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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.

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 has led to large volumes of plastic being released into the environment, where it persists and accumulates (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 2.2×10^7 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 (Kelly et al., 2021; Martínez-Campos et al., 2021; Junaid et al., 2022; Moresco et al., 2022). Once discharged from the WWTP, microplastics often pass through different environmental

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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, which will also influence biofilm formation and plastisphere communities (Duan et al., 2021). 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; Xue et al., 2020; Boni et al., 2021) and increase the potential for dispersal into new ecosystems (Sooriyakumar et al., 2022). Plastics recovered from the environment often harbour potential pathogens and FIOs (Rodrigues et al., 2019; Shi et al., 2021; Kelly et al., 2021; Metcalf et al., 2022b); although most of this evidence has come from 16 S 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.

2. Materials and methods

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.

2.1.1. Mesocosm set-up

Effluent and water samples were collected from three sites within the Forth Catchment (Dunblane, Bridge of Allan and Kirkaldy) in Scotland, UK between 4-6th of June, 2022 (shown in map of Fig. 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; 0.8×

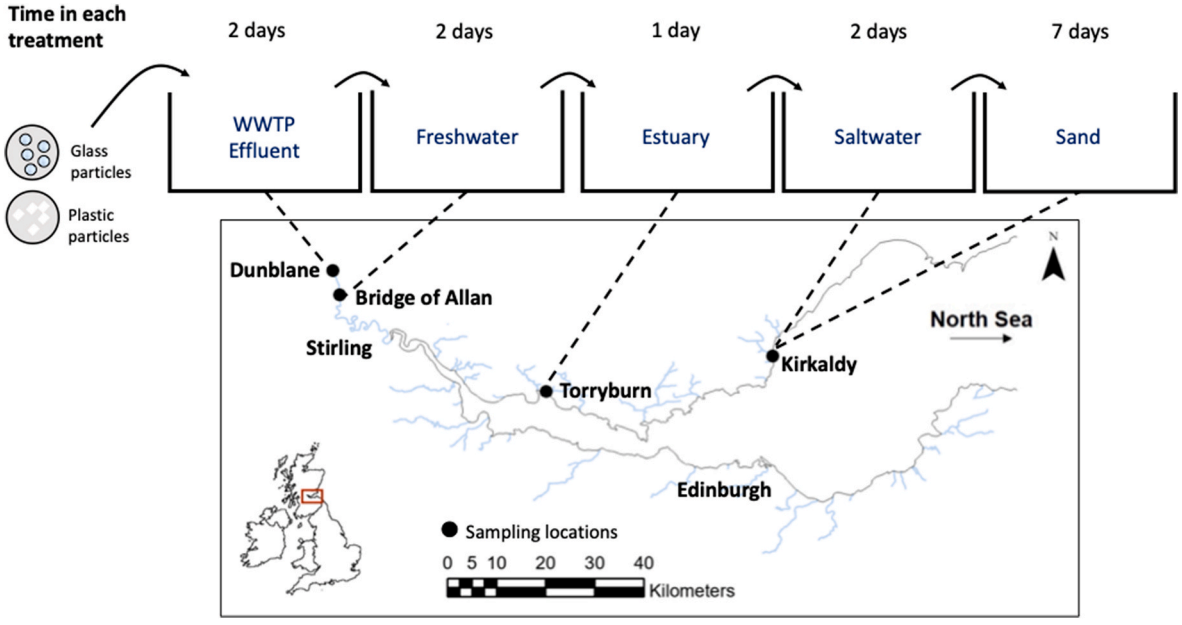

Fig. 1. Map of water collection sites along the Forth Catchment, Scotland, and the mesocosm setup for Experiment 2 showing the simulated transfer through the effluent-freshwater-estuary-marine-beach continuum.

magnification) to visualise differences in size, shape and surface characteristics (Fig. S1). 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 × 30 × 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*, 1.13×10^7 CFU/ml; *E. faecalis*, 8.11×10^5 CFU/ml; *P. aeruginosa*, 1.00×10^7 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).

2.1.2. Sample processing

At each timepoint (1, 2, 3, 4, 5, 6, 7, 9, 12, 17, 22 and 27 days) 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 1500 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.

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 12-16th of April, 2022 (Fig. 1). Salinity, EC, turbidity, and initial background concentrations of target bacteria in all water samples were quantified as described above.

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*, 4.36×10^7 CFU/ml; *E. faecalis*, 6.84×10^6 CFU/ml; *P. aeruginosa*, 2.40×10^7 CFU/ml. Following the initial 48 h 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. 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.

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 × 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. 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).

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):

$$\mathrm{Log}_{10}(C) = A + Be^{-\lambda t} \quad \text{(Eq. 1)}$$

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):

$$\mathrm{Log}_{10}(C) = \mathrm{Log}_{10}(C_0) - kt \quad \text{(Eq. 2)}$$

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. Results

3.1. Experiment 1

3.1.1. Physicochemical characteristics

FIO and *P. aeruginosa* concentrations were highest in the effluent samples at the time of sampling (Table 1). The initial EC in the freshwater (ranging from 0.27 to 0.31 mS) was significantly lower than the seawater (55.5–56.5 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).

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* (Table S1). The mean concentration of *E. coli* remained consistently higher on plastic compared to glass particles throughout the experiment (Fig. 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 (Fig. S1). 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 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 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.

3.2. Experiment 2

3.2.1. 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). *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); 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; 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. 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. 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. 4).

Table 1
Background bacterial and physiochemical properties of water types used in Experiment 1. The mean was calculated from three 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

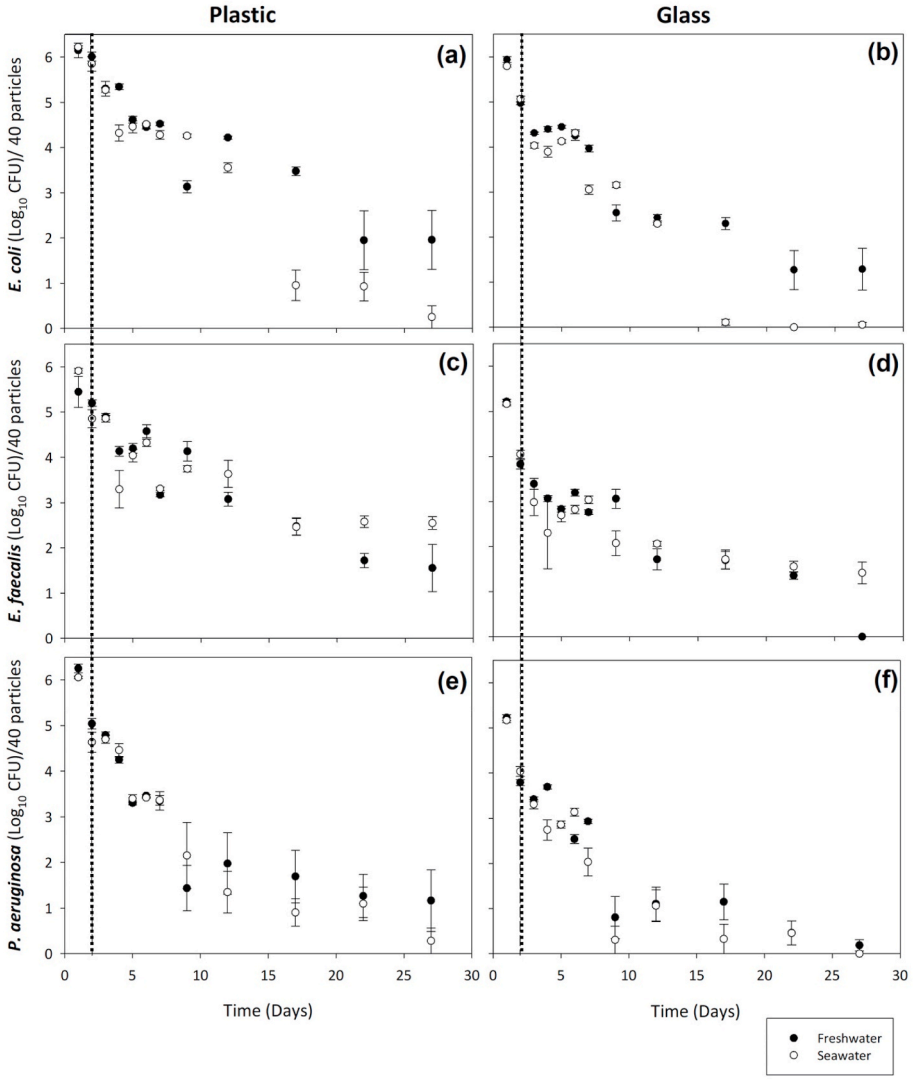

Fig. 2. Concentration of *E. coli* (a, b), *E. faecalis* (c, d) and *P. aeruginosa* (e, f) on microplastic beads (a, c, e) and glass beads (b, d, f) in freshwater (filled circles) and seawater (empty circles). The mean was calculated from four replicates, ± the standard error. Cages containing the beads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

Table 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 λ (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

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). 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).

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 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. 4) and biofilm quantity (Fig. 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

Table 3
Background bacteria and physicochemical properties of water types used in Experiment 2. The mean was calculated from three 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

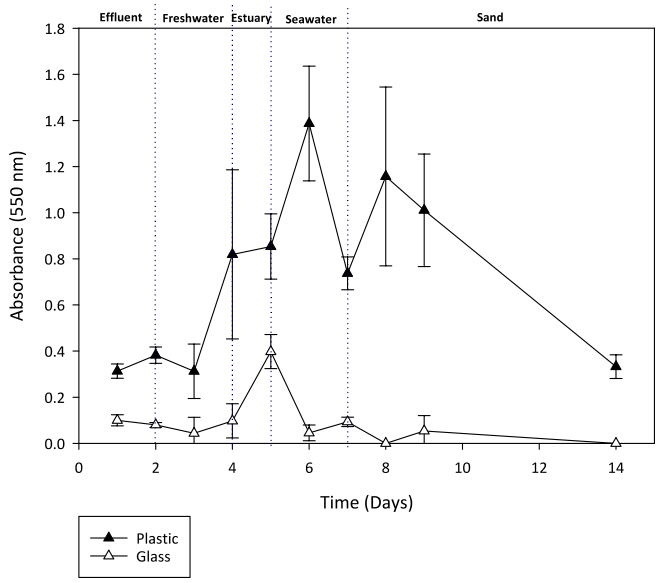

Fig. 3. Crystal violet absorbance values (A550) for biofilm on microplastic beads (filled triangles) and glass beads (empty triangles). The mean was calculated from four replicates, ± the standard error.

species; ANOVA, $F_{2,21} = 1.961$, $p = 0.166$).

4. Discussion

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, and 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 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,

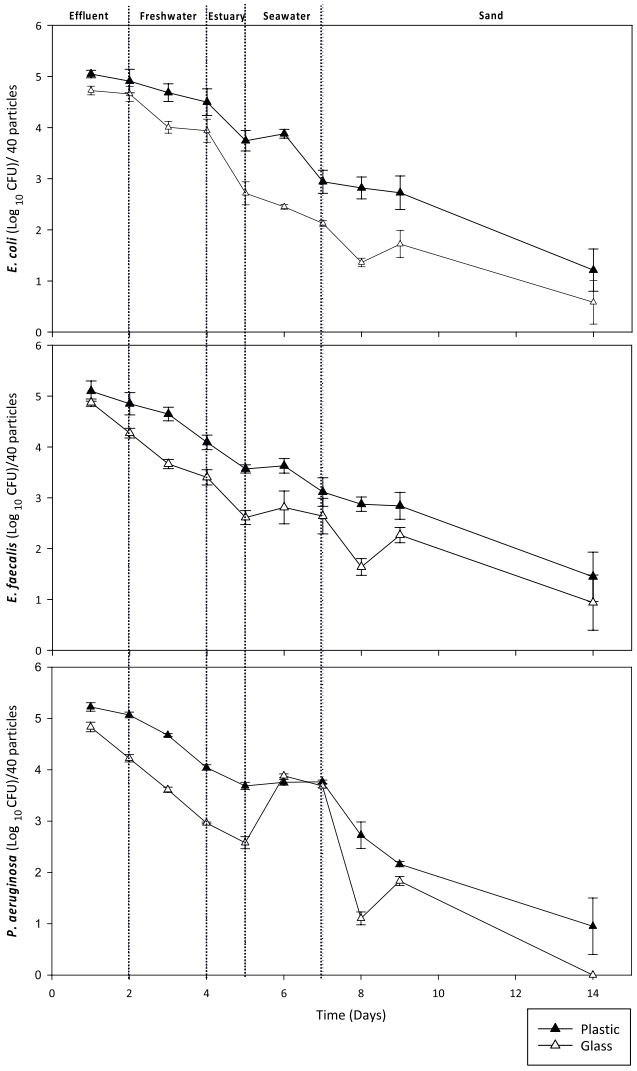

Fig. 4. Concentration of (a) *E. coli*, (b) *E. faecalis*, and (c) *P. aeruginosa*, on microplastic beads (filled triangles) and glass beads (empty triangles). The mean was calculated from four replicates, ± the standard error. The dashed vertical lines represent the transition to the next treatment.

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).

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 microplastic and glass particles decreased with

Table 4
Parameter values for bacterial die-off associated with linear models from Experiment 2.

		Modelled Linear Decline Rate (Day^{-1})[a]		*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

[a] Linear decline rate constant = (2.303 x Fig. 4 slope gradient).

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 UV irradiance (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 more 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., 2022b), 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 human health risk, 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.

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 (2018) 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.

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.

Author contributions

Conceptualisation: RM, HLW, MJO, DMO, RSQ. Methodology: RM, HLW, MJO, DMO, RSQ. Formal analysis: RM. Investigation: RM, HLW, MJO. Writing – Original Draft: RM. Writing – Review & Editing: RM, HLW, MJO, DMO, RSQ. Supervision – RSQ. Project administration - RSQ. Funding acquisition - RSQ.

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.

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Appendix A. Supplementary data

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