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Research Paper

Toxigenic *Vibrio cholerae* can cycle between environmental plastic waste and floodwater: Implications for environmental management of cholera

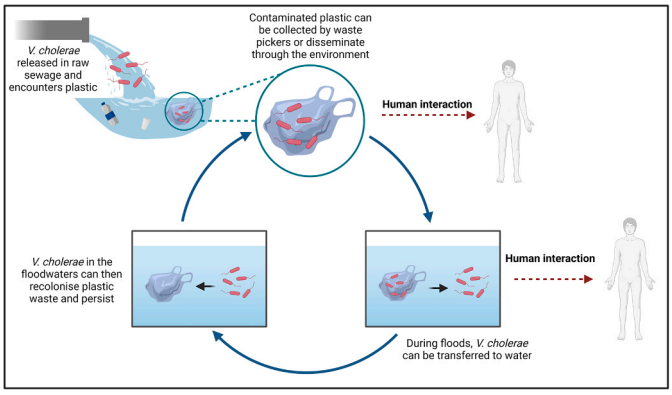
Michael J. Ormsby *, Luke Woodford, Hannah L. White, Rosie Fellows, David M. Oliver, Richard S. Quilliam

Biological and Environmental Sciences, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA. UK

HIGHLIGHTS

- Toxigenic *V. cholerae* can colonise and persist on plastic waste for at least 14-days.
- *V. cholerae* on plastic waste can transfer to, and persist in, floodwater.
- *V. cholerae* can survive on plastics, at concentrations capable of human infection.
- Pathogens colonising plastic pose a heightened environmental and public health risk.

GRAPHICAL ABSTRACT



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ABSTRACT

Globally, there has been a significant rise in cholera cases and deaths, with an increase in the number of low- and middle-income countries (LMICs) reporting outbreaks. In parallel, plastic pollution in LMICs is increasing, and has become a major constituent of urban dump sites. The surfaces of environmental plastic pollution can provide a habitat for complex microbial biofilm communities; this so-called 'plastisphere' can also include human pathogens. Under conditions simulating a peri-urban environmental waste pile, we determine whether toxigenic *Vibrio cholerae* (O1 classical; O1 El Tor; O139) can colonise and persist on plastic following a simulated flooding event. Toxigenic *V. cholerae* colonized and persisted on plastic and organic waste for at least 14 days before subsequent transfer to either fresh or brackish floodwater, where they can further persist at concentrations sufficient to cause human infection. Taken together, this study suggests that plastics in the environment can act as significant reservoirs for *V. cholerae*, whilst subsequent transfer to floodwaters demonstrates the potential for the wider dissemination of cholera. Further understanding of how diseases interact with plastic waste will be central for combating infection, educating communities, and diminishing the public health risk of plastics in the environment.

* Corresponding author.
E-mail address: Michael.ormsby1@stir.ac.uk (M.J. Ormsby).

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1. Introduction

There are an estimated 2.9 million cases, and 95,000 deaths attributed to cholera annually [25], with the majority of these occurring in low- and middle-income countries (LMICs) [58]. The number of countries reporting outbreaks of cholera has increased dramatically in recent years. Twenty-nine countries reported cholera epidemics in 2022, with 13 of these countries reporting their first known cases in 2022 [64]. In Africa, the number of cholera cases recorded in January 2023 was 30% of the total recorded cases for the whole of 2022. This represents an average case fatality ratio of almost 3%, which is an increase from the 2.3% reached in 2022, both case fatality ratios far exceeding acceptable levels according to WHO [64]. Malawi is currently experiencing its worst cholera outbreak in two decades, with over 56,000 cases and 1,712 associated deaths reported between March 2022 and March 2023 [40,65]. Recent outbreaks have also been reported in Bangladesh (January to April 2022), with over 495,000 cases and 29 deaths. In January to March 2023, Pakistan reported over 77,000 cases and Afghanistan over 22,800 cases including seven deaths [17].

Cholera is an acute diarrheal infection caused by the bacterium *Vibrio cholerae*, and often occurs through ingestion of contaminated food or water [16]. Cholera infection is characterised by extreme watery diarrhoea (often called 'rice water stool') that can lead to fatal dehydration. Two serogroups of *V. cholerae*, O1 and O139, are responsible for most outbreaks, although serotype O139, first identified in Bangladesh in 1992, is confined to South Asia. Serotype O1 isolates are further categorised into two biotypes: classical and El Tor. The El Tor biotype displaced the classical biotype as the predominant epidemic strain in 1961 and is responsible for the longest, and most severe, seventh pandemic [6]. Transcriptomic studies have shown that genes encoding proteins required for biofilm formation, chemotaxis, and transport of amino acids, peptides, and iron are expressed more highly in the El Tor biotype than the classical O1 biotype; however, the expression of genes encoding virulence factors are typically higher in the classical O1 biotype than in the El Tor biotype [8]. El Tor strains are considered to have greater environmental fitness than the classical strains, but classical strains can lead to a more severe form of cholera [14].

The required infectious dose of cholera is often high (approx. 1×10^8 colony forming units [CFU]), although much lower doses (between 100 and 1×10^4 CFU) can cause infection when intake is accompanied by a buffering agent, for example, following the use of acid-reducing medications, or with food [53]. Despite being easily treatable with rehydration solution, cholera remains a global threat due to its high morbidity and mortality in vulnerable populations that lack access to adequate health care. The increase in global cholera outbreaks in recent years is thought to have been driven by several factors, including climate change (resulting in increased flooding and droughts); limited healthcare and vaccine availability; and resource depletion due to outbreaks of other pathogens (such as COVID-19, dengue, chikungunya, measles, and monkeypox) [12,64].

V. cholerae naturally forms biofilms in the environment, e.g., seawater, estuaries, rivers, and lakes [15,54], and is frequently found associated with other aquatic organisms, e.g., zooplankton, phytoplankton, [2], and with abiotic materials including wood, cellulose, or glass [35]. Biofilm formation allows tolerance of abiotic and biotic stressors, such as nutrient limitations, pH changes, temperature, and salinity and can enhance environmental persistence of *V. cholerae* [52]. Biofilm-like aggregates of *V. cholerae* have been recovered from patient stool and are known to exhibit a hyper-infectious phenotype, suggesting that biofilms can play a role in transmission from the environment to the host, and in the spread of cholera between hosts [20,44].

Plastic pollution is increasing concurrently with both economic development and urbanisation in many cities in sub-Saharan Africa [3], and has now become a major constituent of landfills and urban dump sites [9]. Distinct microbial populations can colonise the surface of environmental plastic debris in what is collectively known as the 'plastisphere' [67], with increasing evidence that environmental plastic waste can become colonised with human pathogenic bacteria and viruses [37,39]. During the rainy seasons of many LMICs, plastic wastes block urban drainage systems, which can lead to localised flooding and an increased risk of human exposure to raw sewage and thus the spread of waterborne pathogens within highly populated areas [27,31]. Recently, enteric human pathogens such as *Escherichia coli* and *Salmonella* spp. have been found to persist in biofilms on environmental plastics and remain pathogenic [47,46].

In this study, we aimed to: (1) quantify the potential for toxigenic *V. cholerae*, including pandemic isolates of serotypes O1 classical, O1 El Tor and O139, to colonise and persist on plastic waste under simulated environmental conditions; (2) determine the ability of these strains to cycle between the plastisphere and floodwater; and (3) determine the pathogenic potential of these strains following their recovery from floodwater and the plastisphere. We quantified *V. cholerae* survival on the surface of two plastic polymers (polyethylene [PE] and polypropylene [PP]), and on the surface of excised banana leaf (BL) as a typical organic waste, under conditions characteristic of sub-Saharan Africa and South Asia. To determine the state of pathogenicity, a *Galleria mellonella* model of infection was employed to determine the infectious dose (ID50) for each strain, allowing comparison with the concentrations of *V. cholerae* recovered at each timepoint.

2. Materials and methods

Our experimental set-up represents a controlled simulation of a typical cholera outbreak scenario in sub-Saharan Africa and South Asia, whereby floodwaters contaminated with toxigenic *V. cholerae,* and sewage encounter plastic waste (and organic waste) in the environment. Following removal of floodwater, we determine if isolates of *V. cholerae* associate with the plastisphere, and how long they persist under environmental conditions of varying temperatures and periods of desiccation. Finally, the ability of *V. cholerae* to detach from the plastisphere and re-contaminate fresh floodwater is tested. In this way, we aim to establish the cyclical potential of *V. cholerae* for exploiting non-host environments for survival and onward transmission, and the implications of this novel habitat for wider environmental management of cholera.

2.1. Bacterial strains and growth conditions

The pathogenic strains of *Vibrio cholerae* used in this study were, serotype O1 classical strain 8021; Serotype O1 El Tor Strain V06/009; and Serotype O139 strain NCTC12945 (Table 1). Strains were serotyped using a MASTring (MASTring, Biomerieux, UK) slide agglutination assay, according to the manufacturer's instructions, to confirm their identity. Prior to use, isolates were grown in Luria-Bertani (LB) broth (Invitrogen, UK) at 37 °C and shaken at 120 rpm, unless otherwise stated. Following recovery from plastic or banana leaf surfaces (described later), isolates were grown on TCBS selective media (Merck, Germany), prepared according to the manufacturer's instructions. Periodically, throughout the experiment isolates were confirmed as *V. cholerae* by PCR (data not shown).

2.2. PCR for Vibrio cholerae identification

The presence of *V. cholerae* specific genes (*ompW* and *epsM*); the cholera toxin gene (*ctxA*); the toxin coregulated pilus gene (*tcpA*); and serotype specific O1 and O139 genes (*rfbO1*; *rfbO139*) were confirmed by PCR. Amplification reactions consisted of 12.5 µl master mix (New England Biolabs, UK), 1 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: 5 min initial denaturation at 94 °C, followed by 35 cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 1 min, with a

final extension of 72 °C for 5 min. All PCR products were assessed with a 1.5% agarose gel using GelRed® staining (Biotium, USA) and visualised with UV light. Primers used and amplicon sizes for each product are listed in Table S1.

2.3. Formation of natural biofilm on high density polyethylene, polypropylene and banana leaf

Forty-eight metal frames containing either blue high-density polythene (PE) bags (20 μm; Thali outlet, Leeds, UK; 16 frames), blue foil-backed polypropylene (PP) crisp packets ('Wotsits', Walkers Crisps, UK; 16 frames), or banana leaf (BL; Lakshmi stores, UK; 16 frames) were prepared in the same way as [46]. Briefly, materials were housed in bespoke high-grade stainless-steel frames (200 mm × 140 mm x 1.5 mm; frames were sterilised by autoclaving prior to the material being inserted) and submerged in either fresh surface water (subsequently called 'FW') or brackish water (subsequently called 'BW') to allow generation of a natural biofilm (Fig. 1a). FW was collected from the Allan Water river (Bridge of Allan, Scotland, UK); BW was prepared by mixing seawater collected from Kirkcaldy (Scotland, UK) with the FW (50:50 v/v). Eight frames of PE, PP and BL were submerged into one of six replicate glass tanks containing either FW (three tanks) or BW (three tanks), with each tank containing only one material type. Each tank contained seven litres of water, supplemented with a trace element solution to enhance biofilm formation (CuCl2.2H2O, 15 mg/L; NiCl2. H2O, 25 145 mg/L; Na2MoO4.2H2O, 25 mg/L; ZnCl2, 70 mg/L; MnCl2.4H2O, 100 mg/L; CoCl2.6H2O, 120 146 mg/L; FeCl3, 4 g/L; EDTA, 2 g/L; HCl [25%], 6.5 ml/L) [18]. The frames were arranged in such a way that the depth at which they were held did not differ between materials, ensuring the distribution of particles was not biased towards any particular material. Tanks were aerated continuously using a Pond Air Pump to enhance water movement (Swell, UK). The tanks were covered to prevent evaporation and all frames submerged for 7 days at ambient room temperature (ca. 18–21 °C) to allow a natural biofilm to develop on each material. Salinity, turbidity, and pH of the water in each tank were measured using a portable probe (Combo pH and EC, Hanna Instruments Ltd., UK) and a HI-88703–02 Bench Top Turbidity Meter (Hanna Instruments Ltd., UK) (Table S2).

2.4. Inoculation of frames

Eight individual replicate cultures (four to be used in FW; four to be used in BW) of each *V. cholerae* isolate were grown overnight in LB. Overnight cultures were then diluted 1:100 into fresh, pre-warmed LB (to 37 °C) and grown to an $OD_{600\ nm}$ of 0.5 (equating to approximately 4×10^8 CFU/ml). During this growth period, frames were removed from the tanks (Fig. 1b). Each large square in the frame (4 cm × 4 cm) was subsequently cut into four individual squares (2 cm × 2 cm), gently rinsed with PBS to remove loosely adhered cells, and placed into individual wells of a 12-well flat-bottomed polystyrene plate (Greiner, UK). Individual squares (2 cm × 2 cm) were taken from different frames to constitute the replicate samples. Each 12-well plate contained four individual replicates of PE, PP and BL. Once cultures had reached an $OD_{600\ nm}$ of 0.5, 9 ml were pelleted by centrifugation (4000 rpm, 10 min, 4 °C). Human faecal material (which had been stored frozen, and subsequently sterilised by autoclaving after thawing) was added to 80 ml of either FW or BW (with individual tubes for each replicate and each isolate) at a concentration of 10 mg/ml and thoroughly homogenised (these faecal suspensions are subsequently referred to as 'FW-FS' and 'BW-FS'). Bacterial pellets were then resuspended in either FW-FS or BW-FS. Individual wells of the 12-well plate were then flooded with 3 ml of either FW-FS or BW-FS containing individual isolates of *V. cholerae* ('contaminated floodwater'), placed into incubators at either 20 or 30 °C and left for four days. The exact concentration of bacteria in each inoculum was determined retrospectively by serial dilution and enumeration of CFU. This equated to approximately 1.4×10^7 CFU/ml. The FW and BW were screened to ensure the absence of *V. cholerae* prior to their use. Glycerol stocks (40%) of each initial inoculum were made and stored at − 80 °C. Contaminated floodwater was then removed by pipette, and plates were returned to the incubator until their respective sampling timepoint (24 h; 7 days; 14 days; 35 days). Herein, these samples are referred to as 'desiccated'. Temperatures were recorded every 1 h for the duration of the experiment using an i-Button temperature logger chip (iButtonLink, WI, 176 USA; data not shown).

2.5. Persistence of V. cholerae on PE, PP and BL

Twenty-four hours, and 7, 14 and 35-days after inoculation (DAI), 12-well plates were removed from the incubator, and individual pieces of PE, PP and BL moved to fresh 12-well plates to ensure that any *V. cholerae* recovered was from the material itself and not the well of the plate (Fig. 1c). Similarly, each piece of material was gently rinsed with PBS first to remove any loosely attached cells from the surface. Three hundred microlitres of PBS was added to the material, and left for 15 min. Using a sterile plastic loop, the PBS was agitated, and the surface of the material gently scraped to disperse the biofilm. From this suspension, 20 μl was serially diluted in PBS and plated onto selective agar (TCBS; Merck, Germany) for *V. cholerae* enumeration. The remaining suspension was added 1:1 with 80% glycerol and stored at − 80 °C.

2.6. Transfer of V. cholerae from PE, PP and BL to water and subsequent persistence in water

At each of the timepoints where colonisation of PE, PP and BL were measured by scraping (24 h, 7, 14, and 35 DAI), an additional set of replicate samples of each of the three materials were also moved to fresh floodwater not containing *V. cholerae* (10 ml; FW-FS or BW-FS) to measure transfer of *V. cholerae* from the material surface to the surrounding water (Fig. 1c). Floodwater was prepared as above with 10 mg/ml sterile faecal material. Tubes were held statically at 20 or 30 °C in the dark. At 24 h, 72 h, 7-day, 14-day and 21-DAI, 20 μl was serially diluted in PBS and plated onto selective agar (TCBS) for *V. cholerae* enumeration. A further 100 μl was added 1:1 with 80% glycerol and stored at − 80 °C.

2.7. Recolonisation of PE, PP and BL from contaminated water

To determine if any *V. cholerae* cells were able to recolonise new material following their survival in water, material (PE, PP and BL; 14 DAI sample, as generated in section 2.6) was removed from the floodwater at the 21-day timepoint, and new squares of PE, PP and BL with native biofilms (generated as previously) were added and left submerged for three days. Following this, the materials were recovered and added to a fresh 12-well plate, gently rinsed with PBS, before the biofilm

Table 1
Strain characteristics.

Strain	Designation	Slide agglutination			PCR					
		O1 Inaba	O1 Ogawa	O139	*ompW*	*epsM*	*ctxA*	*tcpA*	*rfbO1*	*rfbO139*
8021	O1 classical	-	+	-	+	+	+	+	+	-
V06/009	O1 El Tor	+	-	-	+	+	+	+	+	-
NCTC12945	O139	-	-	+	+	+	+	+	-	+

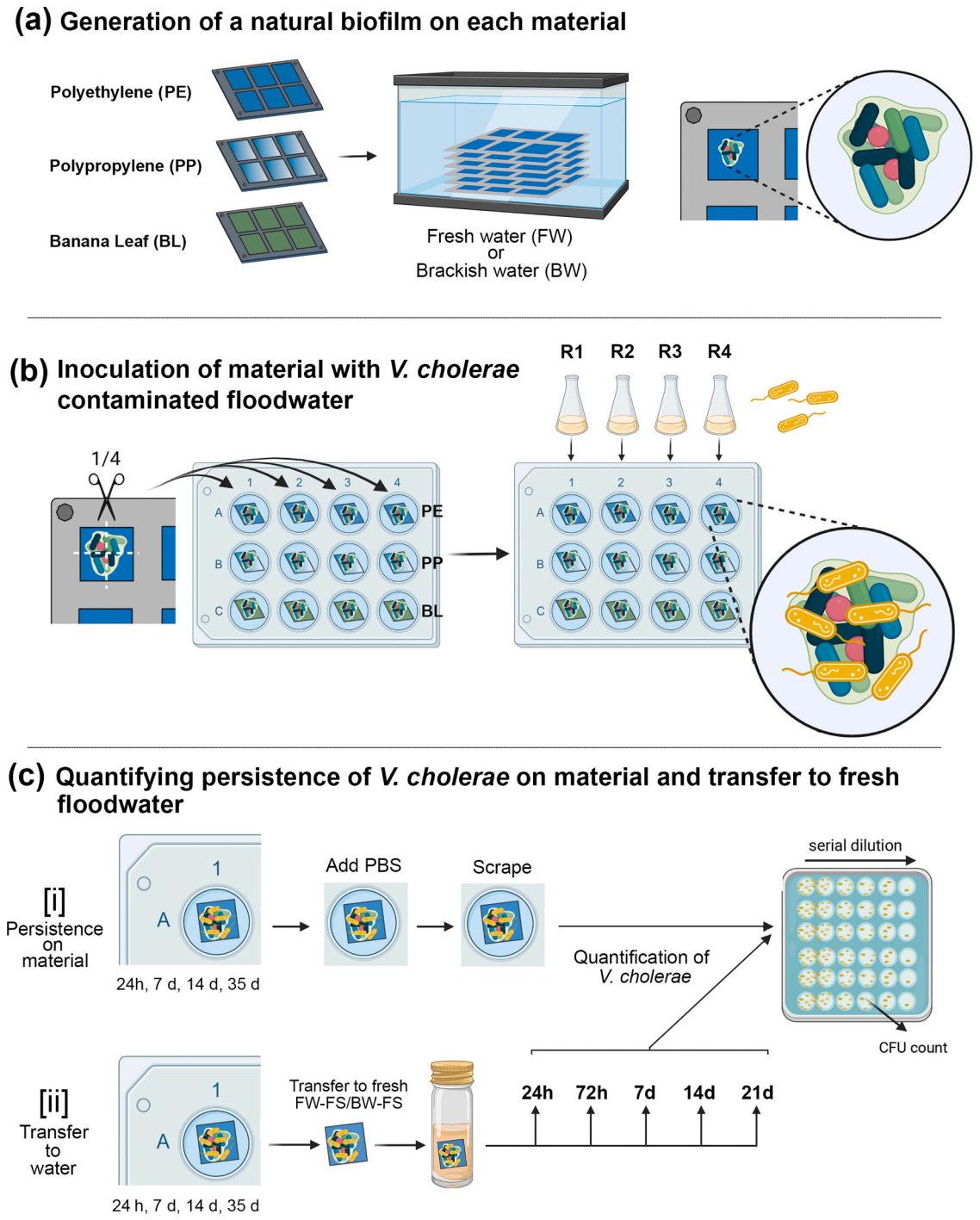

Fig. 1. Schematic overview of the sampling procedure for *V. cholerae* persistence on material and in water. Steel frames housing polyethylene (PE), polypropylene (PP) or banana leaf (BL) were submerged in either fresh water (FW) or brackish water (BW) for 7 d to allow a natural biofilm to form on the material (**a**). Individual squares were excised from the frame and added to individual wells of a 12-well plate (**b**). Bacterial cultures of each *V. cholerae* strain were added to a faecal suspension (FS) in either FW or BW and used to flood each individual square of PE, PP or BL. Floodwater was removed by pipette, before [i] persistence on material; or [ii] persistence in water were determined at designated timepoints (**c**). Figure was generated using BioRender (https://biorender.com/).

was removed and processed as described previously.

2.8. Challenge of Galleria mellonella larvae with V. cholerae

G. mellonella larvae (Livefood, Axbridge, UK), were kept in the dark at 15°C and used a maximum of one week after their delivery. Healthy larvae, measuring 2.0 – 2.5 cm in length and showing no signs of melanisation were used for all experiments. Using a pure culture of each *V. cholerae* strain (O1 classical, O1 El Tor and O139), a dilution series was generated for challenge into *Galleria* larvae, to determine the minimum infectious dose necessary for each strain. Concentrations ranged from 1×10^3 to 10^{12} CFU, at 10-fold increments. *V. cholerae* cultures were grown in LB, washed and resuspended in PBS as described above. A 10 µl sample was injected into each larva (in groups of 10 larvae for each concentration), directly into the hemocoel via the last right pro-limb. Following challenge, larvae were placed in an incubator at 37 °C and survival assessed for 72 h, with larvae considered dead when non-responsive to touch. Experiments were conducted in biological

triplicate. For all challenge experiments, an inoculation of PBS was used as a negative control ($n = 10$ larvae per experiment) to account for mortality caused by physical injury. In order to ensure that the quantity of bacteria was not responsible for lethality in the *Galleria* model, non-pathogenic *E. coli* (DH5α) were also included as a control. Plots of percentage survival vs. challenge dose were generated allowing for a comparison with the concentrations of *V. cholerae* recovered at each time point.

2.9. Statistical analysis

Statistical analyses were conducted using GraphPad Prism Software. Kruskal-Wallis test with Dunn's post-hoc tests were used to compare persistence over time, with P values < 0.05 considered significant. To calculate die off rates, CFU counts were normalised by transforming to $\log_{10}$ CFU/ml. Linear regression analysis, carried out in Minitab version 18 (Minitab Inc.; State College, PA, USA), was used to establish relationships describing the pattern of bacterial decline, and subsequently used to determine the die-off characteristics of *V. cholerae* under the

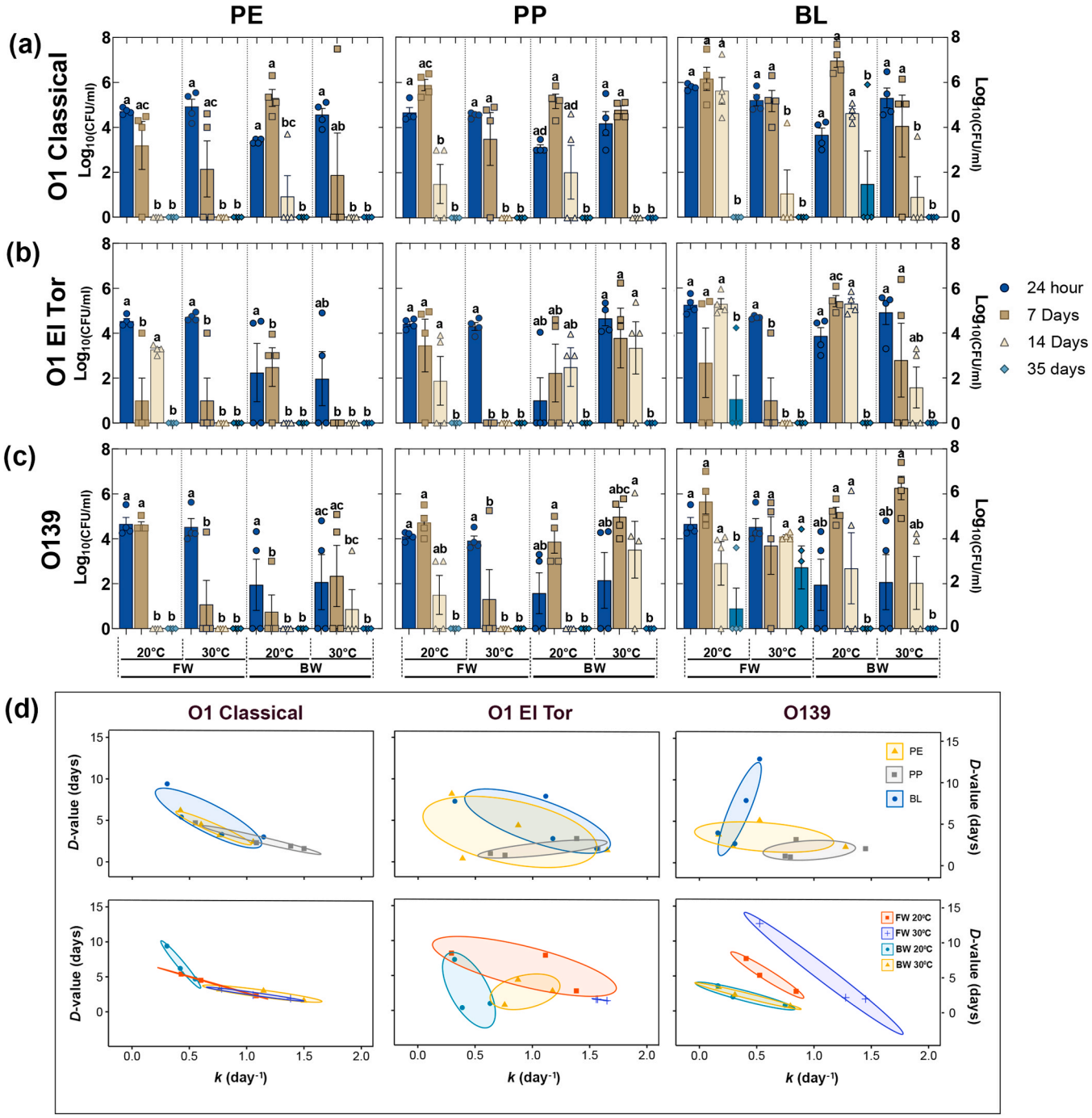

Fig. 2. The effect of temperature and water type on the persistence of different strains of *Vibrio cholerae*. Persistence on PE, PP and BL of *V. cholerae* (a) O1 classical [8021]; (b) O1 El Tor [VO6/009]; and (c) O139 [NCTC12945) at 20 °C or 30 °C in fresh water (FW) or brackish water (BW) supplemented with faecal material. Bars with different letters differ significantly from each other (Kruskal Wallis test, with uncorrected Dunn's post-test). Bars represent the mean (± SE) of four biological replicates. (d) linear decay analysis was performed to identify rates of decline dependent on temperature, water type and material, with data points grouped as 95% confidence ellipses plots [29].

different treatments using the same approach as Afolabi et al., (2020). The decrease in bacterial concentrations followed a linear decline over time, and so a log-linear regression model was fitted to the $\log_{10}$ transformed data and is described by the equation:

$$\mathrm{Log}_{10}(C) = \mathrm{Log}_{10}(C_0) - kt$$

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. Analysis of variance (ANOVA) was used to assess the effect of treatments on k values, and Tukey post-hoc tests used for mean comparisons. Confidence ellipses were calculated and generated using R [29,50].

3. Results

3.1. Persistence of V. cholerae on PE, PP and BL

Serotype O1 strains 8021 (O1 classical) and VO6/009 (O1 El Tor); and serotype O139 strain NCTC12945 were able to persist on PE, PP and BL at 20 °C in both FW and BW, with recovery observed in the samples that had undergone 24 h and 7 days of desiccation (Fig. 2). However, O1 classical and O139 isolates were not recovered from PE beyond 7 days in FW (O1 classical was recovered at day 14 in BW); while isolate El Tor was recovered after 14 days in FW. On PP, all three isolates were recovered after 14 days from the FW sample, however O139 was not recovered after 7 days in BW. On BL, isolates persisted with all strains recovered after 14 days irrespective of the water type. No isolates were recovered at the 35 DAI timepoint on PE or PP; however, single replicates of 8021 (20 °C, BW); VO6/009 (20 °C, FW); and NCTC12945 (20 °C, FW) were recovered on BL.

The persistence of isolates at 30 °C was more varied, with more isolates recovered at later timepoints on PE and PP in BW at 30 °C than at 20 °C; while in FW, isolates were less persistent at 30 °C than at 20 °C. On BL, most isolates were less persistent at 30 °C than at 20 °C, except for O139 isolate NCTC12945, which persisted for longer at 30 °C in FW than at 20 °C. At 30 °C, samples were visibly more desiccated 35 DAI than at 24 h and 7 DAI, with the banana leaves in particular, more shrivelled than at 20 °C.

Log linear regression models were applied to all replicates (R^2 ranged from 0.236 to 1.000) to determine modelled linear decline rate constants (k) and decimal reduction times (D-values) (Table 2; Fig. 2d). The material type did not have any impact on the survival rate of O1 classical isolate, 8021, with no significant differences observed between any material type. Similarly, temperature and water type (FW or BW) did not significantly affect the ability of isolate 8021 to persist. On PE, EL Tor isolate VO6/009 declined at a significantly quicker rate at 30 °C in FW than at 20 °C ($P < 0.001$); and O139 isolate NCTC12945 declined significantly quicker at 30 °C in FW than at 30 °C in BW ($P < 0.05$).

3.2. Transfer of V. cholerae from plastic to water

All three strains of *V. cholerae* were able to be transferred into fresh floodwater following dispersal from the plastisphere of desiccated samples at 24 h, 7-, 14- and 35-days (Fig. 3; Supplementary Figs. S1, S2 and S3). In some cases, isolates that were not detected following scraping of the desiccated materials were subsequently identified in the water, indicating resuscitation or proliferation. After 24 h of desiccation, almost all samples were able to transfer to FW-FS and BW-FS and persisted for at least a further 14-days. The O1 classical isolate readily persisted in FW-FS at 20 °C with cells recovered at the 21-day timepoint; O1 El Tor isolate VO6/009 showed comparable persistence at both temperatures; while O139 isolate NCTC12945 persisted for longer at 30 °C than 20 °C, with cells recovered on both PP and BL at the 21-day timepoint (in both FW-FS and BW-FS). Similar patterns were observed for the samples desiccated on PE, PP and BL for 7 and 14-days; however, samples persisted for longer in FW-FS than BW-FS (Fig. 3; Supplementary Figs. S1, S2 and S3). Although samples did not persist for as long in the BW-FS, they were recovered in greater concentrations than in the FW-FS, suggesting more favourable conditions for initial survival. Samples that had been desiccated onto each material for 35 days, where not recovered as regularly, with El Tor isolate VO6/009 recovered more frequently than the O1 classical and O139 strains.

Table 2
Linear decline rates and decimal reduction times for *V. cholerae* isolates.

Strain	Treatment			K (day^{-1})		D-value (days)	R^2
				Mean[a]	SEM		
O1 classical (8021)	**PE**	FW	20 °C	0.601	0.131	4.5	0.728
			30 °C	1.056	0.234	2.4	0.825
		BW	20 °C	0.421	0.085	6.2	0.505
			30 °C	0.763	0.093	3.2	0.664
	PP	FW	20 °C	1.084	0.223	2.3	0.896
			30 °C	1.383	0.300	1.9	0.941
		BW	20 °C	0.551	0.087	4.7	0.522
			30 °C	1.499	0.199	1.6	0.873
	BL	FW	20 °C	0.429	0.014	5.4	0.844
			30 °C	0.782	0.127	3.3	0.820
		BW	20 °C	0.305	0.061	9.4	0.539
			30 °C	1.147	0.458	3.0	0.844
O1 El Tor (VO6/009)	**PE**	FW	20 °C	0.294	0.033	8.2	0.622
			30 °C	1.651	0.067	1.4	1.000
		BW	20 °C	0.388	0.388	0.4	0.250
			30 °C	0.874	0.403	4.4	0.818
	PP	FW	20 °C	1.384	0.360	2.8	0.996
			30 °C	1.569	0.240	1.6	0.973
		BW	20 °C	0.631	0.407	1.0	0.469
			30 °C	0.758	0.463	0.8	0.500
	BL	FW	20 °C	1.114	0.513	7.9	0.953
			30 °C	1.558	0.233	1.6	0.974
		BW	20 °C	0.321	0.022	7.3	0.657
			30 °C	1.176	0.344	2.8	0.918
O139 (NCTC12945)	**PE**	FW	20 °C	0.526	0.113	5.1	0.812
			30 °C	1.276	0.151	1.9	0.918
		BW	20 °C	0.293	0.169	2.0	0.236
			30 °C	0.168	0.097	3.4	0.365
	PP	FW	20 °C	0.844	0.050	2.8	0.782
			30 °C	1.450	0.149	1.7	0.984
		BW	20 °C	0.748	0.437	0.8	0.500
			30 °C	0.794	0.470	0.7	0.500
	BL	FW	20 °C	0.408	0.137	7.5	0.724
			30 °C	0.526	0.339	12.5	0.834
		BW	20 °C	0.161	0.094	3.6	0.332
			30 °C	0.310	0.209	2.3	0.274

[a] Linear decline rate constant = (2.303 x slope gradient). Some R^2 values of 1.000 were generated due to there being only two data points.

3.3. Recolonisation of PE, PP and BL from contaminated water

To determine if any *V. cholerae* cells were able to recolonise new material following their survival in water, material (PE, PP and BL; 14 DAI sample, as generated in section 2.6) was removed from the floodwater at the 21-day timepoint, and new squares of PE, PP and BL with native biofilms were added. Each strain was only able to colonise PP in the FW-FS (albeit not in all replicates), with the O1 classical isolate 8021 being the most able to recolonise (4/16 recolonised) (Fig. 4). Only one replicate of El Tor isolate VO6/009 (FW-FS; 20 °C) and two replicates of O139 isolate NCTC12945 (FW-FS; 20 °C and 30 °C) were recovered. No recolonisation was detected in BW-FS on PP. Similar rates of recolonisation were observed on PE, with O1 classical (4/16) recolonising in FW-FS more frequently than El Tor (1/16) or O139 (1/16). However, O1 classical and El Tor were recovered on PE in BW-FS at 30 °C, even though no CFU had been identified in the original water. Significant numbers of CFU were recovered from the BL samples, in which almost all replicates of all strains (in both water types and temperatures) were

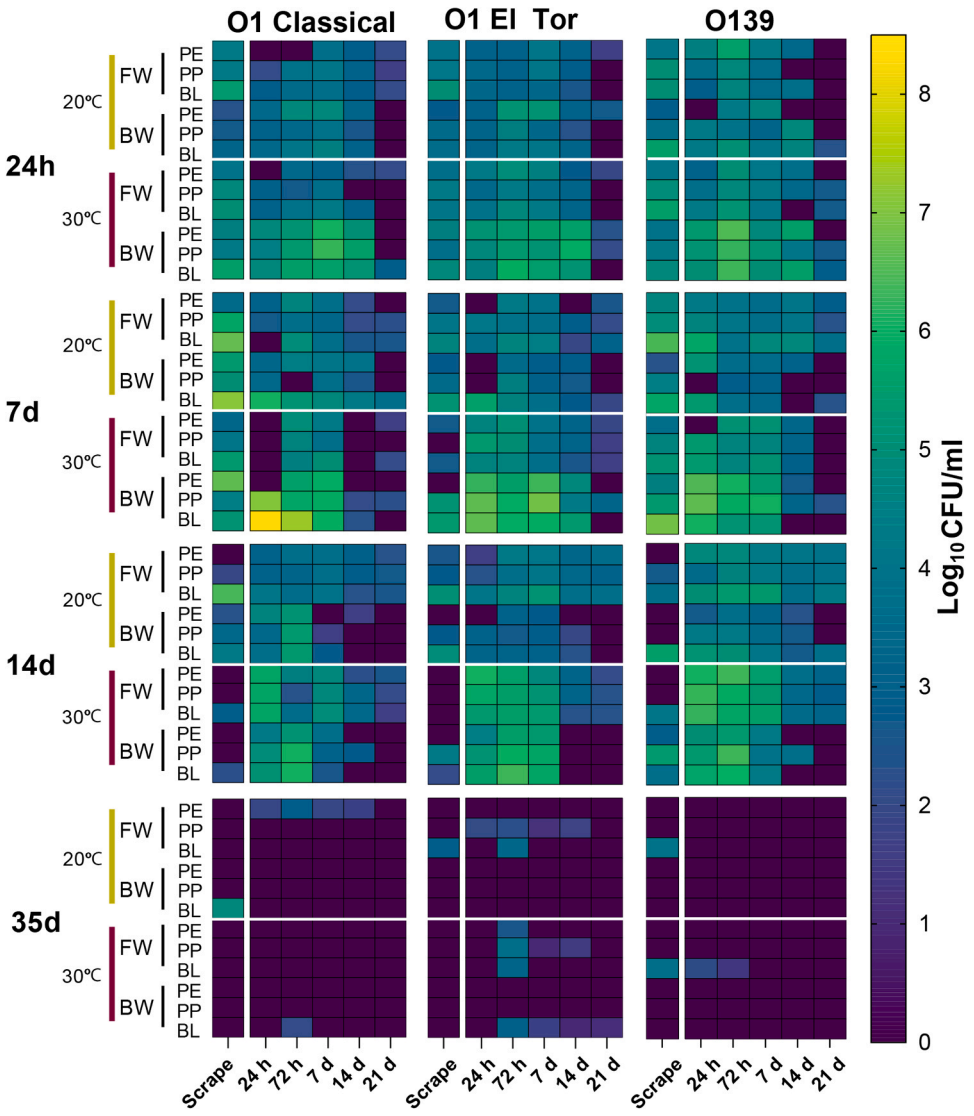

Fig. 3. The persistence of different strains of *Vibrio cholerae* in water following their recovery from the plastisphere. O1 classical [8021]; O1 El Tor [VO6/009]; and O139 [NCTC12945] were recovered from PE, PP and BL after desiccation for 24 h, 7d, 14d or 35d and transferred to fresh water (FW) or brackish water (BW) supplemented with faecal material. Cells of viable *V. cholerae* were enumerated by plate counts at defined intervals. Four biological replicates were included in each experiment and are presented as the mean.

recovered ($P < 0.05$). Viable CFUs were recovered on BL in the BW-FS samples where no viable CFUs were identified in the contaminated water prior to its addition.

3.4. Retention of pathogenicity of *V. cholerae* following long term persistence

In all cases, as the infectious dose of *V. cholerae* increased, the survival rate of *Galleria* larvae decreased (Fig. 5). After 72 h, the required dose to induce 50% lethality (ID50) in the *Galleria* larvae was 1.1×10^9 CFU/ml, 2.1×10^5 CFU/ml and 1.3×10^6 CFU/ml for O1 classical, O1 El Tor and O139, respectively. Analysis of the CFU/ml of each strain following survival in FW-FS and BW-FS revealed that O1 classical did not reach the required CFU on PE, PP or BL to induce 50% mortality, at any timepoint. Sufficient cells of El Tor were recovered in both the FW-FS and BW-FS samples, particularly after growth at 30 °C. Similarly, O139 was recovered at concentrations high enough from both the FW-FS and BW-FS at 30 °C to induce 50% mortality in *G. mellonella*. At the 72 h timepoint, the PBS control did not cause significant larval mortality, whilst the *E. coli* DH5α did not induce more than 40% lethality at the highest dose of 1×10^{12} CFU/ml (Supplementary Fig. S4), indicating that the pathogenicity observed by *V. cholerae* cells was due to virulence and not bacterial load or background material.

The infectious dose of most isolates fell within the human infectious dose range of *V. cholerae* in healthy individuals (between 1×10^3 and 10^8 CFUs/ml [53]) (Fig. 5). Following desiccation onto PE, PP and BL for 7 days, all three strains of *V. cholerae* were recovered in concentrations high enough to cause infection. After 14 days, O1 classical and O1 El Tor isolates were only recovered in sufficient concentrations from samples held at 20 °C, while the concentration of O139 isolate NCTC12945 was much less at this point. After 35 days, only several strains remained at infectious levels, all of which were recovered from PE. Most strains reached infectious levels in FW-FS and BW-FS following their transfer from PE, PP and BL, irrespective of the length of desiccation time prior to inoculation of the water, with isolates inoculated into BW at 30 °C recovered at the highest levels. However, following 21 days in the water, most strains had fallen below the minimum infectious dose required, with only sporadic recovery observed.

4. Discussion

This study has demonstrated that the major toxigenic serogroups of *V. cholerae* are capable of colonising plastic waste under simulated environmental conditions and persisting for extended periods following desiccation. Furthermore, these pathogens can be transferred to water from both plastic and organic waste, remain viable, and in some cases, replicate to levels sufficient to cause disease. This confirms that plastic waste in the environment can act as a reservoir for cholera and

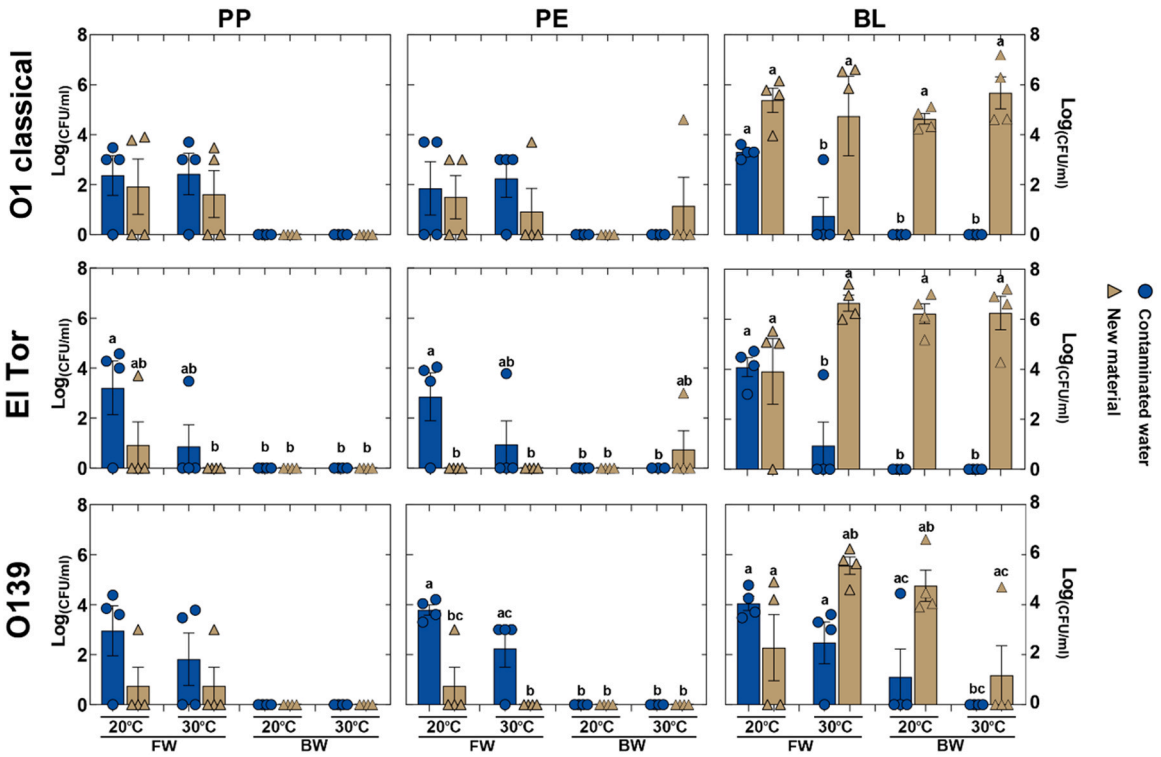

Fig. 4. Colonisation of PE, PP and BL by water contaminated with *V. cholerae* recovered from the plastisphere. Recolonisation of PE, PP or BL from 21-day old fresh water (FW) or brackish water (BW) containing either O1 classical (8021), O1 El Tor (VO6/009) or O139 (NCTC12945) *V. cholerae*. Bars with different letters differ significantly from each other (Kruskal Wallis test, with uncorrected Dunn's post-test). Four biological replicates were included in each experiment and each bar represents the mean ± SE.

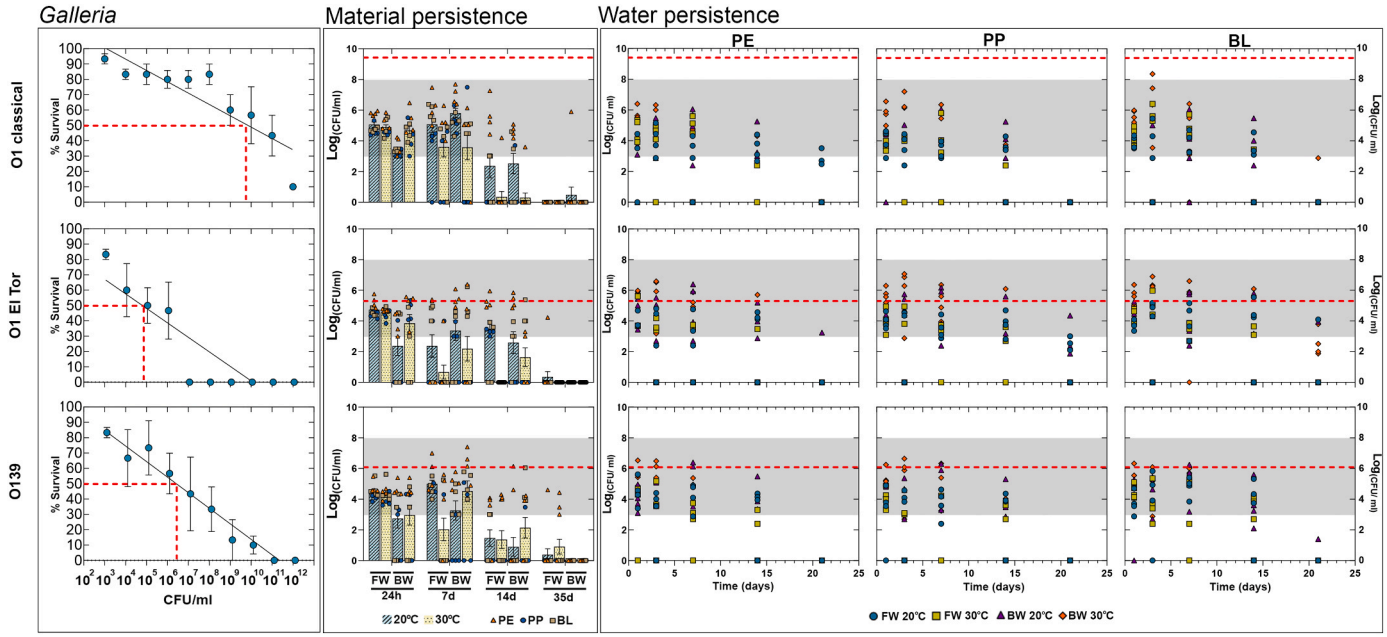

Fig. 5. Calculation of ID50 of each isolate of *V. cholerae* in a *G. mellonella* infection model. *G. mellonella* larvae were challenged with *V. cholerae* O1 classical (8021); O1 El Tor (VO6/009); or O139 (NCTC12945) strains at increasing concentrations, and the survival measured 72 h post challenge. Data points represent the mean ($n = 10$ *G. mellonella* larvae) of three independent biological replicates ± SE. ID50 was calculated using equations generated by linear regression analysis. The ID50 is indicated for each strain through addition of a dashed red line, with points above this line surpassing the ID50 threshold in the *Galleria* challenge model. The greyed box indicates the range of the accepted human infectious dose of *V. cholerae* ($1 \times 10^3 - 10^8$ cells). Persistence in water shows individual replicates recovered, following inoculation of the water with the 24 h colonised material.

represents a significant environmental and public health risk.

It is well documented that the aquatic environment allows for the long-term persistence of *V. cholerae* [15,33], although the environmental reservoir of *V. cholerae* has remained elusive, particularly in the periods between seasonal epidemic outbreaks. Some evidence has indicated that aquatic flora and fauna, including copepods and algae, may act as inter-epidemic reservoirs of *V. cholerae* [26]. Our study has shown that *V. cholerae* can associate with biofilms that have formed on both plastic waste and organic material, and that both the surrounding environment and microbial community composition of the plastisphere can differentially affect the persistence of *V. cholerae* strains following desiccation. The materials employed in this study have inherently different characteristics, which could in part explain the observed differences in persistence and transmission of *V. cholerae* isolates. For example, the differences in hardness, wettability, and zeta-potential between PE and PP has been shown to influence biofilm biomass, with greater total organic carbon observed in PE biofilms than in PP biofilms. Similarly, the microbial community composition has been observed to differ between biofilms associated with each plastic type. Ultimately, this may impact the ability of *V. cholerae* to associate and persist on PE and PP [23,48]. The surface characteristics of the banana leaf will differ greatly from that of PE and PP, suggesting that the biofilm community here will also vary. The nutrient availability will be greater on the banana leaf (as it is an organic material), which likely explains why the *V. cholerae* cells were recovered for much longer following desiccation on BL than on either PE or PP.

The growth of *V. cholerae* is primarily modulated by temperature and salinity, in combination with other factors such as pH, turbidity, organic components, and the chemical composition of water [15]. Environmental survival of *V. cholerae* is also likely to be influenced by strain-specific characteristics, for example, the El Tor strain became unculturable at a much quicker rate than both the O1 classical and O139 strains on PE, PP and BL. Although in some cases, El Tor became undetectable on PE and PP after 24 h, it readily proliferated when transferred to water, suggesting that this isolate transitions to a VBNC (viable but nonculturable) state more rapidly than the O1 classical and O139 isolates employed here. Similarly, El Tor could be resuscitated to a viable state more quickly than the other two strains. Previous work has indicated that the speed of VBNC development in *V. cholerae* is strain dependent, with some El Tor strains entering VBNC more rapidly than others, and some O1 classical strains progressing to a VBNC state faster than El Tor strains [66]. The ability to induce the VBNC state offers an advantage to organisms living in dynamic environments, where sudden fluctuations can potentially threaten survival [43]. The VBNC response to changes in environmental conditions may allow different species to dominate the same geographical location at different times of the year [43], with environmental parameters known to influence the transition of *V. cholerae* to VBNC [66].

Resuscitation of VBNC *V. cholerae* in the environment can be induced by quorum sensing, where non-cholera *Vibrio* spp. overproduce autoinducer molecules. Subsequently, there may be a role for non-cholera *Vibrio* spp. resuscitating toxigenic *V. cholerae* O1 and leading to seasonal cholera epidemics [42]. Although the role of the plastisphere in autoinducer expression has not yet been examined, the substrate surface is likely to induce genes for biofilm formation and virulence. While the O1 classical and O139 strains grew well in fresh floodwater in this study, the El Tor isolate was the only strain that was able to persist and return to a culturable state on PE, PP and BL following a 35-day desiccation period. This tolerance could in part explain why this strain is responsible for the ongoing seventh pandemic and displacing the O1 classical strain.

All strains appeared to favour growth in the brackish floodwater following transfer from biofilms, particularly at the higher temperature of 30 °C. *Vibrio* spp. prefer warm, slightly salty water, with most cholera cases occurring during warmer months [5]. Unlike most *Vibrio* spp., *V. cholerae* can tolerate the absence of salt [30], although it grows more quickly in salty water than fresh water [5]. For the development of VBNC development in *V. cholerae*, important factors include growth phase, oxygen availability, and nutrient availability [66], with strains less able to enter VBNC at higher temperatures [22]. Higher temperatures can, however, induce increased biofilm formation in the El Tor strain through increased expression of mannose-sensitive hemagglutinin (MHSA), which is often correlated with increased growth [57].

Concentrations of El Tor and O139 recovered from the brackish floodwater following transfer from biofilm were above the ID50 thresholds calculated from the *G. mellonella* infection model, which is increasingly being used as a surrogate model to assess the pathogenic potential of human pathogens [11,24,7]. The concentrations of O1 classical, O1 El Tor and O139 persisting on desiccated material, and in the simulated floodwater, were well within the concentrations capable of causing infection (i.e., between 1×10^3 and 1×10^8 CFUs/ml [53]). This indicates that plastic (PE and PP) and organic material (BL) can provide a substrate for infectious concentrations of *V. cholerae* to persist in a desiccated state and can provide enough inoculum to contaminate subsequent fresh or brackish floodwater (such as would happen during the wet seasons and flooding events) and allow *V. cholerae* to proliferate to high enough concentrations to cause infection. Although we have presented concentrations per millilitre for comparative analysis, when scaled up to concentrations that humans would realistically interact with (such as when drinking water), the number of pathogenic organisms consumed would be significantly higher, increasing the risk of infection further. While contact with the aquatic environment is an accepted route for infection by *V. cholerae*, our data highlights that direct human interaction with plastic pollution (and organic wastes) could also provide an alternate route of infection.

Toxigenic *V. cholerae* strains have been isolated from domesticated animals in areas of current cholera outbreaks [26]; however, domestic, feral, and wild animals, are attracted to dumpsites by human faecal material [13,62]), and subsequent contamination of urban waste piles with animal faeces can add further to the potential pathogen reservoir persisting in the plastisphere [47]. There is also some evidence that insects may be able to transmit cholera, e.g., houseflies (*Musca domestica*) [19,21], and Chironomids (non-biting midges) [68]; insects commonly interact with both plastic and organic waste at dumpsites, and if contaminated with *V. cholerae* could be acting as a vector of mechanical transmission [34].

The ability of *V. cholerae* to survive outside of the host is likely due to its intrinsic ability to form biofilms and associate with inorganic materials and aquatic organisms, including planktonic copepods, zooplankton, and freshwater amoebae [1,59]. At certain times of the year, rainfall run-off in coastal and estuarine areas can be high, resulting in fluctuations in nutrient concentrations that often lead to higher phytoplankton abundance, followed by zooplankton blooms [28,36]. Typically, brackish water from transitional zones is highly turbid as it results from the mixing of fresh and salt water coupled with the resuspension of sediments. The high level of mixing could also increase the chitin content of the water (derived from phytoplankton and zooplankton), which has a positive effect on the growth of *V. cholerae* [56]. Recently, it has been hypothesised that eukaryotic organisms form a major component of the plastisphere [45], and such prokaryote-eukaryote interactions could facilitate the adherence and persistence of *V. cholerae* biofilms, with the plastisphere providing subsequent protection against fluctuating environmental conditions and nutrient-depletion.

Although abiotic surfaces like oceanic plastic debris support *Vibrio* spp. biofilm formation (Kirstein et al., 2016), *V. cholerae* is not frequently associated with environmental plastic waste, with few reports of *V. cholerae* being recovered from plastic in the environment [38,51,55]. To form biofilms and adhere to abiotic surfaces, *V. cholerae* strains typically require a type IV pilus, MSHA, and flagellum [61]; however, it has been shown that O139 strains require only the flagellum for such association [60]. For *Vibrio* to develop more structurally advanced biofilms, they produce *Vibrio* polysaccharide (vps); however, strains of

V. cholerae can produce biofilms in a vps-independent manner, in the presence of Ca^{2+}, which is abundant in seawater but limited in freshwater [33]. However, whether the association with different plastics affects expression of vps, flagella, MHSA and type IV pilin, particularly in response to different environmental conditions remains unknown but would provide important information on how different strains persist in the environment.

Colonisation of plastics by *Vibrio* spp. occurs within the first hour of exposure due to their rapid growth rates [32,38,49]. Gradual warming of water favours potentially pathogenic *Vibrio* spp. and the subsequent emergence of infections, with a recent study showing that increases of seawater temperature can influence the adhesion of *V. parahaemolyticus* to plastic [10,4]. This has implications for the effect of climate change on the distribution of cholera over larger geographic areas, e.g., the dissemination of strains of *V. cholerae*, including O139, which are currently confined to Asia. The ability of *V. cholerae* to colonise and persist on plastics, coupled with the lightweight and buoyant properties of plastic could facilitate the transfer of cholera through the landscape, and increase the risk of contaminating areas where direct human exposure can be high. In comparison to organic materials, synthetic polymers like plastics are slowly degraded and could therefore provide an environmental substrate for the continual re-contamination by *Vibrio* spp. over decades or even centuries. Many areas containing less suitable habitats for *V. cholerae*, including parts of Europe, may subsequently become more suitable with projected increasing temperatures and coastal salinity. Increasing rains and longer wet seasons may also encourage the transmission of cholera over longer periods of time and over larger areas.

In October 2017, the Global Task Force on Cholera Control (GTFCC) launched '*Ending Cholera: A global roadmap to 2030*' a strategy to control cholera outbreaks, with the aim of reducing cholera deaths by 90% and to eliminate cholera in as many as 20 countries by 2030 [63]. The strategy focuses on, (1) early detection and quick response; (2) prevention of cholera recurrence; and (3) coordination of technical support, advocacy, resource mobilisation, and partnership at local and global levels. However, current cholera outbreaks and associated deaths in LMICs suggests that further advances are needed for the environmental management of this disease. While practices such as community engagement and increased communication of how to avoid cholera, are crucial, the long-term solution for cholera control lies in economic development and universal access to safe drinking water and adequate sanitation [63]. However, the environmental adaptability of *V. cholerae* has been key to its persistence over seven pandemics, with the current El Tor pandemic having lasted since 1961 [6].

Plastic waste in the environment now represents a potentially emerging and overlooked component in cholera epidemics. Together with the reported increase in the prevalence of circulating antibiotic resistant *V. cholerae* strains [41], the possibility that plastic pollution could enable and concentrate human pathogens particularly in LMICs and areas of poor sanitation, requires urgent attention. Understanding the ability of such isolates to persist undetected in the environment for long periods, and examining all niches in which these pathogens can survive, including association with plastic waste, will be central for combating infection, educating communities, and diminishing the public health risk.

Environmental implications

Plastics colonised by human pathogens can have significant environmental and human health implications. The lightweight and buoyant properties of plastic facilitates enhanced distribution throughout the environment. This persistence and distribution of plastic contamination increases the risk of direct human exposure. This study demonstrates that human pathogenic *Vibrio cholerae* can colonise plastic waste and survive under environmental conditions; importantly, this pathogen can subsequently be transferred to floodwater, increasing the potential for the spread of cholera. Crucially, the concentration of *V. cholerae* recovered from the surface of plastic pollution, and in the surrounding floodwater, remains high enough to pose a significant threat to human health. Our results attest to the increased co-pollutant risk of plastic pollution, and the heightened risk of human exposure to a dangerous human pathogen in the environment.

CRediT authorship contribution statement

MJO, RSQ: Conceptualisation; **MJO, LW, HW, RF**: Data curation; **MJO**: Formal Analysis; **MJO**: Writing-Original draft preparation; **RSQ**: Supervision; **MJO, LW, HW, RF, DMO, RSQ**: Writing, reviewing, and editing; **RSQ**: 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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2023.132492.

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