Research Paper

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

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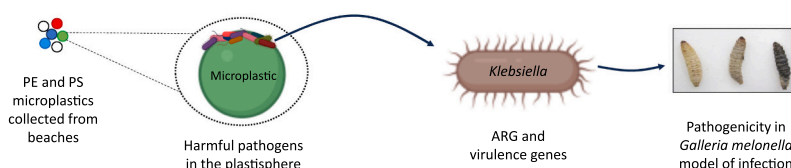
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HIGHLIGHTS

- Potential pathogens detected without the need for a pre-enrichment step.
- *Klebsiella* had ARG and virulence genes, some on plasmids from other species.
- *Klebsiella* isolates showed pathogenicity in a *G. mellonella* model of infection.

GRAPHICAL ABSTRACT

PE and PS microplastics collected from beaches

Microplastic

Harmful pathogens in the plastisphere

Klebsiella

ARG and virulence genes

Pathogenicity in *Galleria melonella* model of infection

ARTICLE INFO

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 pose a public health risk 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.

1. Introduction

Microplastics (particles < 5 mm in diameter) are the most abundant type of plastic pollution in the marine environment [79]. 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 [29,28]. The accumulation of microplastic beads in beach and coastal environments has been reported globally, with beads being distributed

heterogeneously along the high tide mark [1,62]. This is not only unsightly but may also pose a public health risk for recreational beach users [32,44].

Microplastics are rapidly colonised by microorganisms, which form 'plastisphere' communities [84] that are distinct from free-living communities and often contain or even enrich potential human pathogens [44,49]. 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 [86]. Discharge from wastewater treatment plants (WWTPs) is a known hotspot for microplastics and human pathogens [17,60], whilst hospital effluents create an additional risk, with bacteria in these effluents carrying five to ten times more resistant genes per cell than from domestic sources [55]. On their passage through WWTPs, microplastics can become colonised by pathogens, which can facilitate their persistence though the freshwater-estuary-coastal-beach sand continuum [45]. 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.) [46,54,62]. 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 virulence factors [35,75]. The presence of pathogenic-related taxa in the plastisphere does not necessarily correlate with pathogenicity and virulence [12,7]; and there remains uncertainty about whether pathogens attached to microplastics pose a relevant risk to human health [48,9]. 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 [56,59]. Two metaproteomic studies have demonstrated that potential pathogens were less active compared to other plastisphere taxa, with no proteins involved in pathogenicity detected [20,48]. More recently, plastisphere studies have used the insect virulence model *Galleria mellonella* as a proxy for the mammalian innate immune system (e.g., [49]) to address the uncertainty of whether pathogens in the plastisphere are actually pathogenic [7].

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 [44], 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 [6]. *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.

2. Materials and methods

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 (Fig. 1; Fig. S1). 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.

2.2. Sample collection and processing

Microplastic beads (polyethylene [PE] and polystyrene [PS] beads) were collected on five days in November 2022 (Fig. 1. Table S1. Fig. S2) 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; Fig. S2) and measured using callipers (WIHA dialMax ESD, Germany). The beads collected ranged in size from 2 to 5 mm diameter (mean = 4.1 ± 0.1 mm (*n* = 100)). Sand was 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 (Table S2; [58]). Sites where fewer than 10 microplastic beads of each polymer were collected were excluded from further analysis due to too few replicates.

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 [27]. 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 ([27]; Table S3).

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 [45]. 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 μm 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
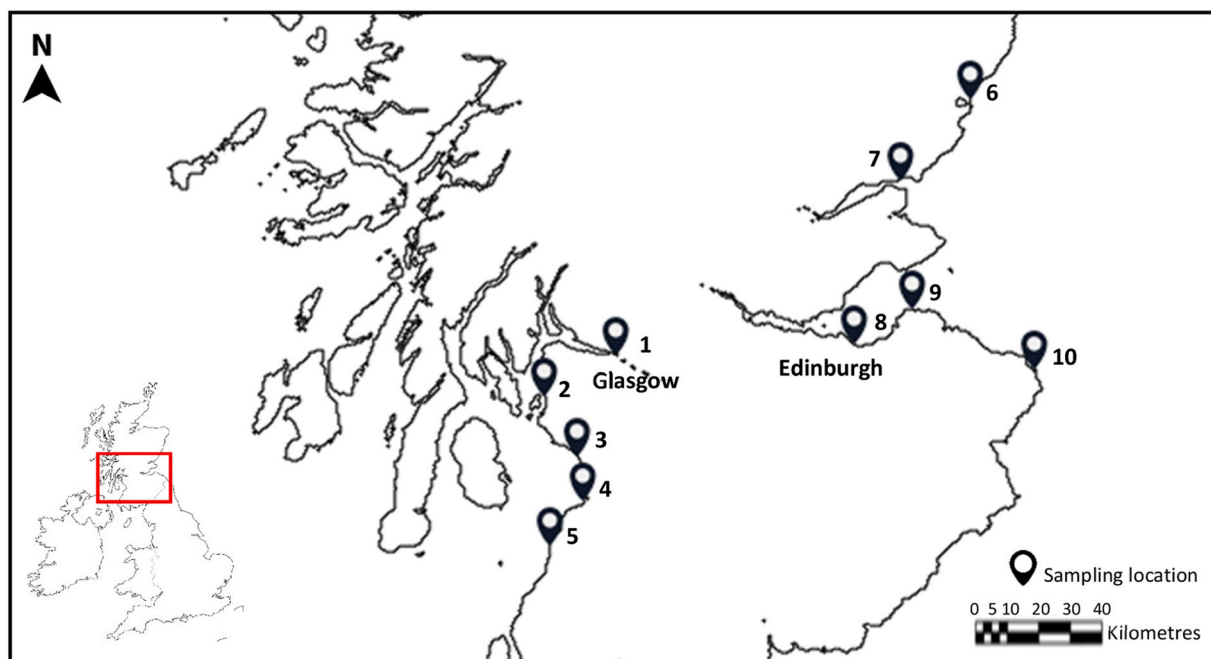
Fig. 1. Map of sampling locations (black points) on the east and west coasts of Scotland numbered according to Table S1.

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 of 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 μm 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.

2.5. PCR for Klebsiella identification

Klebsiella from Broughty Ferry (Site 7; Fig. 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 [72]). 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* [2]. Amplification reactions consisted of 12.5 μl master mix (New England Biolabs, UK), 2 μl primer stock (10 μmol/L) and 1 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 of 72°C for 7 min. All PCR products were run through a 1.5% agarose gel using GelRed ® 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.

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 [33]. 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; [52]), determine species ID (GTDBtk, v 1.7.0; GTDB R06-RS202; [16]), build a phylogenetic tree (SpeciesTree, v 2.2.0; [74]), predict pathogenicity (PathogenFinder, v 1.1; [19]), and determine the presence of virulence genes (VirulenceFinder, v 2.0; [14,25,41]), resistance genes (ResFinder, v 4.1; [11,14,83]), plasmids (PlasmidFinder, v 2.0.1,

Enterobacteriales database; [14,15]) and viruses (VirSorter, v 1.0.5; [65]). 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 (Table S4).

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 and 2.5 cm in length were used for all experiments using methods adapted from Ormsby et al., [49] (Fig. S3). 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 µl 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 µl of bacteria (approximately 10^4 to 10^6 CFU) using a 100 µl Hamilton syringe (Bonaduz, Switzerland) with a 0.6×30 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

µl 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.

2.8. Statistical analysis

Statistical analyses were conducted using R Studio version 3.3.2 (R [18]). 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 [66], followed by Tukey's post-hoc test. Data is reported as mean ± SE. *P* values < 0.05 are considered significant.

3. Results

3.1. Recovery of microplastics beads from coastal beaches

Microplastic beads were verified as either PE or PS (Table 1; Fig. S4; Table S3). PE was present at all sites, whilst PS was collected from nine beaches (Table S1). All PS beads were white, and the majority of PE beads were white (Table S2). 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

Table 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.

present at all sites (Table S1).

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 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 100 g^{-1} dry weight) at site 1, the site near the urban centre of Glasgow (where there was also signs of sewage discharge).

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 (Table S4; Fig. S5); this species is an emerging opportunistic human pathogen, which is closely related to, and lies within, the *K. pneumoniae* complex [64]. According to the Pathogen Finder programme which predicts the pathogenicity of an isolate by identifying proteins with a known involvement in pathogenicity [19], all isolates had a high probability of being a human pathogen (PE = 81%, PS = 89%, Sand = 88%, *K. pneumoniae* = 90%; Table S4), with several virulence and

resistance genes being encoded (Fig. 2).

All isolates possessed between five and six common virulence mechanisms identified using the package VirulenceFinder (Fig. 2; Fig. 3; Table S5). These included the gene *fimH* which was present in all isolates and encodes for type 1 fimbriae, a major adhesion 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 yersiniabactin 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. 2; Table S6). The genes *fosA, OqxA* and *OqxB* were present in all isolates and confer resistance to clinically relevant antibiotics such as fosfomycin, 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.

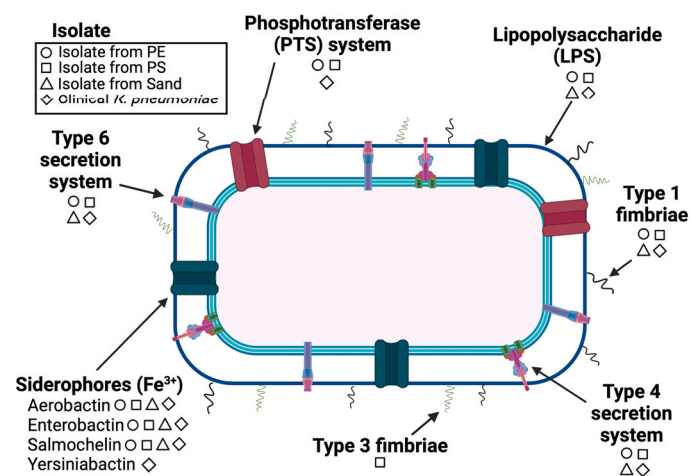Several plasmids were also detected (Fig. 2; Table S7); IncFIB(K) was

Fig. 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.
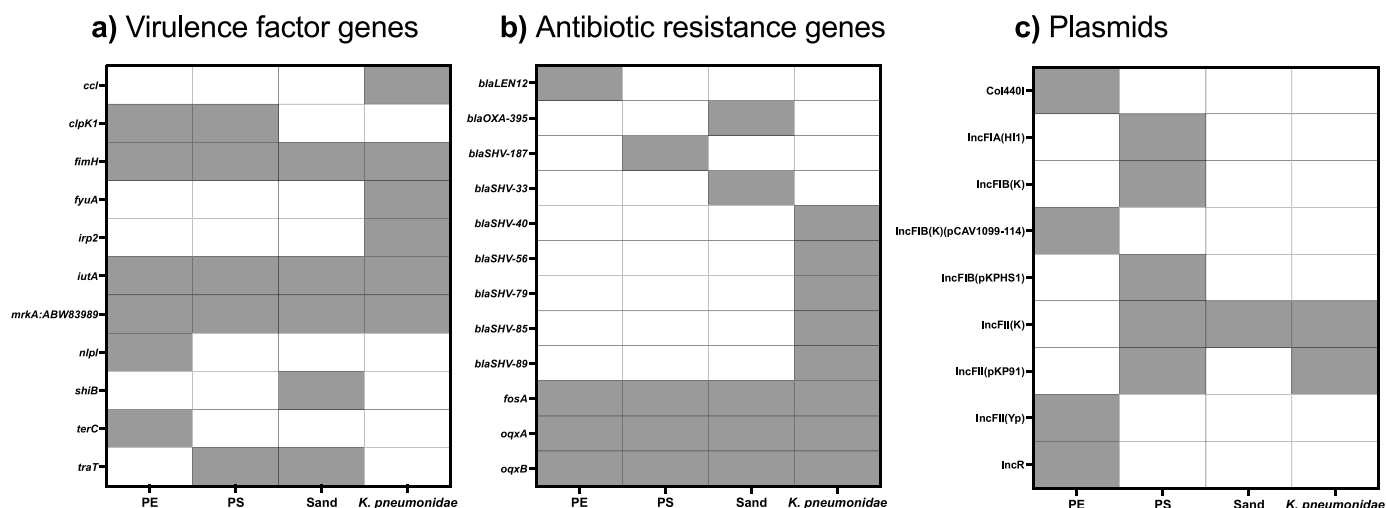

Fig. 2. Presence (grey) and absence (white) of (a) virulence factor genes, (b) antibiotic resistance genes and (c) plasmids, present in strains of *Klebsiella* isolated from PE, PS, and sand. For comparison, the profile of a clinical strain of *K. pneumoniae* has also been included.

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.

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; 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 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. 2) the substrate they were

Fig. 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 $\pm$ SE. * indicates a significant difference between at each time point.

isolated from did not significantly affect virulence gene expression in the *Galleria* model.

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.

4.1. Colonisation of microplastic beads by potential human pathogens

Although previous studies have identified potential pathogens on microplastic beads [44,47,62], 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 [5]. Yang et al., [80] 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., [20] 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 [37,45,31]; 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 [45]. This study was carried out in the winter when concentrations of both *E. coli* and *Pseudomonas* in stormwater are known to be lower [68]. 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 [34]. However, different serotypes and strains have different infectious doses [67]. 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 [34,67]. 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 [21]. 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 [42,43]. Importantly, more than one pathogenic species was present at each site, potentially increasing the likelihood of infection as multi-species biofilms can have higher
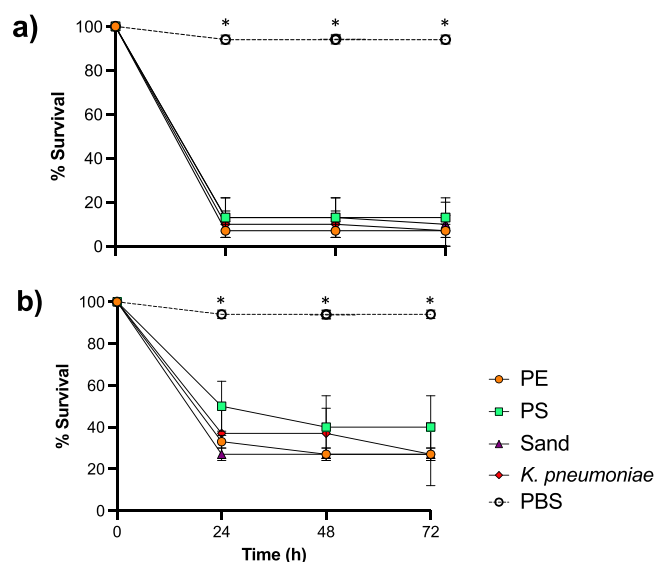
pathogenic potential [69].

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 noso-comial bacterial infections [4]. *Klebsiella* can become abundant in the plastisphere in WWTPs, with ten times greater concentrations on microplastics in effluent compared to microplastics in sewage [30]. All sequenced *Klebsiella* isolates in this study possessed the main factors essential for a high level of, e.g., lipopolysaccharide, fimbriae and siderophores [53,61], and demonstrated pathogenicity in a *Galleria* model of infection. Lipopolysaccharides prevent phagocytosis and inhibit complement-mediated lysis, enabling *Klebsiella* to establish infection [51]. Type 1 fimbriae were present in all isolates, an adhesion factor that contributes towards biofilm formation and stability in *Klebsiella* [61]. 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 [50]. Therefore, it is advantageous for *Klebsiella* to produce a variety of different side-rophores; 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 [72]. Bacterial communities in seawater are dynamic and constantly changing due to tidal flows and currents [81], 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 [85]. 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* [10]; 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.

4.3. 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* [49]. The virulence of plastisphere communities can also differ with polymer type, with communities on LDPE being less virulent than other polymers [37], 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 [7]; 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 [30,59], 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 possess different virulence mechanisms [22,63], it can be just as virulent [39]. 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 [40].

4.4. Klebsiella ARGs and mobile genetic elements

Increasing numbers of *Klebsiella* strains have become resistant to antibiotics [8], 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 [38,46], 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 [38]. 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.; [46,70]); *Pseudomonas* has been previously reported as the dominant host for ARGs, carrying 50% of ARGs on microplastics [73,77]. Silva et al., [71] 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* [23]; 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 plasmids, insertion sequences and bacterio-phages; plasmids remain as extrachromosomal circularised DNA or lin-earised DNA and can be transferred intra- and interspecifically between bacteria within the plastisphere before being integrated into the bacterial genome [13,78]. Plasmid transfer increases in frequency in bacteria associated with microplastics compared to free-living bacteria [3], whilst a higher proportion of plasmid associated ARGs are expressed in the plastisphere compared to in water [76]. Yuan et al., [82] 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 [26]; 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 [57]; this is likely due to the fact that microplastics contain higher metal ion concentrations compared to the surrounding environment [24]. 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.

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 [36]. 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.

CRediT authorship contribution statement

RM: Conceptualisation, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. LFM: Conceptualisation, Methodology, Investigation, Writing – review & editing. HLW: Conceptualisation, Methodology, Investigation, Writing – review & editing. MJO: Conceptualisation, Methodology, Investigation, Writing – review & editing SM: Conceptualisation, Methodology, Writing – review & editing. RSQ: Conceptualisation, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

Data Availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.132567.

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