.1). Overall, the species type and location of site had no significant influence on the MIC (ANOVA, Species: $F_{5, 354} = 1.286$, $p = 0.269$; Site: $F_{6, 353} = 0.428$, $p = 0.86$).

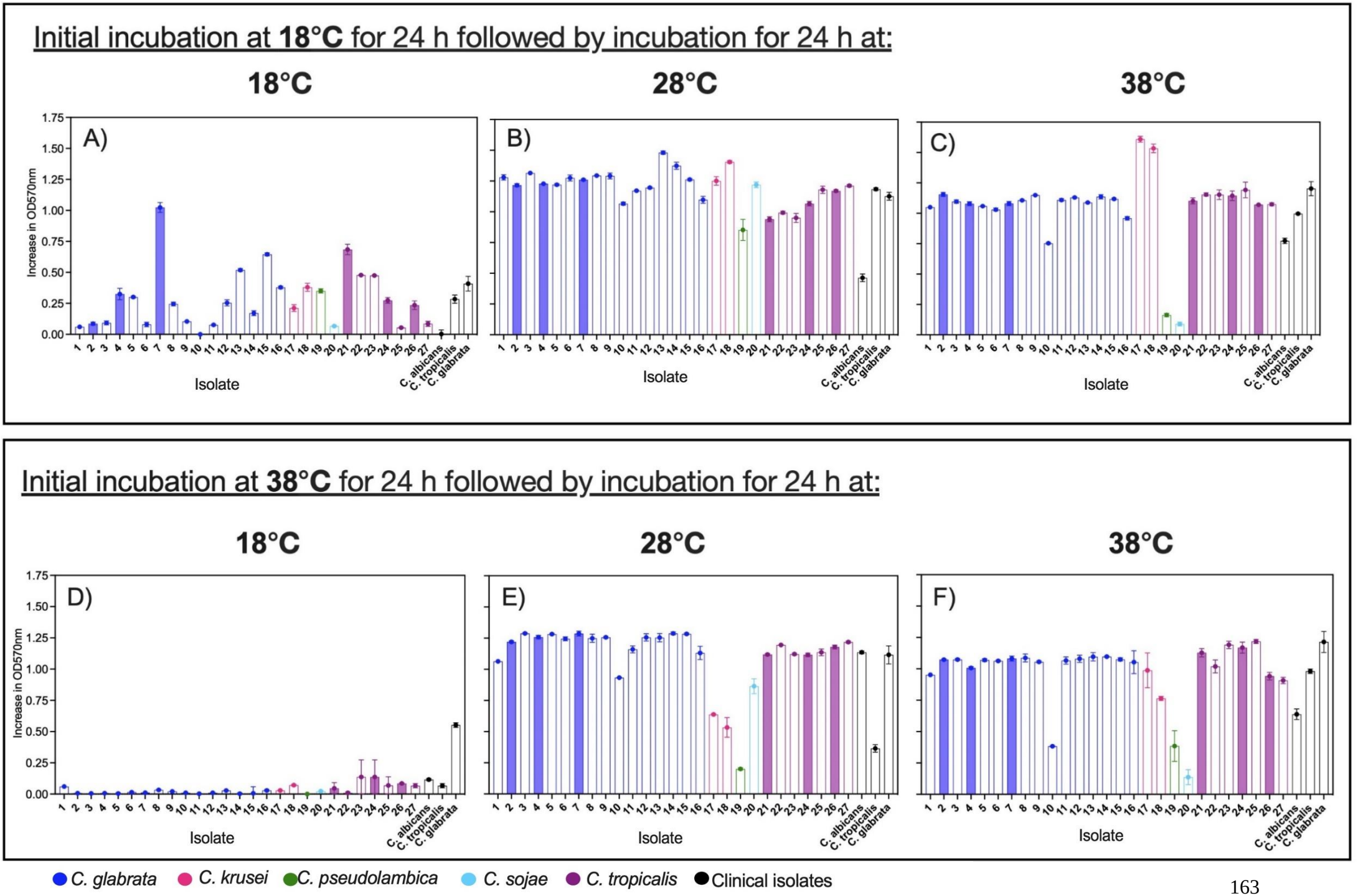

Figure 7.3. Thermotolerance of *Candida* isolates from environmental plastic pollution. Isolates were firstly incubated for 24 h at 18°C (top plots) or 38°C (bottom plots), before subsequent incubation for 24 h at 18°C (A, D), 28°C (B, E) and 38°C (C, F). The different coloured bars represent the different species and clinical controls. The filled bars represent the most virulent isolates (Table 7.2).

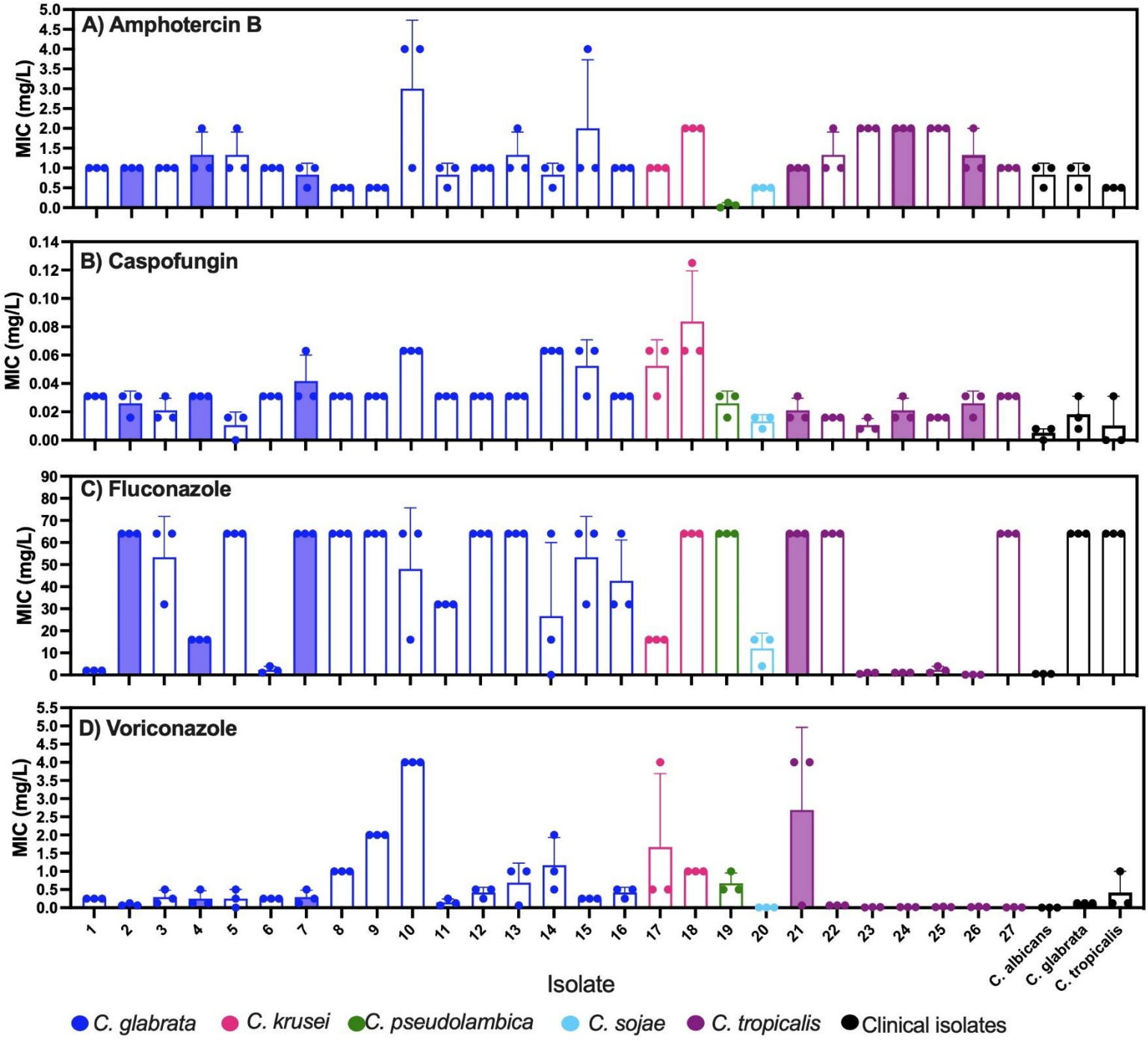

Figure 7.4. Minimum inhibitory concentration (MIC) of *Candida* isolates from environmental plastic pollution. four antifungals were tested at different concentrations: A) amphotericin B (0.008-4 mg/L), B) caspofungin (0.008-4 mg/L), C) fluconazole (0.125-64 mg/L), D) voriconazole (0.008-4 mg/L). The different coloured bars represent the different species and clinical controls. The filled bars represent the most virulent isolates (Table 7.2). There were three replicates per isolate, error bars indicate the range.

7.3.5 Characterisation of the most virulent isolates

C. tropicalis and *C. glabrata* were the most virulent environmental isolates (percentage survival of *Galleria* at 120 h ≤40%; Table 7.2); however, at 120 h the percentage of dead larvae was consistently higher when challenged with the clinical *Candida* isolates (>93% for the clinical isolates, >83% for the environmental isolates; Table 7.2). The most virulent isolates showed varying levels of growth when incubated at different temperatures compared to the clinical controls (Table 7.2; Fig. 7.3). Isolate 7 had the highest growth at 18°C after an initial incubation at 18°C; this isolate was from a different site type (freshwater) compared to the other most virulent isolates. The clinical *C. tropicalis* control also had reduced growth at 38°C, after an initial incubation at 38°C, suggesting that this temperature is above the optimal growth temperature for this species. In contrast, the most virulent environmental isolates of *C. tropicalis* (21, 26 and 24) had higher rates of growth (between 0.942 and 1.169), indicating that these environmental isolates were more tolerant to this temperature. The most virulent isolates also had increased anti-fungal resistance compared to the clinical controls, indicating that these isolates would be more difficult to treat once they have initiated an infection. Increased resistance to amphotericin B and caspofungin compared to the clinical controls was observed for all six of the most virulent isolates. Isolates 21 and 4 also showed increased resistance to voriconazole compared to the clinical controls. There were high levels of resistance to fluconazole in both the clinical controls and the environmental isolates, with three of the most virulent isolates able to grow at the highest fluconazole concentration tested (64 mg/L).

Table 7.2. Most virulent *Candida* isolates from environmental plastic pollution (percentage survival of *galleria* at 120 h ≤40%). Clinical control isolates are also shown.

Isolate	Species	Site	Site type	Virulence (% survival at 120 h)	Mean growth* after incubation at 18°C for 24 h then incubation at:			Mean growth* after incubation at 38°C for 24 h then incubation at:			MIC (mg/L)			
					18°C	28°C	38°C	18°C	28°C	38°C	Fluconazole	Amphotericin B	Caspofungin	Voriconazole
21	*C. tropicalis*	E	Marine	17	0.685	0.939	1.102	0.046	1.119	1.128	64	1.00	0.021	2.690
4	*C. glabrata*	B	Estuarine	33	0.325	1.228	1.079	0.007	1.258	1.007	16	1.33	0.031	0.250
26	*C. tropicalis*	F	Marine	37	0.236	1.170	1.070	0.019	1.179	0.942	1	1.00	0.026	0.021
24	*C. tropicalis*	E	Marine	37	0.274	1.066	1.144	0.137	1.116	1.169	1	2.00	0.021	0.016
7	*C. glabrata*	C	Freshwater	40	1.024	1.261	1.080	0.011	1.286	1.082	64	0.83	0.041	0.290
2	*C. glabrata*	B	Estuarine	40	0.086	1.216	1.155	0.007	1.220	1.073	64	1.00	0.026	0.086
Clinical	*C. albicans*		-	7	0.003	0.463	0.773	0.067	0.365	0.981	0.5	0.83	0.005	< 0.008
Clinical	*C. glabrata*		-	20	0.409	1.126	1.203	0.552	1.116	1.214	64	0.83	0.018	0.125
Clinical	*C. tropicalis*		-	13	0.284	1.184	0.997	0.116	1.136	0.638	64	0.50	0.010	0.410

* Growth was measured as an increase in OD 570nm

7.4 Discussion

This study is the first to identify viable pathogenic *Candida* spp. on environmental plastic pollution washed up onto beaches. The five species detected (*C. glabrata, C. krusei, C. pseudolambica, C. sojae* and *C. tropicalis*) were all pathogenic, thermotolerant and resistant to at least one antifungal drug, which suggests that plastic pollution could poses a significant additional risk to public health at beach environments.

7.4.1 Candida *can colonise plastic pollution in marine, estuarine, and freshwater environments*

C. glabrata is commonly found in seawater (Chen *et al.,* 2009), freshwater (Brandao *et al.,* 2010) and hypersaline waters (Butinar *et al.,* 2005), and in this study was detected on the surfaces of plastic in all three environments, suggesting that *C. glabrata* is highly adaptable to environmental stress. In contrast, *C. tropicalis* was only detected on plastic pollution at the marine sites. Although *C. tropicalis* has previously been detected in freshwater, this species is known to be osmotolerant and able to survive at high salinities (Zuza-Alves *et al.,* 2017). The protective environment afforded by the plastisphere can increase tolerance to environmental stressors and facilitate pathogen survival as plastics move through the freshwater-marine continuum (Metcalf *et al.,* 2023).

Two of the *C. tropicalis* isolates and the two *C. krusei* isolates had high similarity to accessions of strains originally isolated from a human source. *Candida* are one of the most dominant genera in WWTPs (Assress *et al.,* 2019), and it is here that they can come into contact with plastic pollutants (e.g., wet wipes) before being released into the environment. *C. tropicalis* isolated from wet wipes had higher levels of antifungal resistance compared to isolates from the other plastic types, and hospital effluents may provide an additional risk of releasing drug-resistant species of pathogenic *Candida*, e.g., *C. auris,* into the environment (Mataraci-Kara *et al.,* 2020). In a clinical setting, the potential for biofilm formation by *Candida* on plastic surfaces can be influenced by the polymer type (Estivill *et al.,* 2011); however, in this study, we found that the type of plastic that environmental isolates of *Candida* were colonising had no influence on their subsequent virulence, thermotolerance, or drug resistance of the *Candida* isolates.

7.4.2 Virulence of environmental isolates of Candida

C. glabrata and *C. tropicalis* were the most virulent species isolated from environmental plastic pollution. *C. glabrata* is considered the second most pathogenic species, with invasive candidiasis caused by *C. glabrata* causing 20 – 50% mortality at 30 days (World Health Organisation, 2022). *C. tropicalis* is also highly pathogenic, responsible for 7% of candidiasis infections, with mortality as high as 60% in adults (Turner and Butler, 2014; World Health Organisation, 2022). Importantly, *C. albicans* which is responsible for 65% of candidiasis infections and considered the most pathogenic species of *Candida* (Turner and Butler, 2014), was not detected in this study. *C. albicans* is a common member of the human microbiota but survival on surfaces outside of the human body is limited compared to other *Candida* species (Wißmann *et al.,* 2021). Globally, the proportion of *C. albicans* infections is decreasing in parallel with an increasing incidence of *C. glabrata* and *C. tropicalis* (Berkow and Lockhart, 2017). At a time when global research is primarily focused on *C. albicans* it is also important that we consider the environmental reservoirs and life-cycles of non-albicans species of pathogenic *Candida*.

C. sojae is an emerging pathogen; despite its physiological similarities to *C. albicans* and *C. tropicalis*, it was only in 2022 that the first human infection was reported, maybe as a result of being previously misidentified (Abdel-Hag *et al.,* 2022; Chrenkova *et al.,* 2022). However, in this study *C. sojae* had lower levels of virulence and antifungal resistance compared to the other species, implying that this species presents a lower risk to public health. Although there are currently no reports of *C. pseudolambica* causing human infection, environmental isolates did have high levels of virulence in the *G. mellonella* model. This species is also closely related to the pathogenic *C. krusei* (Ebadi *et al.,* 2022), indicating that there is the evolutionary potential for this species to emerge as a human pathogen.

Adhesion strategies are widely recognised as major virulence factors in pathogenic *Candida* species, with several *Candida* species possessing GPI-modified cell wall proteins (Sundstrom, 2002). The degree of adhesion varies between species, with *C. tropicalis* and *C. krusei* demonstrating greater adhesion than *C. glabrata* (Modrzewska *et al.,* 2015). In this study, we have quantified virulence of *Candida* isolates in the *G. mellonella* model; however, whether biofilm formation on environmental plastic surfaces increases the expression of adhesion factors of environmental isolates of *Candida* and whether this influences subsequent mechanisms of pathogenicity is unknown. Understanding colonisation of plastic surfaces, and the influence of the plastisphere for altering virulence and drug

resistance profiles of fungal pathogens is urgently needed to more fully quantify the potential human health risks of plastic pollution (Ormsby et al., 2023; Ekdahl et al., 2023).

7.4.3 Thermotolerance of Candida *isolates*

Human body temperature (37°C) is a potent nonspecific defence against fungal pathogens, above which fungi are unable to grow and establish an infection. Robert *et al.,* (2009) analysed 4,802 fungal strains and showed how every 1°C increase in the 30°C-40°C range prevented an additional 6% of fungal isolates from growing. However, we found that several environmental isolates of *Candida* could grow at 38°C, indicating that they are more likely to cause human infection. After an initial incubation at 18°C before being moved to 38°C (representing the movement of *Candida* from the environment to the human body), most isolates showed high growth. *C. krusei* showed the highest growth rates, indicating that they were able to acclimate quickly to temperature changes. Liu *et al.,* (2005) showed how *C. krusei* could survive heat shock up to 45°C by shifting the metabolic pathways from glycerol to trehalose synthesis. Similarly, *C. glabrata* is able to tolerate high temperatures due to changes in the calcineurin pathways (Chen *et al.,* 2012). Some factors enabling thermotolerance (e.g., heat-shock protein 90 and calcineurin) can also enhance antifungal resistance (Berman and Krysan, 2020).

C. pseudolambica and *C. sojae* showed less growth at 38°C, and both species have lower maximum growth temperatures (below 40°C) compared to other *Candida* species (Nakase *et al,* 1994; Ebadi *et al.,* 2022). There are only a handful of cases of *C. sojae* causing human infection (Abdel-Hag *et al.,* 2022; Chrenkova *et al.,* 2022), and *C. pseudolambica* is yet to be reported causing human infection, indicating that temperature may be the main barrier against infection by these two species. Less growth was seen when isolates were initially incubated at 38°C before incubation at 18°C (representing the movement of *Candida* from the human body to the environment), indicating lower levels of survival in the environment. However, the protective environment provided by the plastisphere may increase survival of fungi at these temperatures (Lacerda *et al.,* 2020). For all environmental isolates, growth at 28°C was higher, indicating that in warmer locations *Candida* is more likely to survive the transition from the human body to the environment.

7.4.4 Antifungal resistance of Candida *isolates*

Globally, pathogenic fungi are evolving resistance to all licensed antifungal drugs (Fisher *et al.,* 2022). Azoles, especially fluconazole, are the most widely used antifungals because of their high effectiveness, low toxicity and ability to be taken orally (Partha *et al.,* 2022). However, the environmental isolates screened in this study had high levels of resistance to fluconazole, with several isolates resistant to concentrations of 64 mg/L. Despite *C. albicans* being fairly susceptible to fluconazole, other species of *Candida* such as *C. tropicalis* and *C. glabrata* have relatively high rates of fluconazole resistance (Hendrickson *et al.,* 2019). For example, in South Korea fluconazole resistance in *C. glabrata* increased from 0 to 8.3% between 2008 and 2018 (Won *et al.,* 2021), whereas *C. auris* can be highly drug resistant, with 93% of isolates resistant to fluconazole (Lockhart *et al,* 2017). Although yet to be isolated from environmental plastic pollution, *C. auris* has already been identified in WWTP effluents (Barber *et al.,* 2023), and can persist and remain pathogenic in the plastisphere in simulated environments (Akinbobola *et al.,* 2024).

Voriconazole is an alternative azole, used to treat candidiasis and fluconazole resistant strains of *Candida*; however, there is concern that voriconazole resistance can emerge following exposure to fluconazole, particularly in cases involving *C. glabrata* (Pfaller *et al.,* 2011). Polyenes and echinocandins are more recently developed antifungals, with higher treatment success rates than azoles (Tashiro *et al.,* 2020). Despite its toxic potential and common side-effects (e.g., nephrotoxicity), amphotericin B is an effective antifungal treatment against progressive and drug-resistant fungal infections with low resistances rates compared to azoles and other antifungals (Cavassin *et al.,* 2021). However, resistance to all anti-fungal drugs is increasing in *Candida*, with evidence of an increase in multi-drug resistance (Khan *et al.,* 2020; Kilburn *et al.,* 2022). There are also eco-evolutionary links between environmental and clinical resistance due to the increasing use of agricultural fungicides (particularly azoles), which have been hypothesises to drive increased resistance of *Candida* to antifungal drugs in the clinic (Fisher *et al.,* 2022). Although in this study, it was impossible to quantify how long these *Candida* isolates had been in the environment, several isolates did have greater levels of antifungal drug resistance than clinical isolates, which may have been caused by persistent low-level exposure to agricultural fungicides or antifungal drugs commonly found in wastewater.

The antifungal drug caspofungin is often only used to treat serious fungal infections in critically ill patients on intensive care units, and most environmental isolates in this study were susceptible to

caspofungin, corresponding with the globally low levels of caspofungin resistance (Pfaller *et al.,* 2006); however, in general, environmental isolates of *Candida* in this study did show a slightly higher level of resistance to caspofungin compared to the clinical isolates, and therefore a greater understanding of how drug resistance in strains of *Candida* is affected by the plastisphere and the environment is urgently required (Ormsby et al., 2023).

7.4.5 Horizontal gene transfer

Increased AMR of bacterial pathogens is common in the plastisphere, for example, through horizontal gene transfer (HGT), which can selectively enrich for antibiotic resistance genes (ARGs) in the plastisphere (Liu *et al.,* 2021). HGT is less common in eukaryotes, although the transfer of chromosomal azole resistance has been observed in *Aspergillus fumigatus* (Morogovsky *et al.,* 2022). There is also evidence for interkingdom HGT occurring between bacteria and *Candida parapsilosis* (Fitzpatrick *et al.,* 2008), indicating that ARGs and mobile genetic elements (e.g., plasmids, insertion sequences) have the potential to be shared between members of the plastisphere community including pathogenic strains of *Candida*.

7.5 Conclusion

Species of *Candida*, including several high priority species on the WHO 'Fungal priority list', were isolated from environmental plastic pollution from marine, estuarine, and freshwater beaches. Most of these isolates were virulent, thermotolerant, and drug resistant, and in many cases more pathogenic than comparable clinical isolates. This highlights the potential public health risk at the beach and the now urgent need to improve public awareness, monitoring, and environmental management to prevent human exposure to plastic pollution colonised by *Candida*. With the proportion of global infections caused by non-albicans species of *Candida* increasing in prevalence (Berkow and Lockhart, 2017), it is vital that we increase our focus on these emerging and recently emerged pathogens and continue to improve our understanding on the transfer routes to humans through the colonisation of environmental plastic pollution.

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8. Policy Brief: The Hidden Risks of Beach Plastic Pollution

Policy brief compiled for circulation to health professionsls, policy makers and environmental regulators.

Author contribution:

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- **Davey Jones, David Oliver, David Thomas, Joseph Christie, Peter Robins, Sabine Matallana-Surget:** Writing – review & editing.
- **Richard S. Quilliam:** Conceptualisation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

The hidden risks of beach plastic pollution

Plastic debris on beaches is unsightly and can also pose a human health risk. This policy brief summarises the sources and human exposure routes of plastic pollution in the environment, as well as highlighting the hidden risks associated with beach plastic pollution through their colonisation by disease causing organisms.

Overview

- Plastic pollution, including wet wipes and microplastic beads and fibres, continue to pollute our beaches.
- This plastic pollution is colonised by microorganisms including harmful bacterial, fungal and viral pathogens.
- Pathogens colonising plastics can survive the downstream transfer from freshwater to seawater, moving pathogens into areas where they are more likely to come into contact with humans, such as on beaches or in recreational waters.
- Potential pathogens colonising plastics can be virulent, antimicrobial resistant and thermotolerant and therefore, pose a heightened risk to human health at the beach.

Background

Plastics are extremely versatile, being used for a diverse array of products and applications. This has resulted in an increase in the global production of plastics, from 35 million tonnes in 1950 to 390 million tonnes in 2021[1]. However, less than a 10% of plastics are recycled globally[2]. This results in large amounts of plastic continuously being released into the environment, and due to their longevity, they can accumulate in both terrestrial and aquatic ecosystems. Estuaries in particular act as a temporary sink for plastics for days to weeks depending on plastic type and subtle compounding hydrodynamics that are poorly characterised. Plastic pollutants can negatively impact ecosystems and can become rapidly colonised by microbial biofilms known as 'plastisphere' communities[3]. Most microbes are harmless to humans, fulfilling a variety of important ecological functions, including recycling organic matter and digesting food in animal guts. However, pathogenic microbes can be harmful to humans and cause disease. Plastisphere communities can contain or even enrich human pathogens, which can then be dispersed within environments and pose a risk to human health[4].

Sources of plastics

- Plastics include a diversity of polymer types, colour, shapes and sizes.
- Plastics enter the environment through a variety of pathways (Fig. 8.1): Most plastic pollution originates from terrestrial sources, whilst 20% is from marine sources[5].
- Plastic pollution can be directly released into the environment from littering, accidental spills and inappropriate waste management. Other sources include wastewater treatment plants (WWTPs), industrial activity and shipping.
- Once in the aquatic environment plastics can wash up onto beaches; 77% of beached litter is plastic[6] (Fig. 8.2). Two of the most commonly found types of plastic pollution on beaches are microplastics and wet wipes.

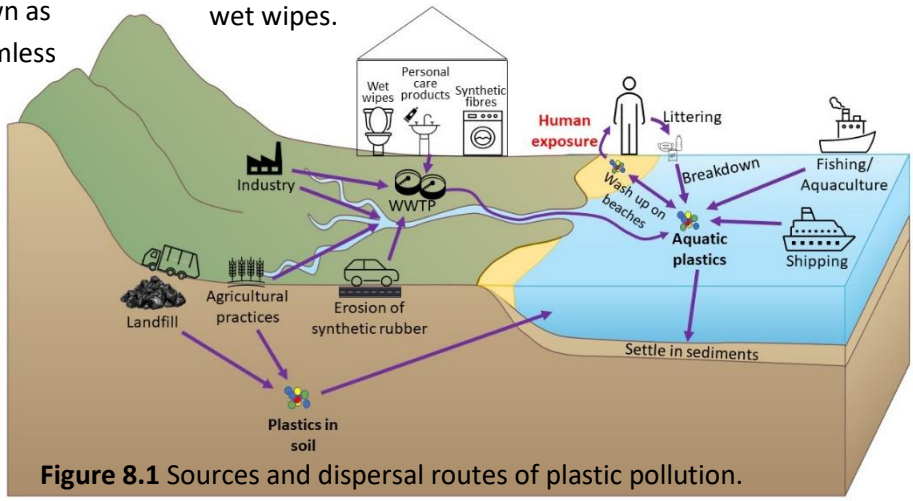

Figure 8.1 Sources and dispersal routes of plastic pollution.
WWTP: Wastewater treatment plant

Microplastics

Microplastics are the most abundant type of plastic pollution in the marine environment[7]. They can either be manufactured (e.g., virgin preproduction microplastic beads, and beads used in consumer care products and clothes fibres), or are derived from the degradation and fragmentation of larger items of plastic pollution. Virgin pre-production microplastic beads are produced by the petrochemical industry for use as the raw material in the production of many thermally moulded plastic products. During manufacturing or other processes in the plastics supply chain (e.g., transport), microplastic beads can be spilled and released into the environment[8]. Microplastics are also released from WWTPs; although WWTPs capture between 60 and 99.9% of plastics in biosolids, they are not designed to retain microplastics and a significant proportion are still released into the environment in sludge and effluent[9].

Wet wipes

Reports of illegal sewage discharges and combined sewer overflow (CSO) events are becoming more common in the UK. The disposal of wet wipes in toilets causes sewerage blockages, that also lead to an increase in CSO discharges[10]. Wet wipes are responsible for 75% of sewerage blockages which, in the UK costs around £100 million a year to clean up[10]. Collectively, this results in an increasing number of wet wipes washing up onto our beaches. A recent survey reported 63 wet wipes for every 100 m of beach in Scotland, a 150% increase compared with 2021[11]. Along with wet wipes, other sewage-associated plastic waste, including cotton bud sticks and sanitary products (e.g., sanitary pads and tampon applicators), are also being discharged directly into the aquatic environment and washing up onto British beaches.

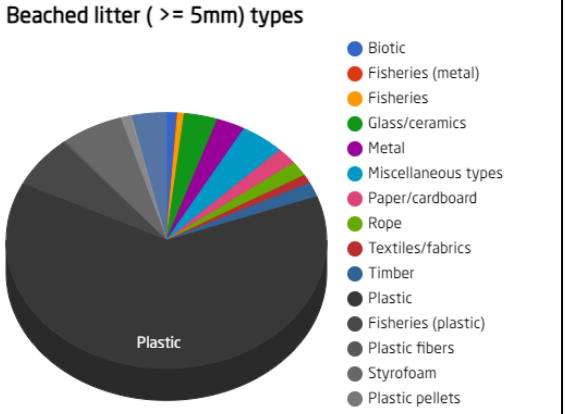

Figure 8.2 Composition of beached litter (Source[6]).

Human exposure routes

Human exposure to environmental plastic pollution is inevitable due to its ubiquity in the environment[12]. There are several routes of exposure which can lead to the transfer of pathogens on plastics. Beaches are one of the main human exposure routes due to recreational use of beaches occurring year-round (e.g., swimming, walking, playing). Coastal beaches have long been used for recreation, whilst recreation along rivers and estuaries is increasing, especially with the implementation of designated bathing rivers. As plastics increase in prevalence, there are increasing opportunities for humans to come into physical contact with them and any pathogens on their surfaces to be transferred and cause disease[13].

Pathogens colonise plastic pollution

Human pathogens have been identified within the plastisphere of several plastic polymers[4] (Fig. 8.3). Recent research has focused on identifying pathogens in the plastisphere, and understanding their survival dynamics, pathogenicity and subsequent risk to human health. The main groups of pathogens which have been detected on plastics are bacteria, fungi and viruses.

Bacteria

- *Escherichia coli* and *Enterococcus faecalis* are faecal indicator organisms (FIOs), which are the key monitoring parameters used for regulating and classifying microbial bathing water quality. Both have been frequently identified in the plastisphere in numerous environments[4].
- *E. coli, E. faecalis* and *Pseudomonas aeruginosa* survived on the surfaces of microplastic beads for at least 25 days in freshwater or seawater[14]. During this time plastics can be transported into areas with increased human exposure. These pathogens also survived in the plastisphere when moving downstream between different environmental matrices.
- Microplastic beads from Scottish beaches were colonised by a variety of bacterial pathogens, including *Klebsiella, Vibrio* spp. and *Salmonella*[15].
    - Although concentrations were low, some bacteria only have a small infectious dose (e.g., certain strains of *E. coli* have an infectious dose of only 10 cells[16]).
- Pathogens were more often associated with sewage-associated plastic waste (wet wipes and cotton bud

sticks) than with seaweed, suggesting that plastics pose a higher risk than natural materials at the beach[17].

- *E. coli* persisted on wet wipes in beach sand for 15 weeks, providing numerous opportunities for human exposure and pathogen transfer during this time[18].

Fungi

- Multi-drug resistant *Candida auris* persisted for 30 days on microplastic beads in freshwater, seawater and on the surfaces of sand[19].
- Several pathogenic *Candida* species were detected on plastic pollution from freshwater, estuarine and marine beaches[18].

Viruses

- Multiple viral pathogens have been detected on the surfaces of plastics, including norovirus[20].
- Enteric viruses, including rotavirus, were more stable and had enhanced survival in the plastisphere compared to in the water[21].

Pathogens colonising plastic pollution pose a risk to human health.

Pathogen presence does not guarantee expression of virulence genes and a cause of disease. However, recent research has shown how pathogens colonising plastics possess and express genes that enable them to successfully cause infection and provide resistance against antimicrobial treatments.

Virulence

- *Klebsiella* isolated from microplastic beads from a public beach possessed virulence genes which were expressed in a *Galleria mellonella* model of infection[15].
- *Candida* isolated from plastic pollution at the beach expressed virulence in a *Galleria mellonella* model of infection[22].

Antimicrobial Resistance

- Antimicrobial resistance is an emerging crisis worldwide, causing serious issues to successful prevention and treatment of disease. For example, in 2019, nearly 5 million deaths were associated with bacterial antimicrobial resistance[23]. Multiple examples of antimicrobial resistance in plastisphere pathogens have now been demonstrated.
- Bacteria colonising wet wipes showed evidence of antimicrobial resistance to common antibiotics including ampicillin. Resistance to multiple antibiotics was seen in several isolates and there were cases of increased resistance in isolates from wet wipes compared to from seaweed[17].
- *Klebsiella* isolated from beach microplastic beads had antimicrobial resistance genes, providing resistance against common antibiotics such as amoxicillin and chloramphenicol[15].

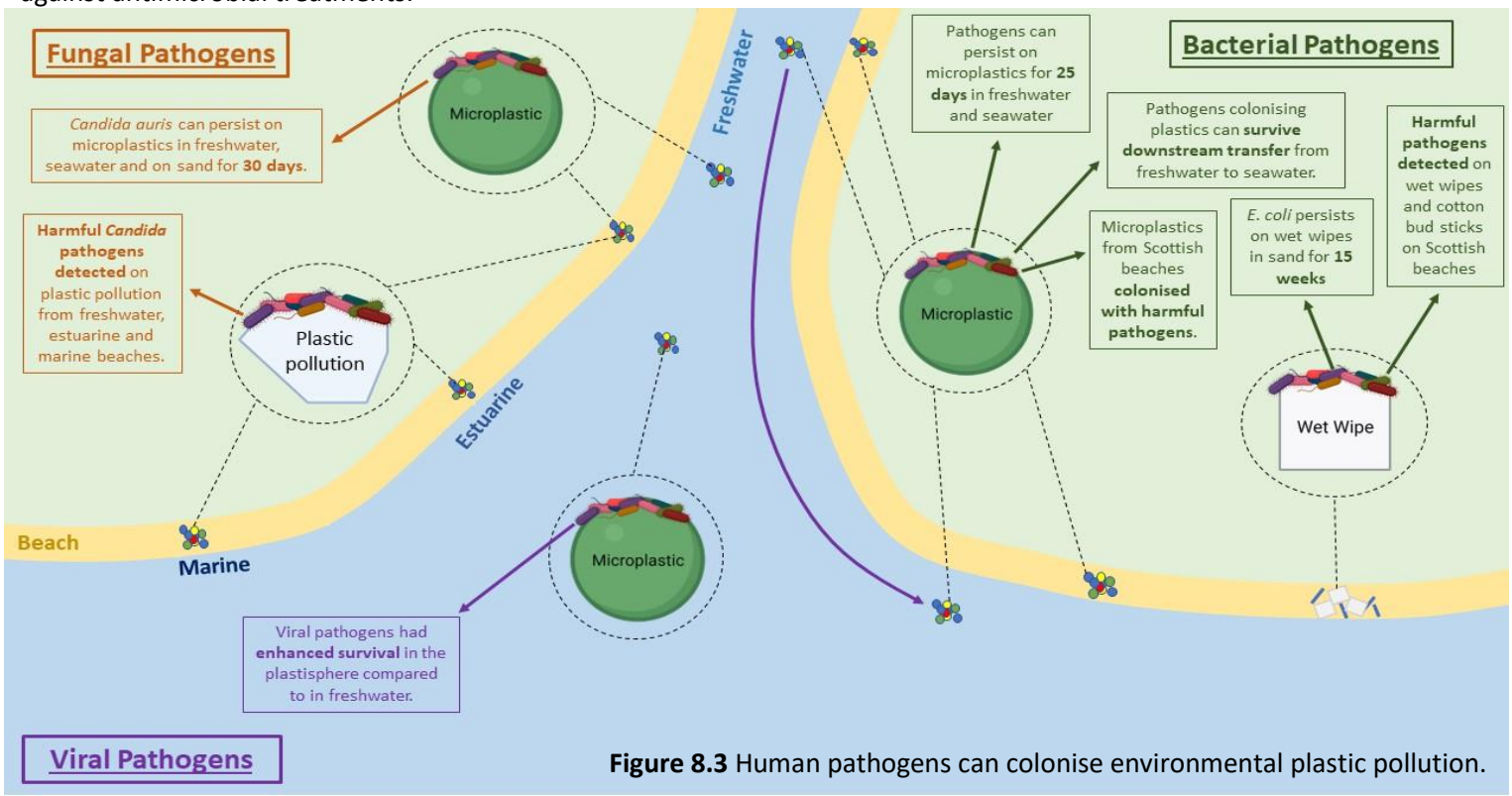

Figure 8.3 Human pathogens can colonise environmental plastic pollution.

Glossary

- **Antimicrobial resistance (AMR):** Changes in pathogenic microbes that cause the drugs used to treat infections to become less effective.
- **Combined sewer overflow events (CSOs):** An outlet from the public sewer that is designed to spill excess rainwater and sewage into the environment during periods of high rainfall to reduce the risk of sewage backing up.
- **Gastroenteritis:** Inflammation of the stomach and intestines, typically resulting from bacterial toxins or viral infection and causing vomiting and diarrhoea.
- **Microbe:** A biological living system that is microscopic in size, which may exist in its single-celled form or as a colony of cells.
- **Microplastics (MPs):** Small spheres or fragments of plastic debris less than 5 mm in length.
- **Pathogen:** An organism causing disease to its host. Includes bacteria, fungi and viruses.
- **Plastisphere:** The microbial communities colonising plastic debris.
- **Thermotolerant:** The ability of an organism to survive changing temperatures.
- **Virulence:** A pathogen's ability to cause disease. The degree of damage caused by a microbe to its host.

- *Candida* isolates from plastic pollution showed resistance to several antifungals, including fluconazole and caspofungin. Isolates showed increased resistance to amphotericin B compared to clinical *Candida* isolates[22].

Thermotolerance

- Temperature influences an organism's ability to grow and establish infection. Thermotolerant organisms can adapt and grow at a wide range of temperatures.
- Thermotolerance is particularly important for pathogenic fungi. Human body temperature (37°C) is the human body's main defence against fungal pathogens, above which fungi are unable to grow.
- *Candida* isolates from plastic pollution could successfully grow at a range of temperatures, including at 38°C, indicating that they are more likely to cause human infection[22].

Recommendations

Recent research has highlighted the human health risk that pathogen colonised plastic pollution can pose. Therefore, we provide several recommendations to policy and practitioner communities and other interested stakeholders to help reduce the presence of plastic pollution on our beaches and reduce the risk to human health.

- WWTPs are one of the main sources of microplastics and other sewage-associated plastic pollution. Tackling this source will reduce the quantity of pathogen colonised plastics entering the environment.
    - More effective wastewater treatment methods are required to reduce the survival of harmful pathogens in WWTP effluent.
    - Improved infrastructure and associated funding provision, together with increased monitoring and management is needed to prevent both legal and illegal release of untreated sewage into the environment.
    - Engineering solutions are required to reduce microplastic discharge from homes and WWTPs.
    - The flushing of plastic containing wet wipes is one of the primary causes of sewage blockages, a greater investment in public education and awareness-raising programmes is required to eliminate wet wipes entering sewerage systems.
- Improved monitoring and enforcement of regulations on the plastics supply chain, preventing the release of microplastics from all parts of the chain, particularly focussing on transport.
- Improved public education and awareness on the ability of plastic pollution to transfer harmful pathogens, highlighting the importance of handwashing after coming into contact with plastic pollution.
- Encourage the public to take part in beach cleans and reporting through citizen science projects to reduce the presence of plastic pollution on beaches.

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9. Synthesis and Conclusions

9.1 Summary

Keswani *et al.* (2016) first highlighted plastic pollution as a potential reservoir for pathogens harmful to human health. Since then, numerous studies have identified pathogens in the plastisphere and there has been a push to determine the real human health risk this could have (Junaid *et al.,* 2022). The aim of this thesis was to improve our knowledge and understanding on the potential human health implications that pathogen colonised plastic pollution could have, with a particular focus on beach environments (Table 9.1; Table 9.2).

9.2 Implications

Humans are continually polluting the environment. Here it has been shown how plastic pollution can become multi-pollutants due to their ability to be colonised by and transfer harmful pathogens. The physical presence of plastics in beach environments is already known to negatively impact ecosystems (Keswani *et al.,* 2016; Bucci *et al.,* 2020), and human mental health (Wyles *et al.,* 2016). But the ability of plastics to potentially transfer harmful pathogens provides additional implications to human health. Together, the evidence in this thesis could be used to help inform environmental management and policy recommendations and reduce the implications to human health (Table 9.2; Chapter 8).

Table 9.1 Research findings from each chapter.

Research Findings	**Chapter**				
	Chapter 3: From wastewater discharge to the beach: Survival of human pathogens bound to microplastics during transfer through the freshwater-marine continuum	***Chapter 4:*** Evidence of interspecific plasmid uptake by pathogenic strains of *Klebsiella* isolated from microplastic pollution on public beaches	***Chapter 5:*** Sewage-associated plastic waste washed up on beaches can act as a reservoir for faecal bacteria, potential human pathogens, and genes for antimicrobial resistance	***Chapter 6:*** Persistence of 'wet wipes' in beach sand: an unrecognised reservoir for *localised E. coli* contamination	***Chapter 7:*** Screening environmental isolates of human pathogenic *Candida* spp. colonising plastic pollution
Colonising group					
Bacteria	X	X	X	X	-
Fungi	-	-	-	-	X
Survival					
Freshwater	X	-	-	-	X
Marine	X	-	-	-	X
Beach	X	X	X	X	X
Transfer between matrices	x	-	-	-	-
Characterisation					
ARGs	-	X	-	-	-
AMR in MIC test	-	-	X	-	X
Thermotolerance	-	-	-	-	X
Virulence genes	-	X	-	-	-
Pathogenicity in *Galleria* model of infection	-	X	-	-	X

Table 9.2 Research priorities.

Research Priorities (Objectives)	Key Findings	Future Work	Policy Implications
Evaluate the load of potential bacterial and fungal pathogens **colonising** different types of environmental plastic pollution.	• Several potential bacterial pathogens can colonise environmental plastic pollution (Chapter 2). • *E. coli, E. faecalis* and *P. aeruginosa* sucessfully colonised PE microplastic beads (Chapter 3). • Potential pathogens were detected on microplastic beads from Scottish beaches without the need for a pre-enrichment step (Chapter 4). ○ Urban sites had higher bacterial concentrations. • FIOs more often associated with wet wipes and cotton bud sticks than with seaweed (Chapter 5). • *E. coli* colonised wet wipes when incubated in effluent (Chapter 6). • Several *Candida* species colonised plastic pollution from freshwater, estuarine and marine beaches (Chapter 7).	• Further comparisons to control materials. • Evaluate the load of eukaryotic and viral pathogens on environmental plastic pollution.	• Need to prevent pathogen colonised plastics from entering the environment e.g., from WWTPs and the plastics supply chain. • More governmental support for beach cleans to reduce the presence of plastics on our beaches.
Quantify the **survival dynamics** of potential pathogens on plastics during the movement between different environments.	• *E. coli, E. faecalis* and *P. aeruginosa* survived on the surfaces of microplastic beads for at least 25 days in freshwater or seawater (Chapter 3). ○ Viable *E. coli, E. faecalis* and *P. aeruginosa* still detected in the plastisphere after moving downstream between different environments. • *E. coli* persisted on wet wipes in beach sand for 15 weeks (Chapter 6).	• Pathogen transfer from plastics to humans. • Survival dynamics of eukaryotic and viral pathogens. • Influence of other pollutants (e.g., chemical toxins) on the survival of pathogens in the plastisphere. • Influence of climate change (e.g., increasing temperature, increased UV, ocean acidification) on the survival of pathogens in the plasitpshere.	• Regulatory monitoring of plastic along with levels pathogens in the plastipshere as part of the bathing water directive.

Characterise the AMR, **pathogenicity** and thermotolerance of potential pathogens.	• *AMR*
	  ○ *Klebsiella* isolated from beach microplastic beads had ARGs, some on plasmids from other species (Chapter 4).
	  ○ FIOs and *Vibrio* spp. colonising wet wipes showed evidence of resistance to common antibiotics (Chapter 5).
	  ○ *Candida* isolates from plastic pollution showed evidence of resistance to several antifungals, with some isolates showing increased resistance compared to clinical *Candida* isolates (Chapter 7).
	• *Pathogenicity*
	  ○ *Klebsiella* isolates from beach microplastic beads possessed virulence genes which were expressed in a *Galleria melonella* model of infection (Chapter 4).
	  ○ *Candida* isolates from plastics showed pathogenicity in a *Galleria melonella* model of infection (Chapter 7).
	• *Thermotolerance*
	  ○ *Candida* isolates from plastics could grow at a range of temperatures, including (38°C) above human body temperature (Chapter 7).
		• Evidence of horizontal gene transfer in the plastipshere.
		• Can transferred pathogens cause disease in humans?
		• Thermotolerance of prokaryotic pathogens.
		• Does pathogenicity and AMR of plastipshere pathogens increase when exposed to increased temperatures?
			• Provides evidence that pathogens are not only present but also have features that make them more likely to cause disease, which should lead to a greater impetus for change e.g., education campaigns, improved regulations and management.
			• Evidence to support improving management and regulations on the disposal and release of antimicrobials.

9.3 Future work

Whilst this thesis has advanced our understanding on the potential human health risks that pathogen colonised plastics provide, there are still several knowledge gaps and future work which needs to be carried out before the actual risk can be calculated (Table 9.2). We are still yet to fully understand whether pathogens can transfer from plastics within humans. To date there has only been one published study directly demonstrating pathogen transfer via microplastic ingestion, with *E. coli* being transferred to the gut tissue of the northern star coral (Rotjan *et al.,* 2019). Metcalf (2019) also found that more *Vibrio* was transferred to oysters when in the plastisphere compared to planktonic *Vibrio*, suggesting higher rates of transfer when there is a vector. Despite Beloe *et al.* (2022) highlighting the importance of understanding whether pathogens can be transferred from plastics to the human host and remain viable once within the host, there is still yet to be conclusive evidence on whether this does occur.

We must also determine whether the transferred pathogens are able to cause disease. Rotjan *et al.* (2019) did demonstrate mortality in those star corals which had ingested microplastic beads colonised by *E. coli* compared to no mortality in those which had ingested microplastics with no biofilm, suggesting that the *E. coli* transferred was the cause of disease and mortality. Whilst no transfer of *E. coli* was demonstrated, Fabra *et al.* (2021) did show increased oxygen consumption and respiration rate of oysters exposed to *E. coli* coated microplastics compared to no measurable physiological responses in those oysters exposed to virgin microplastics, suggesting that the presence and potential transfer of *E. coli* resulted in the changing physiological responses. Further measures of lethal and immunological responses, including stress markers and haemocyte function, should be incorporated into future studies to further our understanding on whether disease is really caused by plastisphere pathogens. However, it is likely to occur; the movement of pathogens from the plastisphere to the human body coincides with a move towards more optimal conditions (e.g., increased temperature), which could in turn increase the pathogens growth rate and the likelihood of disease. Although it would be difficult to carry out experiments in humans, additional models (e.g., mussels, mice) could be used to improve our understanding on whether this transfer process and causation of disease does occur.

Even though there are now numerous studies identifying and characterising human pathogenic bacteria in the plastisphere, studies are still lacking on eukaryotic and viral pathogens (Ormsby *et al.,* 2023). Initial research demonstrates that eukaryotic and viral human pathogens are capable of surviving and persisting in the plastisphere (e.g., *Candida auris* [Akinbobola *et al.,* 2024]; *Cryptococcus* spp. [Gkoutselis *et al.,* 2021]; Rotavirus [Moresco *et al.,* 2022]). Eukaryotic and viral human pathogens represent some of the most important disease-causing agents globally (e.g., *Aspergillus* spp. [3 million infections annually], *Candida* spp. [1.5 million infections annually], SARS-CoV-2 [7 million deaths]). This highlights the importance of expanding our understanding on the risks that pathogens other than just prokaryotes can pose to human health. Although this thesis has focused on human pathogens, plastics also have the ability to transfer other pathogens which are pathogenic to other species (e.g., fish [Naudet *et al.,* 2023], coral [Feng *et al.,* 2020], shellfish [Xiao *et al.,* 2023]). This could indirectly effect humans e.g., reduced food supply, reduced tourism. Therefore, it is important that future work focuses on additional pathogenic species along with those known to be human pathogens.

Chapter 2 highlighted the importance of including an appropriate control material in plastisphere studies. Since this paper was published, an increasing number of studies are including control materials (e.g., Cheng *et al.,* 2024; Magalhaes *et al.,* 2023); but there are still conflicting results on whether pathogens are more abundant on plastics or control materials. However, there is consensus that pathogens are enriched on plastics compared to substrates such as sand (Pham *et al.,* 2021; Magalhaes *et al.,* 2023); with sand being the main material humans will come into contact with at beach environments, which highlights how this plastics could pose a heightened public health risk at the beach. This signifies the continued importance of incorporating control materials in future plastisphere pathogen studies.

9.4 Conclusion

As plastic demand continues to grow and production increases, the volume of plastic pollution entering the environment will also rise, with 53 million metric tons of plastic predicted to enter the environment annually by 2030 (Borelle *et al.,* 2020). This will lead to a corresponding increase in human exposure to pathogen colonised plastics. This thesis has shown how potential pathogens are able to colonise and survive on the surfaces of plastic pollution in single environmental matices or during the

transfer between different environmental matrices. These pathogens can also possess and express genes which cause antimicrobial resistance, virulence and thermotolerance, meaning they will be more likely to cause infection and could be harder to treat. Therefore, it is vital that we continue to study the ability of plastics to act as novel vectors of disease, as well as educating policy makers and members of the public.

9.5 References

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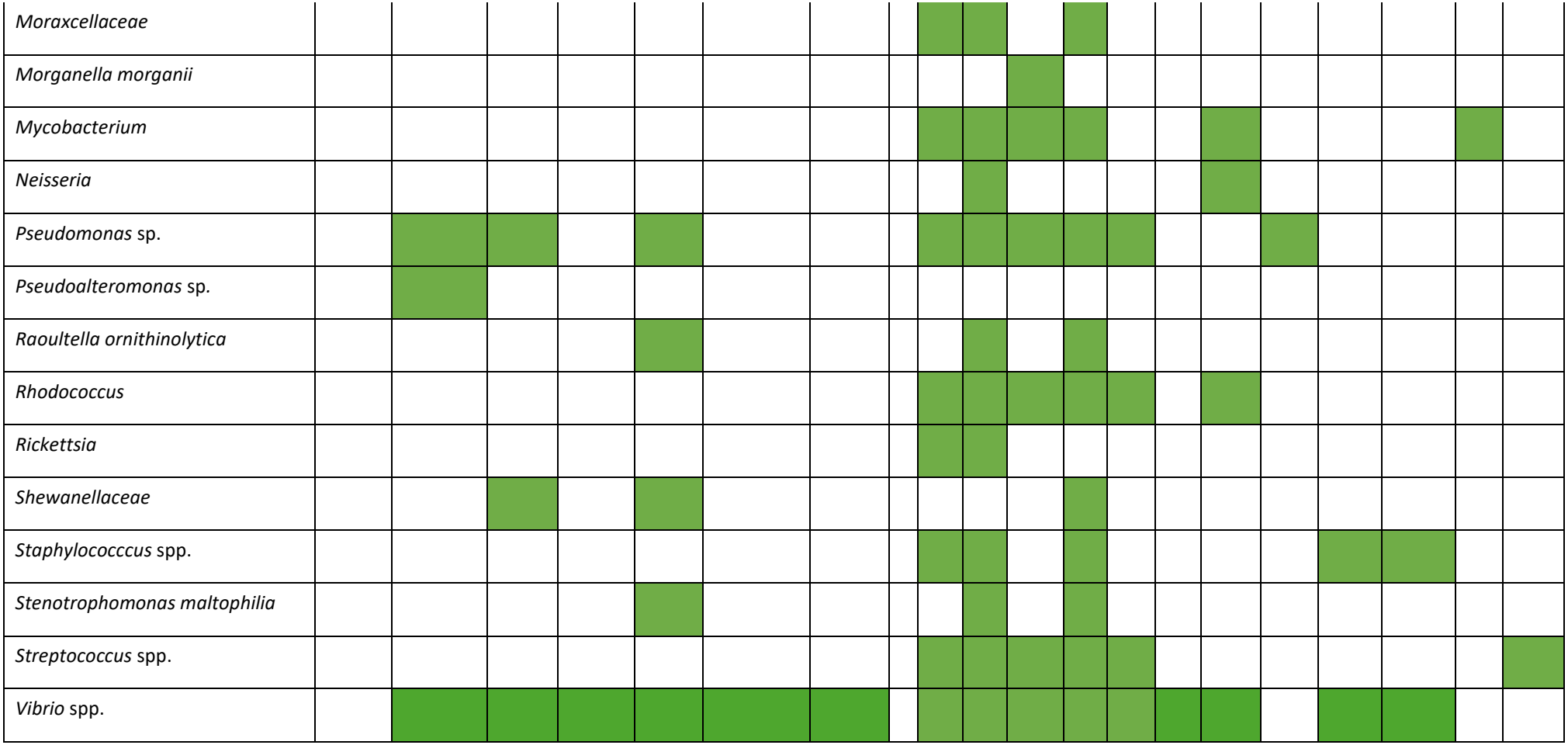
10. Appendices

Appendix A

Table A1. The different species of human pathogenic bacteria detected on control materials and plastic polymers.

	Control Materials								Plastic Polymer											
Pathogen	Chitin	Feather	Glass	Metal	Sand	Seaweed	Wood		PP	PE	PET	PS	PU	PC	PVC	PBT	OXO	PHBV	PA	PAN
Acinetobacter oleivorans									■	■	■									
Arcobacter sp.	■		■		■				■	■		■			■					
Alteromonas		■																		
Bacillus spp.									■	■	■	■	■		■					
Burkholderia sp.										■		■			■				■	
Campylobacteraceae									■	■		■								
Clostridium perfringens										■										
Coxiella sp.									■	■	■	■								■
Escherichia coli					■	■				■										
Enterococci					■	■														
Enterobacteriaceae			■		■	■			■	■		■								
Enterococcus faecium										■		■			■				■	
Francisella									■	■										
Heliobacter spp.										■										
Klebsiella pneumoniae									■	■	■	■			■				■	
Legionella									■	■	■	■			■					■
Listeria monocytogenes										■		■			■				■	



* PP-polypropylene, PE-polyethylene, HDPE-high density polyethylene, LDPE-low density polyethylene, PET-polyethylene terephthalate, PS-polystyrene, PU-polyurethane, PVC-polyvinyl chloride, PC – Polycarbonate, PBT- Polybutylece terephthalate, OXO-Additivated PE with pro-oxidant, PHBV-poly(3-hydroxybutyrate-co-3-hydroyvalerate), PAN- polyacrylonitrile

Appendix B

Table A2. Material properties of the different plastic polymers and control materials. A dash indicates where the information was not available.

Material	Material Property			
	Density (g/cm^3)	Hydrophobicity (°)	Surface Charge (mV)	Surface Roughness (nm)
Plastic Polymer				
Polypropylene	0.86-0.95	93	-10	14
Polyethylene	0.91-0.97	83	-9	21
Polystyrene	0.96-1.04	85	-40	92
Polyurethane	1.20	80	-20	-
Polyvinyl chloride	1.38	87	-10	14
Polyethylene terephthalate	1.37-1.45	90	-8	7
Organic Control Material				
Feather	0.04	88-95	-50	-
Wood	0.04	70-90	-2 - -10	350
Leaf	0.3	55-140	-60	50-275
Seaweed	0.3	47-62	-19	-
Fabric (cotton)	0.4	145	-12	12
Cardboard	0.7	110	Negative	5
Chitin	1.4	66-75	1-7	-
Cellulose	1.5	45-53	-24 - -42	0.3
Rope	7.5	>90	-	-
Inorganic Control Material				
Pumice	0.3	-	-26 - -60	-
Paint	1.5	>90	Negative	-
Rubber	1.5	>90	-45	200
Sand	1.7	<90	Negative	370-720
Ceramic	2.5	<90	-	50-400
Glass	2.6	55	-60	5
Rock	2.6	50-80	-50	15-950
Metal	2.7-7.8	55-97	-30	120

Appendix C

Table A4. Bacterial concentrations at day 1 in experiment 1. The mean was calculated from 8 replicates, ± the standard error. * denotes significance.

	Bacteria concentrations (log_{10} CFU/40 particles)		*t* test Result
	Plastic	Glass	*P* value
E. coli	6.19 ± 0.08	5.87 ± 0.04	< 0.01*
E. faecalis	5.68 ± 0.18	5.19 ± 0.03	< 0.05*
P. aeruginosa	6.15 ± 0.06	5.20 ± 0.04	< 0.001*

Appendix D

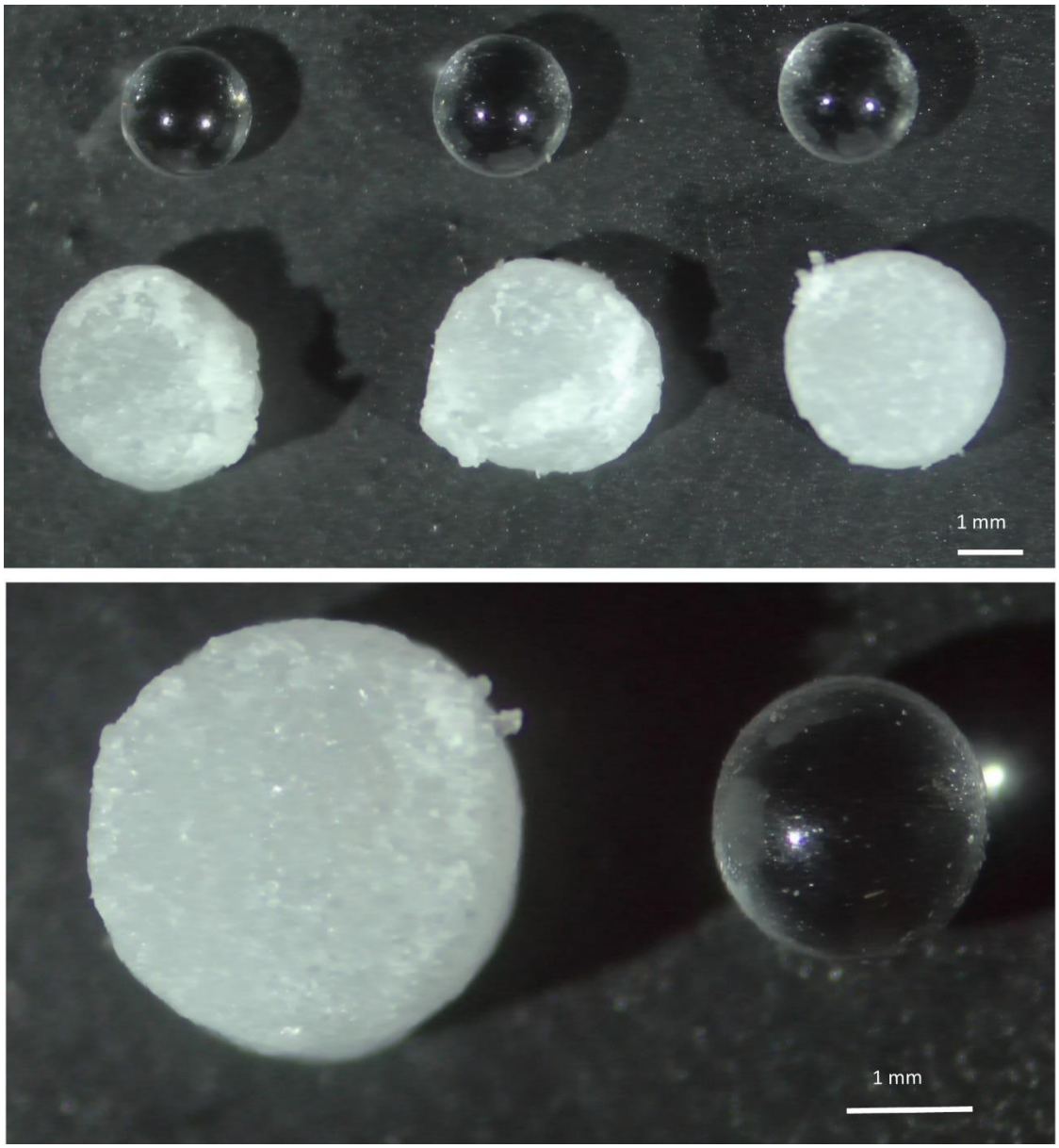


Figure A4. (A) Representative morphology of 2 mm glass particles (top row) and 2 mm plastic particles (bottom row); and (B) gross surface characteristics of plastic particles (left) and glass particles (right).

Appendix E

Table A5. Field observations at each study site.

Site	Bathing Water Beach	Coordinates	Date	Water Temperature (°C)	Salinity (PSU)	Organic Matter (%)* and Substrate Type	Beach users	Number of PE beads collected*	Number of PS beads collected*
1 Erskine	N	55.919° N, -4.464° W	28/11	8.0	2	- <10% OM (sticks) - Sand	7 walkers	52	106
2 Largs	Y	55.783° N, -4.861° W	28/11	10.7	36	- <10% OM (brown seaweed) - Sand, shingle, dunes	>50 walkers	84	0
3 Irvine	Y	55.599° N, -4.684° W	30/11	9.2	34	- <10% OM (brown seaweed, sticks) Sand, dunes	>50 walkers, 2 horse riders	81	8
4 Ayr	Y	55.453° N, -4.644° W	30/11	8.4	34	- <10% OM (brown seaweed, sticks) - Sand	>50 walkers	90	2
5 Turnberry	N	55.312° N, -4.838° W	30/11	9.5	36	- <10% OM (brown seaweed) - Sand, dunes	4 walkers, 3 horse riders	101	5
6 Montrose	Y	57.716° N, -2.448° W	21/11	10.7	34	- <10% OM (brown seaweed and sticks) - Sand, dunes, large rocks	2 walkers	1	54
7 Broughty Ferry	Y	56.466° N, -2.861° W	21/11	8.6	34	- 10% OM (brown seaweed, sticks, leaves) - Sand, dunes	10 walkers	20	45
8 Portobello	Y	55.954° N, -3.108° W	23/11	9.6	34	- <10% OM (brown seaweed, sticks, leaves)	>50 walkers	106	3

						-	Sand			
9 Yellowcraig	Y	56.065° N, -2.775° W	23/11	8.7	35	-	30% OM (brown and red seaweed) - Sand, dunes	12 walkers	49	1
10 Eyemouth	Y	55.874° N, -2.093° W	25/11	9.0	33	-	30% OM (brown seaweed, sticks) - Sand, rocks	20 walkers, 1 swimmer	2	138

***** Sites where fewer than 10 microplastic beads were collected were excluded from further analysis

Appendix F

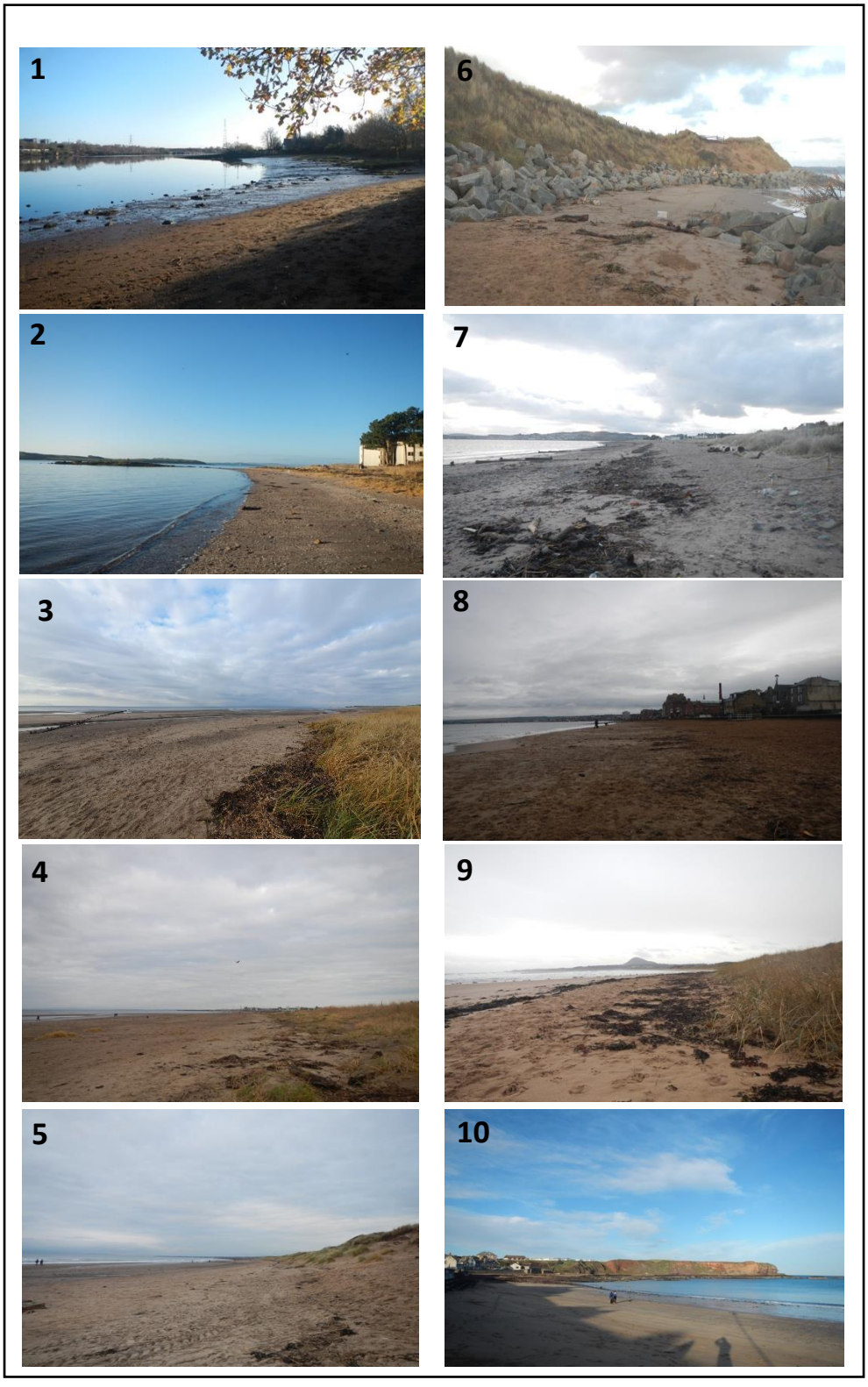


Figure A6. Images of sampling locations numbered according to Appendix E.

Appendix G

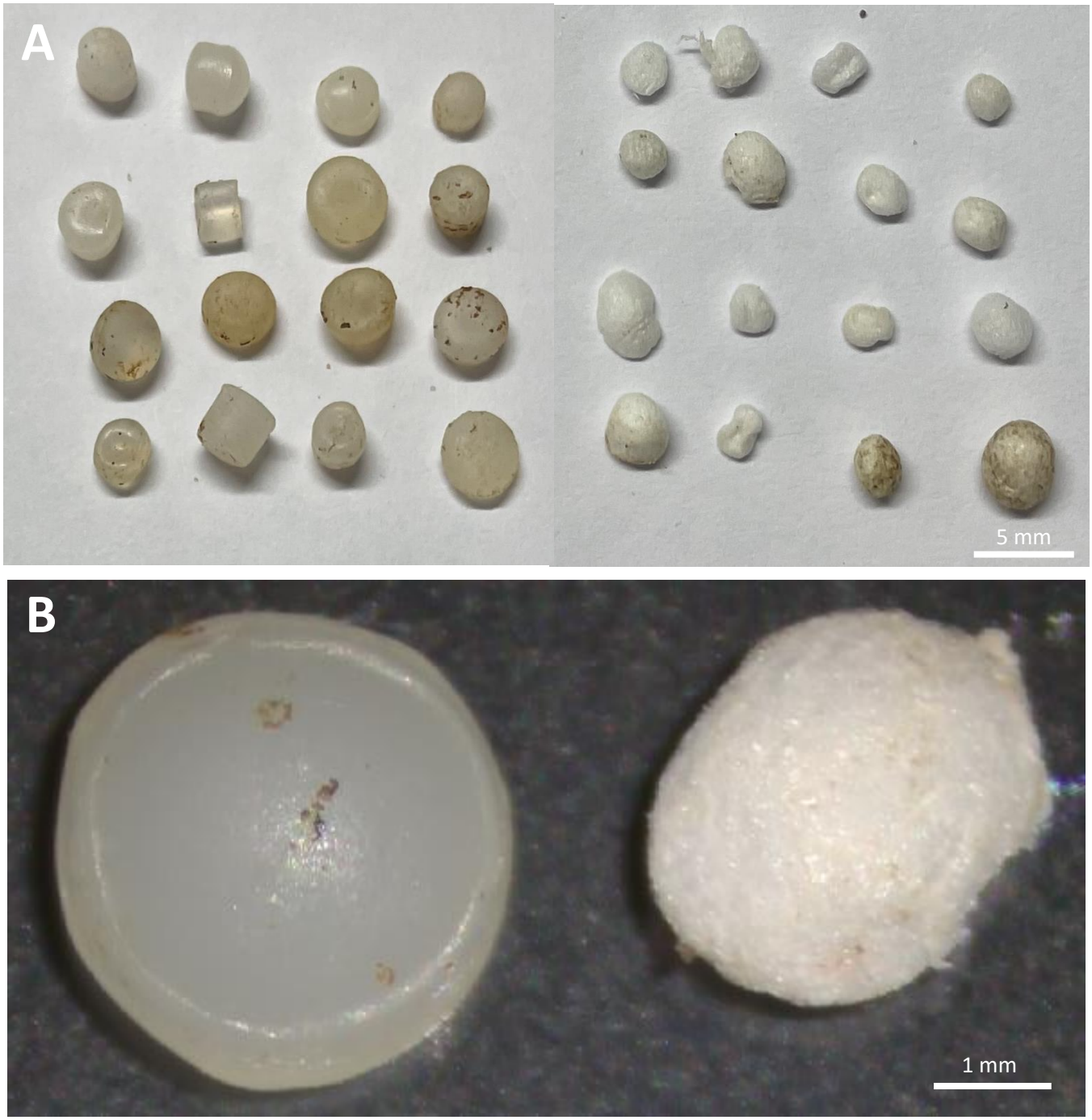


Figure A7. (A) Polyethylene (PE; left) and polystyrene (PS; right) beads collected during sampling. (B) Gross surface characteristics of microplastic beads (PE left; PS right).

Appendix H

Table A8. Total colour composition of PE beads collected from ten beaches in Scotland. Sites are numbered according to Appendix E.

	Colour (%)								
Site	**White**	**Yellow**	**Blue**	**Grey**	**Green**	**Red**	**Purple**	**Pink**	**Black**
1	94	-	2	-	2	-	-	2	-
2	98	-	-	1	-	1	-	-	-
3	70	-	14	10	-	-	1	-	5
4	76	-	1	12	4	1	-	-	6
5	83	1	3	6	3	-	1	-	3
6	-	-	-	-	-	-	-	-	-
7	100	-	-	-	-	-	-	-	-
8	95	1	3	-	1	-	-	-	-
9	86	4	10	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-

Appendix I

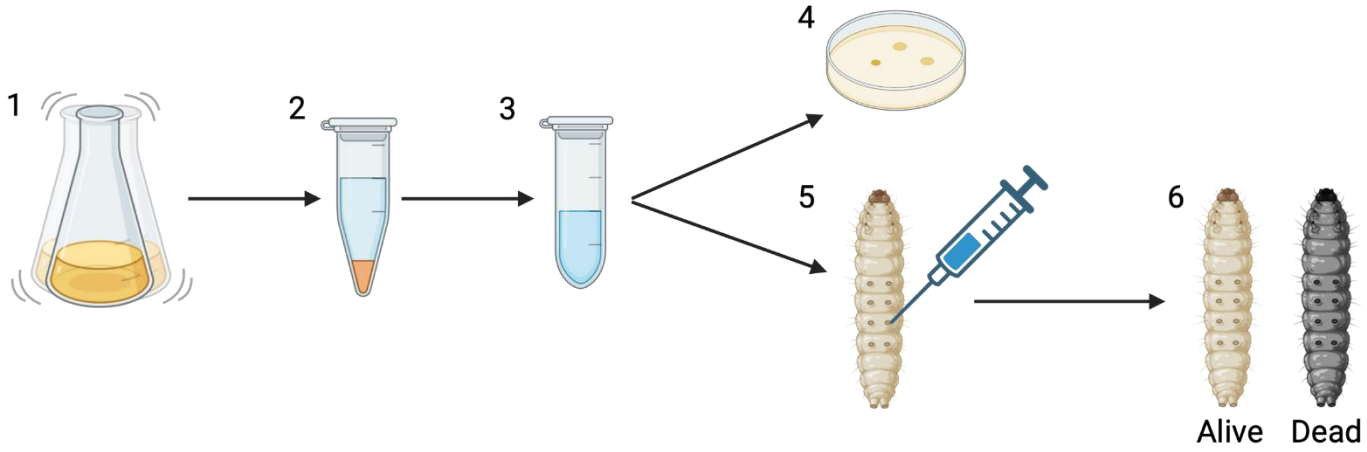


Figure A9. Experimental procedure: glycerol stocks of *Klebsiella* from site 7 were grown overnight in LB (1), cells were centrifuged (2), and resuspended in PBS (3), before CFU enumeration (4) and injection into *G. melonella* (5). After injection, larvae were incubated at 37°C and inspected 24, 48 and 72 h post-injection; larvae were considered dead when they did not respond to touch stimuli (6). Figure Created with BioRender.com.

Appendix J

Table A11. Polymer type was confirmed by comparing to reference spectral libraries (Jung *et al.,* 2018). Specific matching values to the library and their corresponding bonds are detailed below.

Polymer	Chemical structure	Absorption bands (cm^{-1}) used for identification	Assignment
Polystyrene (PS)		2847	C-H stretch
		1601	Aromatic ring stretch
		1492	Aromatic ring stretch
		1451	CH_2 bend
		1027	Aromatic CH bend
		694	Aromatic CH out-of-plane bend
		537	Aromatic ring out-of-plane bend
Polyethylene (PE)		2915	C-H stretch
		2845	C-H stretch
		1472	CH_2 bend
		730	CH_2 rock
		717	CH_2 rock

Appendix K

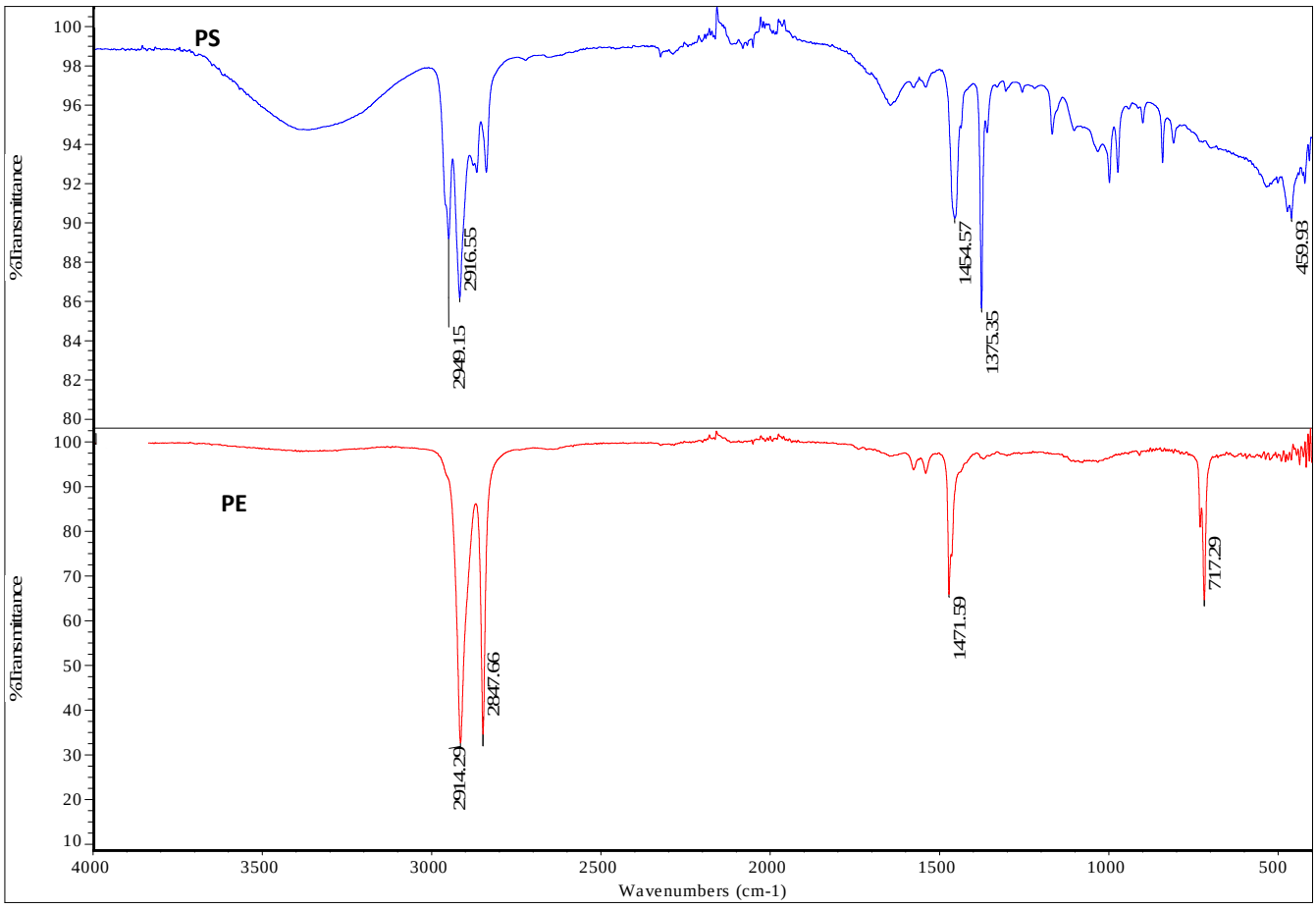


Figure A10. FTIR spectra of polystyrene (PS; blue spectra) and polyethylene (PE; red spectra) beads collected from beaches around Scotland.

Appendix L

Table A12. Comparative genome statistics from *Klebsiella* isolates from PE, PS, sand and *K. pneumoniae.*

Isolate	Species	Strain	Completeness (%)	Contamination (%)	GC (%)	Coverage	Plasmids	MGE	Contigs	Longest (bp)	5S	16S	23S	tRNA	Probability human pathogen (%)	Matched pathogenic families
PE	*K. variicola*	DSM 15968	98	1.00	57	50x	5	181	11 (6 Circular)	5,614,364	9	8	8	87	81	206
PS	*K. pneumoniae*	ATCC 13883	97	22.91	58	90x	5	142	15 (8 Circular)	5,285,520 (Circular)	9	8	8	89	89	249
Sand	*K. pneumoniae*	ATCC 13883	98	0.39	57	50x	1	55	4 (2 Circular)	5,197,591 (Circular)	1	9	9	92	88	263
K. pneumoniae	*K. pneumoniae*	ATCC 13883	96	0.43	57	32x	2	90	3 (1 Circular)	4,319,548	9	8	8	86	90	231

Appendix M

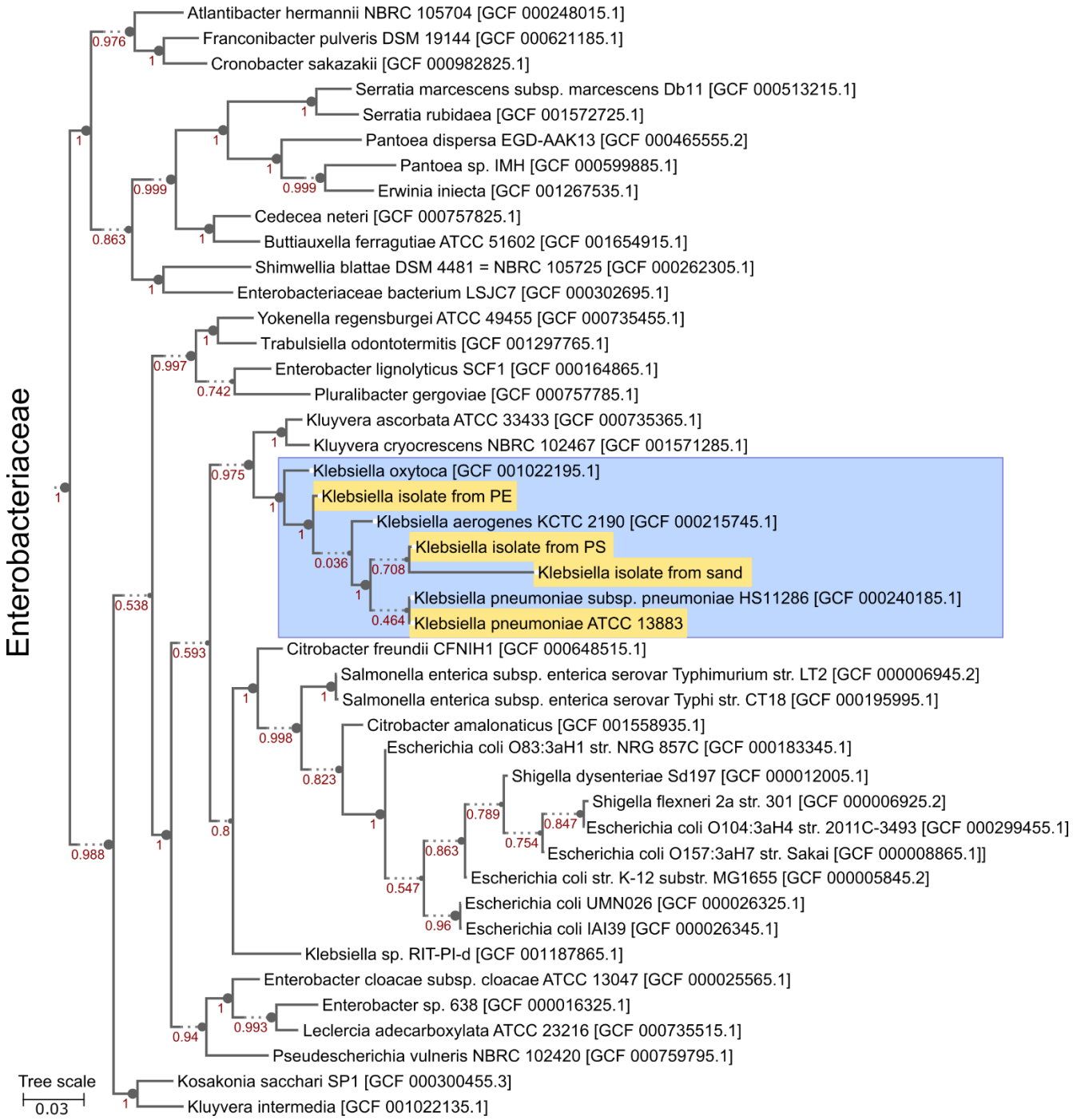


Figure A13. Phylogenetic tree of the *Klebsiella* isolates from PE, PS, sand and *K. pneumoniae.* The *Klebsiella* genus is highlighted in blue and the isolates from this study highlighted in yellow.

Appendix N

Table A14. Virulence genes identified in *Klebsiella* isolates from PE, PS, sand and *K. pneumoniae.*

Isolate	Virulence Factor	Protein Function	Contig	Position in contig	Accession number	Query/ Template length	Identity (%)
PE	*clpK1*	Heat shock survival AAA family ATPase	6	102524.. 105310	WP053271808	2787/2883	99
	fimH	Type 1 fimbrae	5	4619015.. 4619503	NA	489/489	95
	iutA	Ferric aerobactin receptor	5	1991093.. 1993282	FLWH01000001	2191/2190	95
	mrkA:ABW82989		5	4600665.. 4601279	NA	615/597	92
	nlpl	Lipoprotein nlpl processor	5	4878879.. 4879762	CP000243	886/885	90
	terC	Tellurium ion resistance protein	6	31484.. 32524	UDGH01000006	1041/1041	99
PS	*clpK1*	Heat shock survival AAA family ATPase	10	132794.. 135580	WP053271808	2787/2883	99
	fimH	Type 1 fimbrae	9	4314900.. 4315388	NA	489/489	100
	iutA	Ferric aerobactin receptor	9	1995394.. 1997582	FLWH01000001	2191/2190	99
	mrkA:ABW83989		9	4301741.. 4302355	NA	616/597	96
	mrkA:ABW83989		10	218647.. 219262	NA	615/597	95
	traT	Outer membrane protein complement resistance	5	9992.. 10723	FLWH01000012	732/732	97
Sand	*fimH*	Type 1 fimbrae	1	60804.. 61231	NA	489/489	100

	iutA	Ferric aerobactin receptor	1	3806883.. 3809072	FLWH01000001	2190/2190	99
	mrkA:AB W83989		1	1490765.. 1491379	NA	615/297	96
	shiB	Homologs of the *Shigella flexneri* SHI-2 pathogenicity island gene shiA	1	1477733.. 1478221	AAD44732	428/462	93
	traT	Outer membrane protein complement resistance	3	84715.. 85446	NZ_KZ984345	732/732	99
K. pneumoniae	*ccl*	Cloacin	3	174404.. 176089	NZ_UEMD01000021	1692/1692	93
	fimH	Type 1 fimbrae	1	2996109.. 2996597	NA	489/489	99
	fyuA	Siderophore receptor	1	3932618.. 3934597	UGDD01000002	1980/1980	100
	irp2	High molecular weight protein 2 non-ribosomal peptide synthetase	1	3947820.. 3953921	NZ_NTOW01000002	6108/6108	100
	irp2	High molecular weight protein 2 non-ribosomal peptide synthetase	1	3009145.. 300975	NZ_NTOW01000002	6108/6108	100
	iutA	Ferric aerobactin receptor	1	104811.. 107000	FLWH01000001	2190/2190	99
	mrkA:AB W83989		1		NA	615/597	96

Appendix O

Table A15. Resistance genes identified in *Klebsiella* isolates from PE, PS, sand and *K. pneumoniae.*

Isolate	Resistance Gene	Phenotype	Contig	Position in contig	Accession number	Query/ Template length	Identity (%)
PE	*blaLEN12*	amoxicillin, piperacillin, ticarcillin, ampicillin, cephalothin	5	2529982.. 2530770	AJ635406	789/789	100
	fosA	fosfomycin	5	594514.. 594933	ACZD01000244	420/420	9
	oqxA	trimethoprim, ciprofloxacin, nalidixic acid, cetylpyridinium chloride, benzylkonium chloride, chloramphenicol	5	4266168.. 4269318	EU370913	1176/1176	98
	oqxB	trimethoprim, ciprofloxacin, nalidixic acid, cetylpyridinium chloride, benzylkonium chloride, chloramphenicol	5	4264969.. 4266144	EU370913	3153/3153	97
PS	*blaSHV-187*	unknown beta-lactam	9	2508667.. 2509532	LN51533	867/867	99
	fosA	fosfomycin	9	522386.. 522805	ACW001000079	420/420	100
	oqxA	trimethoprim, ciprofloxacin, nalidixic acid, cetylpyridinium chloride, benzylkonium chloride, chloramphenicol	1	1805795.. 1806969	EU370913	1176/1176	99
	oqxB	trimethoprim, ciprofloxacin, nalidixic acid, cetylpyridinium chloride, benzylkonium chloride, chloramphenicol	1	5183269.. 5183688	EU370913	3154/3153	99
	oqxB	trimethoprim, ciprofloxacin, nalidixic acid, cetylpyridinium chloride, benzylkonium chloride, chloramphenicol	1	1802621.. 1805771	EU370913	3154/3153	99
Sand	*blaSHV-33*	amoxicillin, ampicillin, cephalothin, piperacillin, ticarcillin	1	3297929.. 3298788	AY037779	861/861	100

	blaOXA-395	amoxicillin, ampicillin, meropenem	4	207713..208504	AYO306133	792/789	97
	fosA	fosfomycin	1	5183269..5182688	ACZD01000244	420/420	97
	oqxA	trimethoprim, ciprofloxacin, nalidixic acid, cetylpyridinium chloride, benzylkonium chloride, chloramphenicol	1	1805795..1806969	EU370913	1176/1176	99
	oqxB	trimethoprim, ciprofloxacin, nalidixic acid, cetylpyridinium chloride, benzylkonium chloride, chloramphenicol	1	1802621..1805771	EU370913	3153/3153	99
K. pneumoniae	*blaSHV-40*	cefepime, ampicillin, ceftriaxone, ceftazidime, amoxicillin, cefotaxime, piperacillin, ticarcillin, aztreonam	3	538004..538865	AF535128	862/861	100
	blaSHV-56	ticarcillin+clavulanic acid, ampicillin+clavulanic acid, ampicillin, amoxicillin, piperacillin, piperacillin+tazobactam, ticarcillin, amoxicillin+clavulanic acid	3	538004..538865	EU586041	862/861	100
	blaSHV-79	cephalothin, ampicillin, amoxicillin, piperacillin, ticarcillin	3	538004..538865	AM176554	862/861	100
	blaSHV-85	cephalothin, ampicillin, amoxicillin, piperacillin, ticarcillin	3	538004..538865	DQ322460	862/861	100
	blaSHV-89	cephalothin, ampicillin, amoxicillin, piperacillin, ticarcillin	3	538004..538865	DQ193536	862/861	100
	fosA	fosfomycin	1	1428817..1429236	ACZD01000244	420/420	99

oqxA	trimethoprim, ciprofloxacin, nalidixic acid, cetylpyridinium chloride, benzylkonium chloride, chloramphenicol	1	3320059.. 3320035	EU370913	1176/1176	99
oqxB	trimethoprim, ciprofloxacin, nalidixic acid, cetylpyridinium chloride, benzylkonium chloride, chloramphenicol	1	3316885.. 3320035	EU370913	3154/3153	99

Appendix P

Table A16. Plasmids detected in *Klebsiella* isolates from PE, PS, sand and *K. pneumoniae.*

Isolate	Plasmid	Note	Contig	Contig length (bp)	Position in contig	Accession number	Query/ Template length	Identity (%)
PE	Col440I	*Klebsiella pneumoniae* strain FDAARGOS_440 plasmid unnamed	10	11,540	8338.. 8452	CP023920	116/114	96
	Col440I	*Klebsiella pneumoniae* strain FDAARGOS_440 plasmid unnamed	10	11,540	2573.. 2686	CP023920	115/114	97
	IncFIB(K)(pCAV 1099-114)	*Klebsiella oxytoca* strain CAV1099 plasmid pCAV1099-114, complete sequence	6	283,706	242284.. 242843	CP011596	560/560	100
	IncFII(Yp)	*Yersinia pestis* Pestoides F plasmid MT, complete sequence	9	33,463	7659.. 7886	CP000670	229/230	96
	IncR	*Klebsiella pneumoniae* strain NK245 plasmid pK245, complete sequence	4	70,127	50225.. 50475	DQ449578	251/251	100
PS	IncFIA(HI1)	*Salmonella typhi* R27 plasmid complete sequence	12	96,847	57326.. 57713	AF250878	388/388	97
	IncFIB(K)	*Klebsiella pneumoniae* strain ST258 plasmid pKPN-IT, complete sequence	10	236,500	229058.. 229617	JN233704	560/560	99
	IncFIB(pKPHS1)	*Klebsiella pneumoniae* subsp. pneumoniae HS11286 plasmid pKPHS1, complete sequence	2	113,609	51443.. 52002	CP003223	560/560	99

	IncFII(K)	*Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 plasmid pKPN3, complete sequence	10	236,500	102861.. 103008	CP000648	148/148	98
	IncFII(pKP91)	*Klebsiella variicola* strain 342 plasmid pKP91, complete sequence	12	96,847	17784.. 18013	CP000966	230/230	100
Sand	**IncFIB(K)**	*Klebsiella pneumoniae* strain ST258 plasmid pKPN-IT, complete sequence	3	108,162	24359.. 24918	JN233704	560/560	100
K. pneumoniae	**IncFIB(K)**	*Klebsiella pneumoniae* strain ST258 plasmid pKPN-IT, complete sequence	3	233,177	86165.. 86724	JN233704	560/560	100
	IncFII(pKP91)	*Klebsiella variicola* strain 342 plasmid pKP91, complete sequence	3	233,177	16911.. 169840	CP000966	230/230	100

* The plasmids highlighted in bold contained virulence and resistance genes.

Appendix Q

Table A17. Phage/viral-like sequences detected in *Klebsiella* isolates from PE, PS, sand and *K. pneumoniae.*

Isolate	Contig	Contig length (bp)	Phage hallmark genes	'VirSorter' Prediction	PlasmidFinder?	Plasmid	Plasmid Coverage (%)
PE	11	13	0	'Possible'	No		
	4	82	0	'Possible'	Yes, one plasmid	IncR	100
	5	6,765	12	'Likely' phage	No		
	6	354	0	'Possible'	Yes, one plasmid	IncFIB(K)(pCAV1099-114)	100
PS	11	62,023	10	'Likely' phage	No		
	5	34,649	3	'Likely' phage	No		
	12	96,847	0	'Possible'	Yes, two plasmids	IncFIA(HI1) IncFII(pKP91)	100 100
	13	1,254	0	'Possible'	No		
	14	2,143	0	'Possible'	No		
Sand	1	6,392	5	'Likely' phage	No		
	2	202	2	'Likely' phage	No		
	3	163	0	'Possible'	Yes, one plasmid	IncFIB(K)	100
K. pneumoniae	1	5,362	0	'Likely' phage	No		

2	1,222	7	‘Likely’ phage	No		
3	305	0	‘Possible’	Yes, two plasmids	IncFIB(K) IncFII(pKP91)	100 100

Appendix R

Table A18. Field observations at each study site.

Site	Coordinates	Date	Water Temperature (°C)	Salinity (% salt)	Organic Matter (%)* and Substrate Type		Beach users	Sanitary products (g per 100 m (fresh weight))
					Upper shore/ strandline	Lower shore		
S1 – Culross	56.055° N, -3.631° W	06/12/2021	4.8	2.3	- 50 % OM (brown seaweed) - Sand/shingle	- Medium OM cover - Silt/fine sand	None	734.22
S2 - Torryburn	56.058° N, -3.572° W	06/12/2021	4.1	0.9	- 70 % OM - Rocky shingle	- Medium OM cover - Silt/fine sand	Few dog walkers	132.64
S3 – North Queensferry	56.008° N, -3.393° W	06/12/2021	6.0	2.7	- 100 % OM (brown seaweed mixed with sticks and logs). - Rocky	- Medium OM cover over rocky section. - Sand/silt	None	24.25
S4 – Aberdour (Silver Sands)	56.053° N, -3.287° W	06/12/2021	6.6	3.0	- ~10 % OM (seaweed and leaves) - Fine dry sand	- <5% OM (seaweed and leaves) - Thick mass of brown seaweed along waterline (~2 ft high x 2 ft deep) - Fine dry sand.	Lots of walkers and dog walkers	3.02
S5 – Burntisland	56.060° N, -3.226° W	06/12/2021	5.4	2.8	- Minimal strandline, <5% OM - Fine dry sand	- <5 % OM - Brown seaweed along waterline (~1 ft x 1 ft) - Fine sand	Lots of walkers	2.61
S6 – Portobello	55.951° N, -3.102° W	06/12/2021	5.4	2.9	- Minimal strandline, <5 % OM - Sandy	- > 95 % OM (mix of red and brown seaweed) - Sandy	Lots of walkers, dog walkers and families	30.54
S7 - Leith	55.968° N, -3.137° W	06/12/2021	5.6	3.0	- Strandline made up ~ 60 % of visible beach.		Some dog walkers	18.39

					- OM composed of brown seaweed and sticks/wood.			
S8 – Crammond	55.981° N, -3.298° W	07/12/2021	3.3	2.4	- ~ 30 % OM (brown seaweed, leaves, sticks). - Coarse sand	- <5% OM - Fine sand (compacted)	Lots of dog walkers (~20)	-
S9 – Blackness	56.004° N, -3.520° W	07/12/2021	3.4	2.4	- ~ 30 % OM (brown seaweed). - Coarse sand.	- <5% OM - Silt/mud/sand - Scattered rocks	Some families	-
S10 – Bo'ness	56.013° N, -3.567° W	07/12/2021	4.0	2.3	- ~ 30 % OM (brown seaweed, sticks, leaves) - Coarse sand	- <5% OM - Sand/silt/mud - Scattered rocks	Some dog walkers	21.75

***** Visual estimate of organic matter

Appendix S

Material	***Vibrio* species present**
Seaweed	*V. alginolyticus, V. parahaemolyticus, V. cholera*
Wipes	*V. alginolyticus, V. parahaemolyticus*
Sticks	*V. alginolyticus, V. cholera, V. vulnificus*

Table A19. *Vibrio* species detected on stick, wipe, and seaweed samples.

Appendix T

Table A20. Background *E. coli* concentrations and physicochemical properties of water types used in simulated flush. The mean was calculated from 3 replicates.

		Physicochemical characteristics	
	E. coli concentration (CFU/100 ml or CFU/100g dry weight sand)	Salinity (‰)	pH
Tap water	0	0	7.63 ± 0.10
Effluent	2.24×10^4	0	7.39 ± 0.02
Seawater	2.70×10^1	32.67 ± 0.33	8.04 ± 0.01
Sand	4.12×10^2	-	-

Appendix U

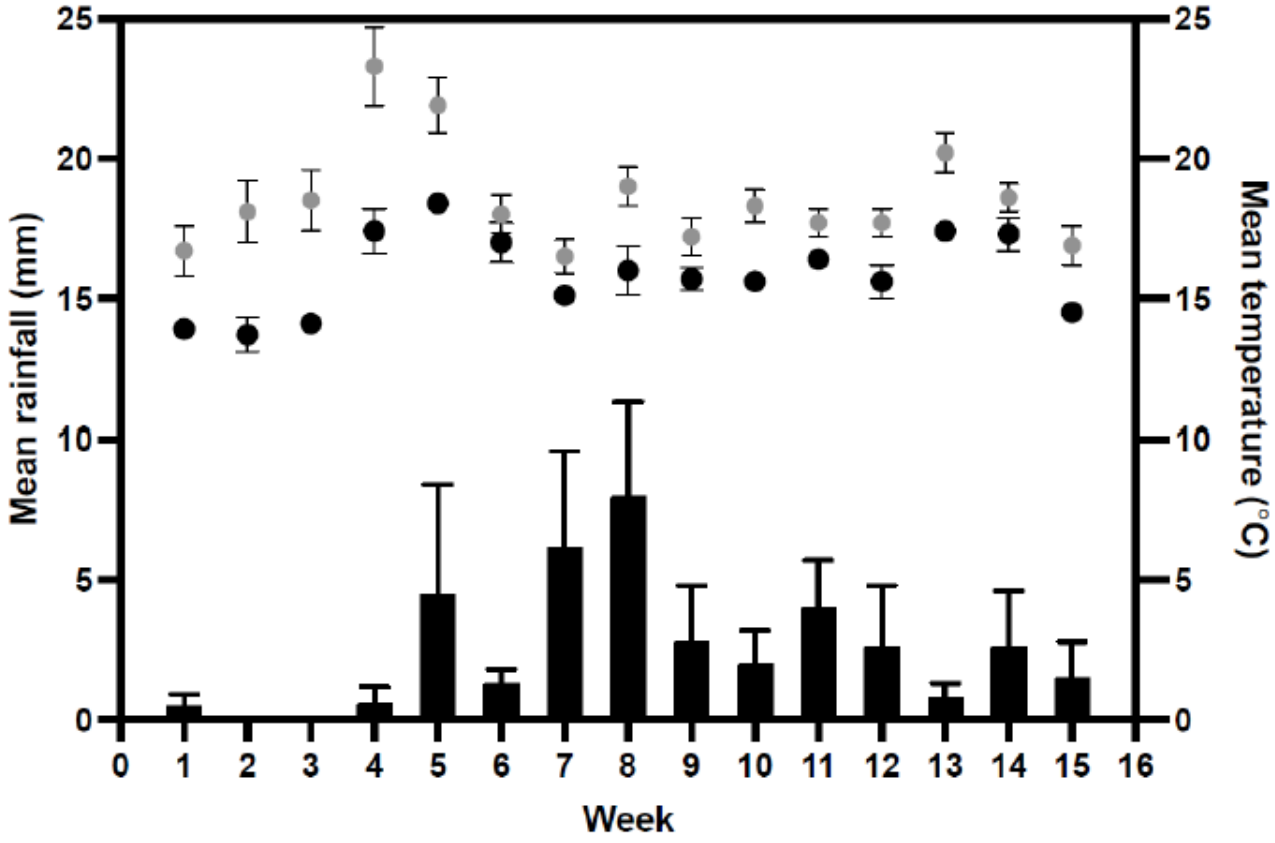


Figure A14. Rainfall (black bars), air temperature (black circles), and mesocosm temperature (grey circles). The weekly mean is displayed ± standard error.

Appendix V

Table A21. Sampling locations.

Site	Site type	Coordinates	Date	Substrate type
A*	Marine	55.640° N, -4.808° W	02/05/23	Sand
B	Estuarine	55.919° N, -4.464° W	02/05/23	Sand/silt
C	Freshwater	55.731° N, -3.906 ° W	02/05/23, 26/05/23	Grass bank, silt riverbed
D	Freshwater	56.021° N, -3.759° W	27/03/23	Mud/silt
E	Marine	55.968° N, -3.136° W	26/05/23	Sand
F*	Marine	55.954° N, -3.109° W	27/03/23	Sand

* Bathing water beaches

Appendix W

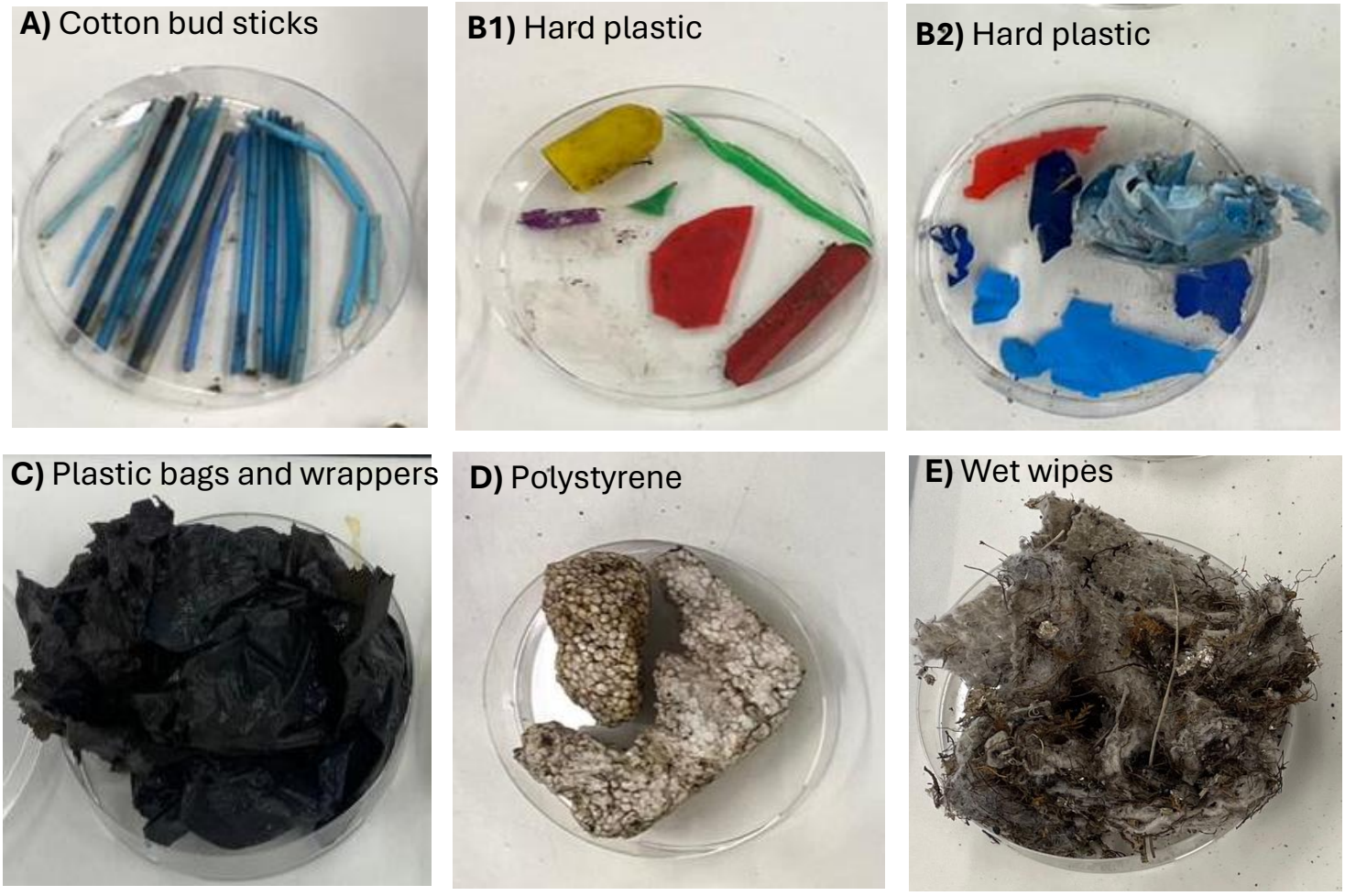


Figure A15. Examples of plastic samples.