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1 **Bacterial colonisation dynamics of household plastics in a coastal environment**

2

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16 **Abstract**

17 Accumulation of plastics in the marine environment has widespread detrimental consequences
18 for ecosystems and wildlife. Marine plastics are rapidly colonised by a wide diversity of bacteria,
19 including human pathogens, posing potential risks to human health. Here, we investigate the
20 effect of polymer type, residence time and estuarine location on bacterial colonisation of common
21 household plastics, including pathogenic bacteria. To do so, we submerged five main household
22 plastic types: low-density PE (LDPE), high-density PE (HDPE), polypropylene (PP), polyvinyl
23 chloride (PVC) and polyethylene terephthalate (PET) at an estuarine site in Cornwall (U.K.) and
24 tracked bacterial colonisation dynamics. Using both culture-dependent and culture-independent
25 approaches, we found that bacteria rapidly colonised plastics irrespective of polymer type. While
26 biofilm community composition changed with colonisation time, no difference was observed
27 between polymer types. Likewise, the presence of pathogenic bacteria, quantified using the insect
28 model *Galleria mellonella*, increased over a five-week period, with no consistent differences
29 observed between polymer types. Pathogens isolated from plastic biofilms using *Galleria*
30 enrichment included *Serratia* and *Enterococcus* species and harboured a wide range of
31 antimicrobial resistance genes. Our findings show that plastics in coastal waters are rapidly
32 colonised by a wide diversity of bacteria, including known human pathogens, independent of
33 polymer type.

34

35 **Introduction**

36 Plastics pollution has huge negative impacts on marine environments (1). In 2017, around 348
37 million tonnes of plastic were produced globally (2). This figure is increasing year on year (2),
38 with a significant percentage of all plastics produced ultimately finding their way into the marine
39 environment (3, 4). Plastics have multiple adverse effects on a wide diversity of wildlife, including
40 entanglement, ingestion and physical damage (e.g. (5-8)). Moreover, it is increasingly recognised
41 that there is potential for plastic pollution to pose a threat to human health (9), from the effects
42 of ingestion of micro- and nano particles (e.g. via shellfish or drinking water (10)) followed by

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43 cellular uptake (11), to decreased psychological wellbeing through pollution of the natural
44 environment (12).

45 The surface of plastic fragments in aquatic environments is rapidly colonised by
46 microorganisms, leading to diverse biofilm communities distinct from that of the surrounding
47 seawater (13-15). Bacterial colonisation of marine plastics is dependent on a variety of abiotic
48 factors, including season, location, colonisation time and polymer type (13, 16, 17). A growing
49 number of studies have used 16S amplicon sequencing approaches to specifically investigate
50 bacterial colonisation of different substrates. Clear differences in bacterial community
51 composition between plastic and other surfaces (e.g. wood or glass) have been reported (e.g. (18,
52 19)) (but see (20)). Differences in plastic colonisation based on beta-diversity metrics have been
53 mixed. Differences have been reported between polystyrene (PS) and polyethylene (PE) and
54 polypropylene (PP) (21, 22), between PS and PP, PE and polyethylene terephthalate (PET) (23)
55 and between PE and PET (24).However, other studies have failed to find biofilm community
56 differences between plastics, e.g. between polyethylene (PE), polypropylene (PP) and
57 polystyrene (PS) (18), between PE and PS (20, 25) or between PE and PP (19).

58 Among bacterial colonisers of marine plastics are pathogenic species known to cause
59 disease in animals (e.g. (26, 27)) and humans (e.g. (28-30)). Plastics could serve as potential
60 vectors for pathogens (30) and/or bacteria carrying antimicrobial resistance genes (31). Plastic
61 pollution could also result in elevated horizontal gene transfer by facilitating close contact (32),
62 and plastic-sorbed contaminants (33) could potentially speed up antimicrobial resistance
63 development (34). Therefore, in addition to direct threats related to ingestion, plastic-borne
64 bacteria may also pose an infection risk to human and wildlife health (35-37). However, previous
65 work has concentrated on indirect quantification of potential pathogens using agar cultivation or
66 amplicon sequencing which cannot quantify the virulence or pathogenicity of bacteria,

67 The aim of this study is to investigate the colonisation dynamics of bacterial pathogens on
68 five of the most common types of household plastics (low-density PE (LDPE), high-density PE
69 (HDPE)), polypropylene (PP), polyvinyl chloride (PVC) and polyethylene terephthalate (PET)), at

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70 multiple coastal sites, and over time. We focus on plastic colonisation in coastal waters where
71 terrestrial inputs of plastics (17) and human-, animal- and soil- associated pathogens is highest
72 (38). We investigate bacterial colonisation dynamics on plastics incubated in the marine
73 environment by quantifying density for subsets of cultivable diversity and testing for differences
74 in taxonomic diversity via 16S amplicon sequencing. We directly tested for the presence of
75 pathogens on biofilm plastics, employing a recently developed selective isolation method using
76 the *Galleria mellonella* insect virulence model (39). We performed two separate experiments: (i)
77 we measured bacterial colonisation after seven weeks across three different locations,
78 quantifying bacterial colonisation through cultivation-based approaches and measuring the
79 presence of pathogens using the *Galleria* assay; (ii) we focused on one location close to shore and
80 in addition to cultivation and the *Galleria* assay, used 16S rRNA amplicon sequencing to quantify
81 bacterial colonisation over time.
82

83 **Materials and Methods**

84 *Plastics*

85 Five plastic types were collected from household recycling: low-density polyethylene (LDPE)
86 from sandwich bags, high-density polyethylene (HDPE) from milk bottles, polypropylene (PP)
87 from peanut butter tubs, polyvinyl chloride (PVC) from roofing material and polyethylene
88 terephthalate (PET) from drink bottles. Plastics were cut into 85mm by 25mm strips with a 5mm
89 diameter hole made at one end using a holepunch. One sample of each type of plastic was attached
90 to a large cable tie via smaller, colour coded cable ties to form a ring (Figure 1a). Rings were then
91 attached to metal mesh bags using cable ties (Figure 1b). Plastics were sterilised using 70%
92 ethanol before environmental incubation.

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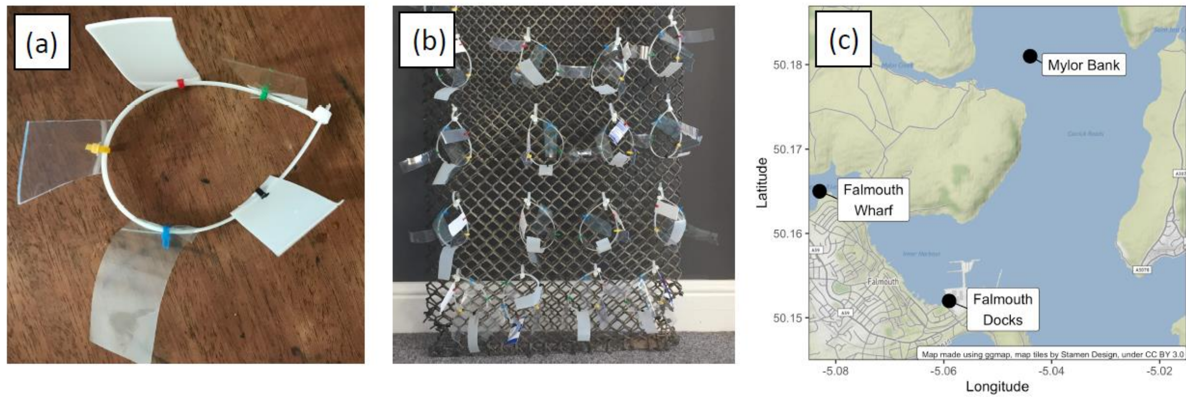

93

94 **Figure 1. Overview of the methods used to submerge household plastics in the Fal**
95 **Estuary.** (a) Strips of LDPE, HDPE, PP, PET and PVC strips were attached to a big cable tie
96 ring using smaller, colour coded cable ties. (b) Each large ring was attached to a metal mesh
97 bag using cable ties which was then submerged at each site (c) Sites used in this study:
98 Falmouth Docks, Falmouth Wharves and Mylor Bank. All three sites were used in the first
99 'Location' experiment whereas the second 'Colonisation' experiment occurred only at the
100 Falmouth Docks site.

101

102 *Location experiment*

103 A total of 18 rings (attached to three mesh bags) were deployed on 10th December 2019 at three
104 sampling sites in the Fal Estuary (Falmouth, Cornwall, UK): Falmouth Docks (50.152, -5.059),
105 Falmouth Wharves (50.165, -5.083) and Mylor Bank (50.181, -5.044) (Figure 1c) with help from
106 Cornwall Port Health Authority. Mylor Bank was further from the shore and was further away
107 human habitation (Figure 1c). The bags were attached to pontoons at Falmouth Wharves and
108 Falmouth Docks and to a buoy at Mylor Bank using a rope, ensuring the bags were fully
109 submerged. Plastic rings were retrieved from each site on 28th January 2020 (after 7 weeks) and
110 placed separately in a sterile plastic box for transportation. Sample processing commenced
111 within one hour of collection.

112

113

114

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115 *Colonisation experiment*

116 To measure differences in the bacterial colonisation of different plastic types through time, 28
117 rings of plastics (four timepoints, seven replicates) were attached to a single metal mesh bag and
118 submerged as described above at the Falmouth Docks site on February 4th, 2020. One set of plastic
119 rings were collected on February 11th, 18th and 24th and March 3rd (weeks 1, 2, 3 and 5) and
120 brought back to the lab.

121

122 *Biofilm processing*

123 Under sterile conditions, each plastic strip was cut in half using sterilised scissors and placed in a
124 50mL falcon tube containing 10mL sterile NaCl buffer (9g/L; Fisher Chemicals, Loughborough,
125 UK) and five sterile glass beads to facilitate removal of biofilm (Millipore Colirollers Plating Beads,
126 Billerica MA, USA). Each tube was vortexed for 60 seconds at 2500 r.p.m. to remove and suspend
127 plastic biofilms. As our focus was on bacteria of terrestrial origin, specifically potential human
128 pathogens, serial dilutions of biofilm suspensions were plated on LB agar (Fisher BioReagents,
129 Loughborough, UK) and coliform agar (Millipore Sigma, Billerica MA, USA) and incubated at 37°C.
130 LB agar selects for relatively fast-growing bacteria; coliform agar was used to quantify both
131 coliform bacteria and *E. coli*. Colony forming units (CFU) were counted after 24 hours of
132 incubation at 37°C. All counts were standardised to CFU/cm^2. Biofilm suspensions were stored in
133 glycerol (Fisher Chemicals, Loughborough, UK) (20% final concentration) at -80°C.

134

135 *Galleria mellonella* virulence assay

136 *G. mellonella* larvae were purchased from Livefood U.K. (http://www.livefood.co.uk) and used
137 within one week of purchase. A 100µL Hamilton syringe (Sigma-Aldrich Ltd, Gillingham, UK) with
138 0.6 x 30mm needles (BD Microlance 3, Becton Dickinson, Plymouth, UK) was used to inject the
139 larvae with 10µL of defrosted biofilm freezer stock into the last left proleg, using 20 larvae
140 (location experiment) or 10 larvae (colonisation experiment) for each sample. The larvae were
141 anaesthetised on ice for 30 minutes before injection. Needles were sterilised between samples by

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142 flushing with 70% ethanol followed by NaCl buffer. Two negative controls for the experiment
143 were used: a buffer control using 10µL of sterile NaCl to control for the impact of physical trauma,
144 and a no-injection control to account for background larvae mortality. After injection, larvae were
145 incubated at 37°C and inspected at 24-, 48- and 72-hours post-injection (location experiment) or
146 24- and 48 hours post-injection (colonisation experiment) to record mortality. Larvae were
147 scored as dead if they did not respond to touch stimuli (39).
148
149 *Isolation of pathogenic bacteria*
150 To isolate pathogens causing *G. mellonella* mortality, we reinjected 20 of the most virulent
151 communities into ten larvae as described above. Larvae demonstrating melanisation – a key
152 indicator of infection – were anesthetised by placing on ice before their haemocoel was extracted.
153 To extract haemocoel, 70% ethanol was used to sterilise the area around the last left proleg before
154 the proleg was removed using sterile micro-scissors and the haemocoel collected using a pipette.
155 This method is advantageous over whole larvae destruction as it minimises contamination with
156 skin and gut bacteria (Hernandez et al 2019). Collected haemocoel (~5-15µL) was diluted in
157 500µL of buffer, plated onto LB agar and incubated for 24 hours. Colonies were then picked and
158 grown in 750µL of LB at 37°C for 24 hours. Cultures were frozen at -80°C in glycerol (at a final
159 concentration of 25%). Defrosted stocks were diluted to (1 x 10^5 CFU/mL) and injected into ten
160 larvae which were then incubated for 18 hours; deceased larvae confirmed that isolated clones
161 were pathogenic and not commensal gut or skin bacteria.
162
163 *16S rRNA amplicon sequencing*
164 DNA was extracted from biofilms using a DNeasy UltraClean Microbial Kit (Qiagen, Hilden,
165 Germany) according to the standard protocol with the only modification to increase the initial
166 centrifugation speed and time (10K x g for 10 minutes) to pellet bacteria suspended in the 20%
167 glycerol stock. DNA concentrations were quantified using the Qubit HS DNA kit (Invitrogen),
168 purity was assessed using nanodrop 260:280 ratios, and integrity was assessed using a 1%

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169 agarose gel. A 251bp conserved fragment in the V4 hypervariable region was targeted using
170 N515f and N806r primers (https://earthmicrobiome.org/protocols-and-standards/16s/) with
171 phasing and a pool of indexed primers suitable for multiplex sequencing with Illumina
172 technology. Sequencing was performed using an Illumina MiSeq V2 500 by the University of
173 Exeter Sequencing Service. Sequencing adapters and any bases below a score of Q22 were
174 removed, alongside any reads <150 bp using '*Cutadapt*' (40). We then processed and analysed the
175 sequence data in R (v4.0.3) (41) using the packages '*dada2*' (42) and '*phyloseq*' (43). Following
176 the standard full-stack workflow, we estimated error rates, inferred and merged sequences,
177 constructed a sequence table, removed chimeric sequences and assigned taxonomy. During
178 processing, the first 25bp of forward and reverse reads were trimmed. Taxonomies were
179 assigned to amplicon sequence variants (ASVs) using the SILVA database (44). We estimated
180 phylogeny using '*fasttree*' (45) to allow for the calculation of Unifrac distances (which take into
181 account the phylogenetic distance between ASVs) between communities. We then removed any
182 reads that had not been assigned to at least the phylum level (613 of 12564 unique ASVs) and any
183 sequences assigned to the phylum Cyanobacteria. Processing and filtering steps resulted in three
184 samples containing fewer than 10,000 reads being removed (one replicate from week 1 LDPE,
185 one replicate from week 1 HDPE, and one replicate from week 2 of PP), with the remaining
186 samples having a maximum read number of 673,525, a minimum of 10,364 and a median of
187 24,920.
188
189 *Whole Genome Sequencing*
190 DNA isolation, Illumina HiSeq sequencing and basic bioinformatics were performed through
191 MicrobesNG, Birmingham, UK. Vials containing beads inoculated with liquid culture were washed
192 with extraction buffer containing lysostaphin and RNase A, and incubated for 25 minutes at 37°C.
193 Proteinase K and RNaseA were added and vials were incubated for a further 5 minutes at 65°C.
194 Genomic DNA was purified using an equal volume of SPRI beads and resuspended in EB buffer.
195 DNA was quantified in triplicate using the Quantit dsDNA HS assay in an Eppendorff AF2200 plate

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196 reader. Genomic DNA libraries were prepared using the Nextera XT Library Prep Kit (Illumina,
197 San Diego, CA, USA) following the manufacturer's protocol with the following modifications: two
198 nanograms of DNA instead of one were used as input, and PCR elongation time was increased to
199 1 minute from 30 seconds. DNA quantification and library preparation were carried out on a
200 Hamilton Microlab STAR automated liquid handling system. Pooled libraries were quantified
201 using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche LightCycler 96 qPCR
202 machine. Libraries were sequenced on the Illumina HiSeq using a 250bp paired end protocol.
203 Sequencing reads were adapter trimmed using '*Trimmomatic 0.39*' with a sliding window
204 quality cutoff of Q15 (46). De novo assembly was performed on samples using '*Unicycler v0.4.8*'
205 (47), and summary stats of assemblies were calculated using CheckM v1.1.3 (48). Genome
206 assemblies were visualised using Bandage (49) and split when there were two large non-
207 contiguous assemblies which were then run separately through the other stages. The assemblies
208 which were likely two or more species were identifiable by a high contamination value (~100%).
209 Contigs were annotated using Prokka 1.14.6 (50). Taxonomic classification of our assemblies was
210 performed using '*MMSeqs2 13.45111*' (51) using the GTDB database (52), and of 16S gene
211 amplicons using '*dada2'* (53) using the SILVA reference database (44). Assemblies were screened
212 for antimicrobial resistance genes and virulence factors using '*AMRFinderPlus v3.10.1*' (54).
213
214 <u>*Statistical analyses*</u>
215 *Analysing differences in culturable abundance between plastics, sites, and time*
216 All analyses were conducted using the statistical programming language R v4.0.3 (41) and all
217 graphs were produced using the '*ggplot2*' package (55). Linear models were used to examine
218 differences in culturable bacterial abundance between locations. The response variable was $\log_{10}$
219 abundance+1 cm^{-2} plastic to normalise the residuals, and the predictors were location, plastic
220 type and their interaction. Separate models were run for each category of culturable bacteria
221 (bacteria culturable on LB, coliform and *E. coli*). Model selection was performed using using
222 likelihood ratio tests. Pairwise multiple comparisons to examine significant differences among

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223 locations or plastics were performed using the R package '*emmeans*' (56). Changes in abundance
224 through time during colonisation were analysed using linear models. The response variable was
225 again $\log_{10}$ abundance+1 cm^{-2} plastic with week of sampling as a continuous predictor and type
226 of plastic as an interaction term. Separate linear models were run for all three categories of
227 culturable bacteria. Model selection and post-hoc multiple comparisons were performed as
228 above.
229
230 *Analysing virulence of plastisphere biofilms*
231 Survival of *Galleria* inoculated with bacteria isolated from biofilms was quantified as a measure
232 of virulence of the plastisphere communities. Survival curves were fitted using Bayesian
233 regression in the package '*rstanarm*' (57) and parameters were estimated using '*tidybayes*' (58).
234 Compared to the popular Cox model, Bayesian implementation can more easily visualise
235 uncertainty and better handle random effects. For survival curves of *Galleria* injected with
236 bacteria isolated from 7-week-long biofilms, we fitted a proportional hazards model with an M-
237 splines baseline hazard, with site, plastic, and their interaction as fixed effects and a random effect
238 of biofilm, as multiple *Galleria* were all inoculated with the same biofilm. For the survival curves
239 through time at Falmouth Docks, we fitted the same model, but with week, plastic, and their
240 interaction as fixed effects and a random effect of biofilm. Models were run for 3000 iterations
241 and three chains were used with uninformative priors. Model convergence was assessed using
242 Rhat values (all values were 1) and manual checking of chain mixing. For both models, log hazards
243 were estimated for each plastic in each site or week. To examine differences in virulence among
244 sites and plastics, hazard ratios were calculated as the exponential of the difference between two
245 log hazards. A hazard ratio above one indicates an increase in virulence compared to the baseline
246 treatment, with a value below one indicating a decrease in virulence compared to the baseline.
247 Median log hazard across treatments was used as the baseline. We calculated median hazard
248 ratios with 95% credible intervals and the probability that the given hazard ratio was above 1.
249 Median hazard ratios with 95% credible intervals that do not cross 1 indicate a significant

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250 difference in virulence between two factors. For visualisation purposes, we show the hazard ratio
251 of each location/polymer type where the baseline is the median log hazard in each experiment,
252 allowing differences in virulence between sites/polymer types to be easily seen.
253
254 *Analysing plastisphere community composition between different plastic types*
255 To investigate how plastisphere bacterial communities changed, we examined changes in
256 community composition, alpha and beta diversity through time. We used weighted Unifrac
257 distance (59) as a measure of compositional dissimilarity between communities, which weights
258 branches in a phylogeny based on the relative abundance of each ASV. As this distance requires a
259 rooted phylogeny, we rooted the tree based on the longest tree branch terminating in a tip.
260 Differences in composition between plastic communities were analysed using the R packages
261 '*phyloseq*' (43) and '*vegan*' (60). We tested whether week of colonisation and plastic type altered
262 community composition using permutational ANOVAs. Permutational ANOVA tests were run
263 using '*vegan::adonis*' with 9999 permutations and differences in group dispersion (which test for
264 differences in beta diversity between treatments) were analysed using '*vegan::betadisper*'. An
265 overall model including plastic type and colonisation week as interacting factors was performed
266 first. We then split the data to examine an overall effect of plastic type by performing a separate
267 PERMANOVA for each week and performing pairwise PERMANOVAs between each plastic type
268 to assess differences. P values were corrected using the *fdr* method (61). When looking at
269 differences in beta diversity, we calculated the distance from the centroid of each plastic by week
270 combination, and then ran a linear model where distance from the centroid was the response
271 variable, and plastic and week were potentially interacting factors. Model selection was
272 performed as above.
273 To investigate differences in alpha diversity and evenness between communities, we
274 rarefied data so that all samples had the same number of reads (11,045). Richness (alpha
275 diversity) was taken as the total number of ASVs and evenness was calculated as Pielou's
276 evenness (62). The amount of change and variation in the number of ASVs across weeks and

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277 across plastic types was tested using linear models. Total ASVs were log_{10}-transformed to
278 normalise residuals, with plastic type and week as potentially interacting predictors. As we did
279 not expect alpha diversity to necessarily change linearly across weeks, we included it as a
280 categorical predictor. For evenness, the analysis was the same, but without any transformation
281 on the response. Model selection was performed as stated earlier.

282

283 **Results**

284 <u>*Bacteria abundance was lowest on plastics further away from human habitation*</u>

285 To determine the effects of location and polymer type on bacterial colonisation, five different
286 plastics were incubated at one-meter depth at three estuarine locations near Falmouth, U.K. After
287 seven weeks of submergence, the abundance of bacteria culturable on LB, coliforms, and *E. coli*,
288 significantly differed between locations (ANOVA between models with and without location as a
289 predictor: bacteria culturable on LB: $F_{2,83} = 69.32$, $p < 0.001$; coliforms: $F_{2,83} = 54.34$, $p < 0.001$; *E.*
290 *coli*: $F_{2,83} = 14.44$, $p < 0.001$) (Figure 2). Across the three quantified groups, abundance was
291 consistently highest at Falmouth Dockyard, which is located closest to human habitation (bacteria
292 culturable on LB: mean =724 CFU cm^2, 95%CI = 490 - 1096; coliform: mean =50 CFU cm^2, 95%CI
293 = 30 – 83 *E. coli*: mean =3.3 CFU cm^2, 95%CI = 2.4 – 4.6) and lowest at Mylor Bank which is furthest
294 from the shore and human habitation (bacteria culturable on LB: mean =25 CFU cm^2, 95%CI = 17
295 – 38; coliform: mean =1.2 CFU cm^2, 95%CI = 0.7 – 2.1; *E. coli*: mean =1.04 CFU cm^2, 95%CI = 0.76
296 – 1.4), although not all contrasts were significant (Table S1).

297 Significant differences in abundance were found between plastics (ANOVA between
298 models with and without plastic as a predictor: bacteria culturable on LB: $F_{4,83} = 4.65$, $p = 0.002$;
299 coliforms: $F_{4,83} = 4.05$, $p = 0.005$; *E. coli*: $F_{4,83} = 3.11$, $p = 0.019$), but there was no significant
300 interaction between location and plastic (ANOVA between models with and without interaction
301 between plastic and location: $p > 0.05$ for LB, coliform and *E. coli*). Abundance was consistently
302 highest on HDPE, but this was only significant across all three quantified groups when compared

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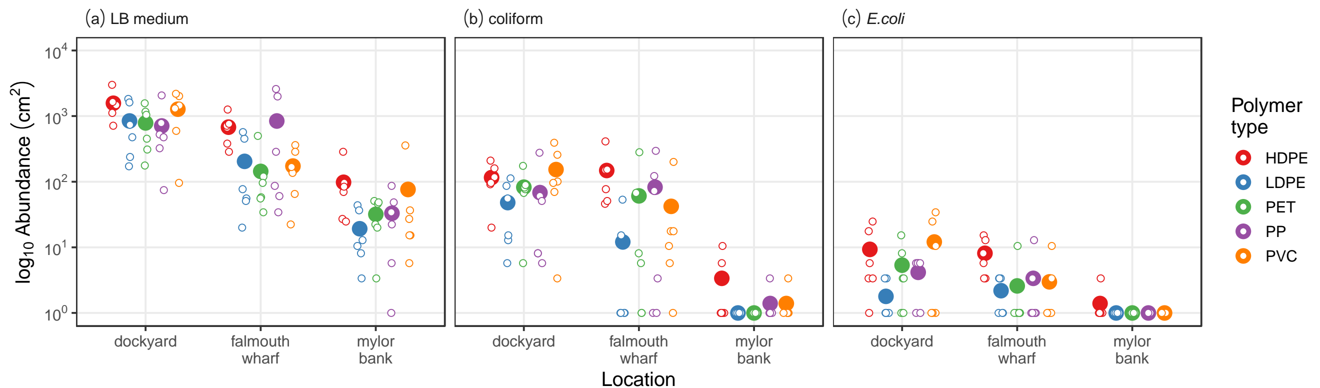
303 to LDPE (Tukey comparison between HDPE and LDPE: bacteria culturable on LB: t = 3.86, p =
304 0.002; coliform: t = 3.88, p = 0.0018; *E. coli*: t = 3.31, p = 0.0118; Table S1).



305

306 **Figure 2. Abundance of (a) bacteria on LB medium, (b) coliform bacteria and (c) *E.*
307 *coli* found across polymer types and locations.** Large points represent mean abundances
308 of plastics at each site and small points represent individual replicates.

309

310 <u>*Rate of bacterial colonisation did not differ between polymer types*</u>

311 To investigate bacterial colonisation of plastics through time, we performed a second experiment
312 at the Falmouth Docks site only, and sampled plastics after one, two, three and five weeks of
313 submergence. During this period, the abundance of bacteria growing on LB, coliforms, and *E. coli*
314 all increased (slope of increase in abundance in best model: bacteria culturable on LB: 0.23,
315 95%CI = 0.17-0.29; coliform = 0.32, 95%CI = 0.25-0.39; *E. coli*: 0.17, 95%CI = 0.13-0.19) (Figure
316 3). For both bacteria culturable on LBand coliforms, the increase in abundance occurred
317 consistently across all types of plastic (ANOVA with and without plastic as a predictor: all p values
318 > 0.05). The only difference in colonisation rate between plastics was found for *E. coli* (ANOVA
319 with and without the interaction between week and plastic: $F_{4,130}$ = 5.95, p = 0.0002), where the
320 rate of colonisation was lowest on LDPE (slope of increase in abundance in best model: *E. coli* on
321 LDPE: 0.05, 95%CI = -0.02 – 0.12), but this was only significantly lower than PET (Tukey
322 comparison between slope of LDPE and PET: t = -4.12, p = 0.0005) and PVC (Tukey comparison
323 between slope of LDPE and PVC: t = -3.92, p = 0.013).

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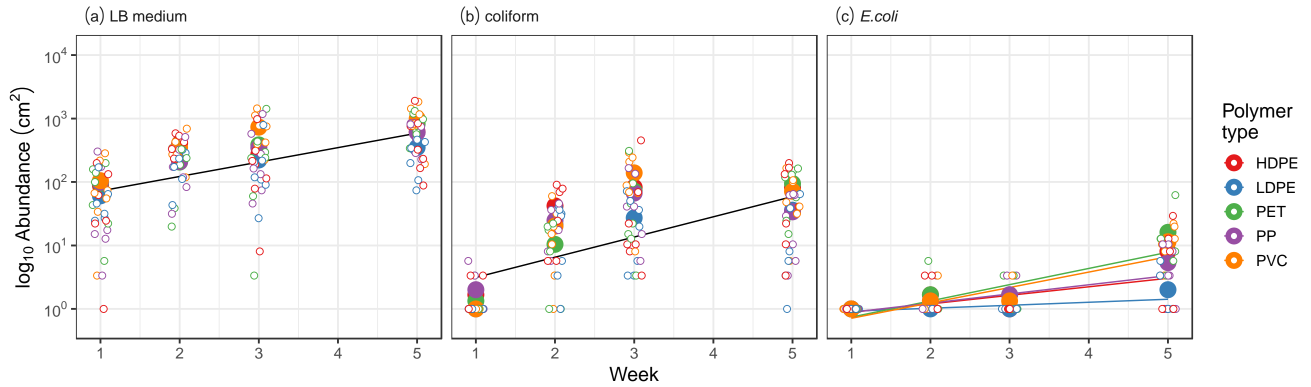

324

325 **Figure 3. Abundance of (a) bacteria on LB medium, (b) coliform bacteria and (c) *E.*
326 *coli*, through five weeks of colonisation at the Falmouth Dockyard site.** Small points
327 represent individual replicates and large points represent mean abundances on each plastic
328 at each week. Lines represent the predicted best fit from the model (see Methods), with the
329 single black line in (a) and (b) indicating that there is no effect of plastic on abundance.

330

331 *Plastic biofilm community composition changes through time, but does not differ between polymer*
332 *types*

333 In addition to culture-dependent approaches, we performed 16S amplicon sequencing across all
334 four timepoints and plastic types for the colonisation experiment. Alpha diversity increased
335 during the first two weeks of colonisation, after which the number of unique ASVs remained
336 stable between weeks 2 and 5 (Figure 4a). This same pattern was seen in the evenness of the
337 biofilm communities, with abundances being more even across species in week 1 compared to
338 weeks 2 to 5 (Figure 4b) which is consistent with a scenario where a subset of taxa increases in
339 frequency over the course of plastic colonisation.

340 Bacterial community composition changed significantly over the course of the experiment
341 (PERMANOVA, $F_{3,108}$ = 23.84, R^2 = 0.38, p = 0.001) (Figure 5). The first principal coordinate
342 explained 22.0% of the total variation and separated the weeks, with the greatest difference
343 between week 1 and week 5. While there was no interaction between plastic and week, plastic
344 type significantly altered community composition when added alongside week (PERMANOVA,
345 $F_{4,108}$ = 1.85, R^2 = 0.04, p = 0.013), although the amount of variation it accounted for was very

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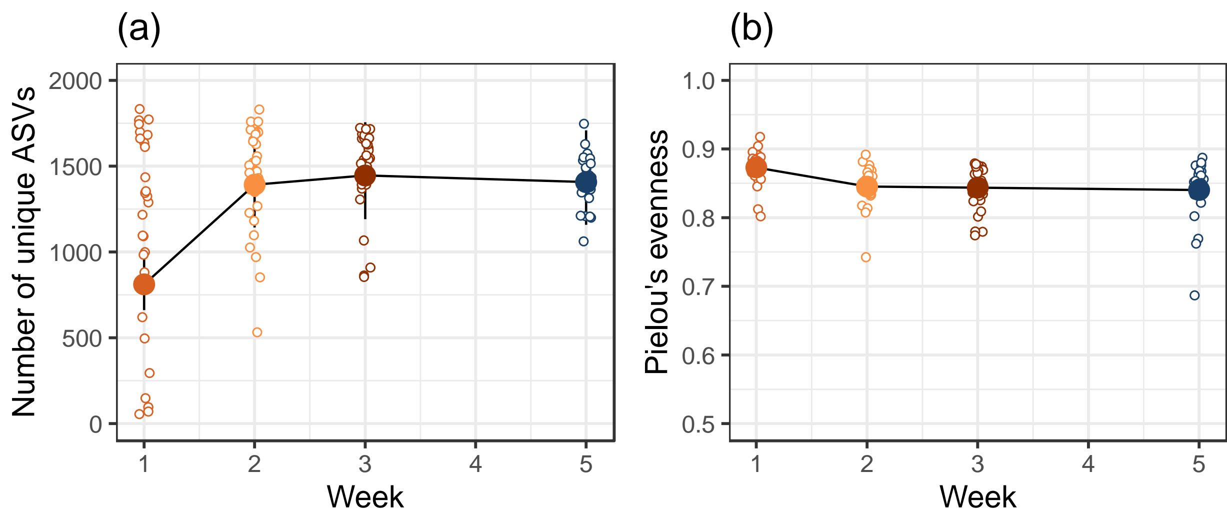
346 small (~4%). To further examine this effect, we ran multiple pairwise permutational ANOVAs on
347 the entire dataset and within each week. We found no significant differences between any two
348 plastics on the whole dataset or within each week (PERMANOVAs: all $p_{adj} > 0.05$), indicating no
349 consistent effects of plastic type. There were no overall differences in between-community
350 diversity (beta-diversity) across weeks or plastics, with the simplest model being one with no
351 predictor (Table S2).



352

353 **Figure 4. Change in (a) alpha diversity and (b) evenness of plastic biofilm**
354 **communities.** (a) Alpha diversity increased from week 1 to week 2 and then remained
355 stable. (b) At the same time, communities became more even, with the only difference in
356 evenness occurring between week 1 and 2. In both panels, small points are individual
357 replicates, large points and line bars represent estimates and 95% confidence intervals
358 from the best model respectively (see Methods), and model estimates are joined by lines to
359 help visualise differences between weeks.

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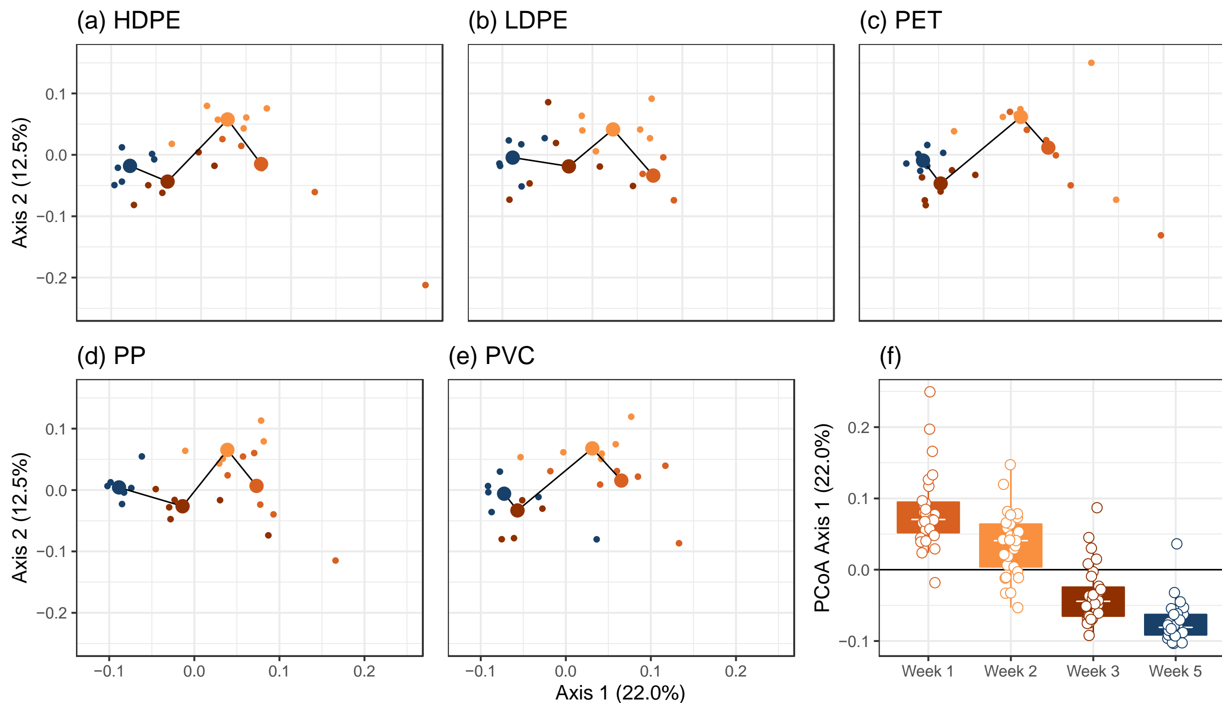

360

361 **Figure 5. Changes in community composition through time (different colours) and**
362 **between plastics (panels a-e).** (a-e) Principal Coordinate (PCoA) plot of communities
363 based on weighted-Unifrac distance. Each panel represents biofilms from different polymer
364 types and shows that community composition changes consistently through time
365 irrespective of plastic type. The percentage of variation explained is shown on each axis
366 (calculated from the relevant eigenvalues). (f) The separation of communities through time
367 along PCoA axis 1 scores**.** In (a-e) small points are individual communities and large points
368 are positions of centroids, with the black lines showing how centroid position changes
369 through time. In (f) points are individual communities, tops and bottoms of the bars
370 represent the 75th and 25th percentiles of the data. White lines are the medians, and the
371 whiskers extend from the respective hinge to the smallest of largest value no further than
372 1.5*interquartile range.

373

374 *Virulence of plastic biofilm communities*

375 We employed a recently developed method where pathogenic bacteria from microbiome samples
376 are selectively enriched using the *G. mellonella* wax moth larva virulence model (39). In the

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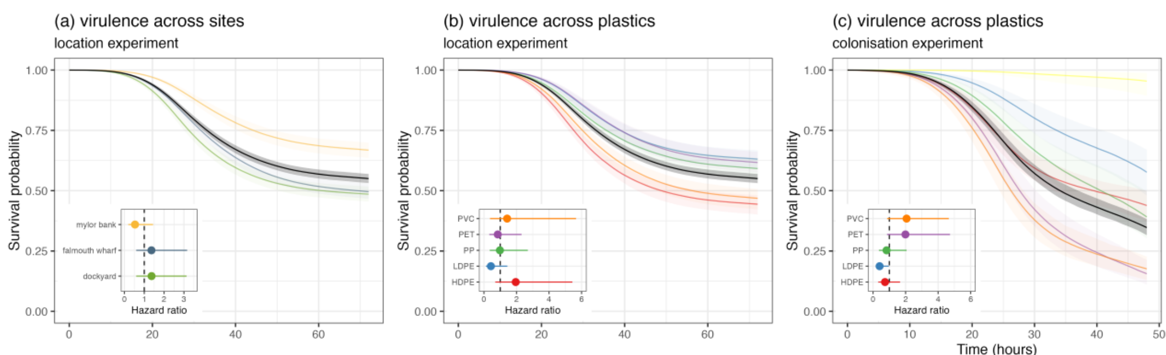
377 location experiment, all hazard ratios between the different sites had credible intervals that
378 crossed one, indicating non-significance at a traditional 0.95 level. However, biofilms from
379 Falmouth Dockyard (mean time to death = 28.5 hours; proportion that died = 0.48) and Falmouth
380 Wharf (mean time to death = 30.8 hours; proportion that died = 0.48) were 2.5 times more
381 virulent than biofilms from Mylor Bank (mean time to death = 34.4 hours; proportion that died =
382 0.32) (Figure 6a, Table S3). There was an 87% probability that the Dockyard and Wharf
383 communities were more virulent than those from Mylor bank for both locations (Table S3).

384 In the colonisation experiment, biofilms from weeks 1, 2, and 3 killed only 4, 7, and 11%
385 of *Galleria* respectively. In contrast, biofilms from week 5 were much more virulent, with 65% of
386 *Galleria* dying during the assay. In both the location and colonisation experiments, we looked for
387 differences in virulence between polymer types (Figure 6a). To classify a polymer type as having
388 more or less virulent communities than others, it would need to have a higher (or lower) hazard
389 ratio than average in both experiments (Table S3). There were no hazard ratios between polymer
390 types that had 95% credible intervals that did not overlap with 1 for both experiments meaning
391 that there were no significant differences in virulence between polymer types. However, we
392 found evidence that LDPE communities are less virulent than other polymer types, with all hazard
393 ratios being above 1 when LDPE was the baseline polymer type (Table S3), with an 89%
394 probability (on average) of the other polymer type being more virulent. The four other polymer
395 types did not have consistent differences in their hazard ratios.



396

397 **Figure 6. Survival curves of *G. mellonella* inoculated with plastic biofilm communities**
398 **from (a, b) the location experiment and (c) the colonisation experiment.** Across all

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399 panels, the black line and shaded region depicts the median virulence of a biofilm
400 community from that experiment, and the median log hazard was used as the baseline for
401 calculating hazard ratios. (a) Effect of location on virulence of biofilm communities. (b)
402 Effect of polymer type on virulence of biofilm communities from the location experiment.
403 (c) Effect of polymer type on virulence of biofilm communities from the colonisation
404 experiment. Lines represent the median prediction and shaded bands represent 95%
405 credible intervals of those predictions. Inset plots are the hazard ratio where the median
406 hazard of the experiment is the baseline. In (c) bright yellow is the survival curve for *G.*
407 *mellonella* injected with water.

408

409 *The identity of putative human pathogens*

410 To obtain a more detailed picture of the taxonomic identity and antimicrobial resistance of
411 individual pathogens in our plastic biofilm samples, we genome-sequenced 16 bacterial clones
412 isolated from some of the most virulent biofilm communities spanning different locations and
413 plastic types (Table 1). Eleven genome assemblies had high completion and low contamination
414 and could be unambiguously assigned to a single bacterial species, but this was not the case for
415 five remaining genome samples – which must have been due to the co-isolation of multiple clones.
416 To retain as much information as possible, these assemblies were not discarded but were
417 bioinformatically separated using the genome viewer Bandage, with assemblies split into non-
418 contiguous sections (46). Using this approach, four contaminated genomes could be split into
419 eight genomes (2 & 3, 6 & 7, 8 & 9, 15 & 16; Table 1). One remaining assembly (17; Table 1) could
420 not be split because it only had one contiguous genome. Retaining a large chimeric assembly was
421 also the case for (split) sample 16 and so for both assemblies multiple identities are presented
422 instead of one (Table 1).

423 The most prevalent pathogen genus isolated was *Serratia* (Table 1), with several clones
424 identified as *S. liquefaciens*, and *S. fonticola*, which are known human pathogens (59) that have
425 previously been reported to occur commonly in soils in Cornwall using the *Galleria* enrichment

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426 method (60). Other species identified were *Enterococcus faecalis* which is known to infect humans
427 (61), and *Bacillus licheniformis* which can cause opportunistic infections (e.g. (62)). Surprisingly,
428 no virulence genes were detected in any of the assemblies, although we used a relatively stringent
429 sequence similarity cut-off of 95%. However, a host of antimicrobial resistance genes belonging
430 to a variety of classes were uncovered (Table 2). These include genes conferring resistance to
431 clinically highly relevant antibiotics such as cephalosporins, aminoglycosides and trimethoprim
432 (in *E. faecalis*) (Table 2).

433

434 **Table 1** Genome characteristics of 20 clones isolated from plastics using the *Galleria*
435 enrichment method.

isolate	plastic	site	contig taxonomic assignment	16S taxonomic assignment	completeness	contamination	GC	genome size (bp)	number of contigs	N50	number of AMR genes
1	HDPE	falmouth wharf	*Serratia liquefaciens*	*Serratia*	100.00	0.89	0.55	5,549,824	78	327,206	2
2	HDPE	falmouth wharf	*Serratia*	*Raoultella/Enterobacter/Serratia*	99.86	0.15	0.59	5,222,268	41	2,939,895	6
3	HDPE	falmouth wharf	*Bacillus licheniformis*	*Bacillus licheniformis*	98.82	0.00	0.46	4,254,100	20	2,234,065	1
4	HDPE	mylor bank	*Serratia*	*Serratia marcescens*	99.86	0.15	0.59	5,218,884	32	2,874,999	6
5	HDPE	dockyard	*Serratia liquefaciens*	*Serratia liquefaciens*	100.00	0.45	0.55	5,413,752	61	318,399	2
6	HDPE	dockyard	*Bacillus licheniformis*	*Bacillus licheniformis*	98.86	0.00	0.46	4,256,127	38	382,110	1
7	HDPE	dockyard	*Enterobacter/Enterobacteriaceae*	*Enterobacter/Cedecea*	98.78	0.60	0.56	5,014,388	240	160,006	3
8	LDPE	dockyard	*Bacillus/Enterobacter*	*Bacillus*	99.45	8.63	0.47	4,977,695	35	1,096,327	1
9	LDPE	dockyard	*Enterobacter/Enterobacteriaceae*	*Enterobacter/Cedecea*	98.78	0.60	0.56	5,014,653	245	160,006	3
10	LDPE	dockyard	*Serratia*	*Serratia marcescens*	99.86	0.15	0.59	5,218,582	30	2,874,999	6
11	LDPE	dockyard	*Serratia fonticola*	*Serratia*	99.94	0.29	0.54	5,756,538	113	185,130	1
12	LDPE	falmouth wharf	*Enterococcus faecalis*	*Enterococcus faecalis*	99.63	0.00	0.37	2,864,632	53	175,637	2
13	PET	dockyard	*Serratia*	*Serratia marcescens*	99.86	0.15	0.59	5,218,760	30	2,874,999	6
14	PP	dockyard	*Serratia*	*Serratia marcescens*	99.86	0.15	0.59	5,218,875	33	2,875,113	6
15	PP	dockyard	*Bacillus licheniformis*	*Bacillus licheniformis*	97.30	0.31	0.46	4,145,137	31	438,169	1
16	PP	dockyard	*Klebsiella/Serratia Enterobacter/Enterobacteriaceae*	*Enterobacter/Serratia*	97.93	188.43	0.57	15,957,883	573	182,752	10
17	PP	dockyard	*Serratia/Enterobacteriaceae*	-	99.66	95.89	0.56	11,471,120	188	336,395	7
18	PP	falmouth wharf	*Serratia*	*Serratia marcescens*	99.86	0.15	0.59	5,219,474	35	2,875,113	6
19	PVC	dockyard	*Serratia liquefaciens*	*Serratia liquefaciens*	100.00	0.45	0.55	5,571,931	37	398,855	2
20	PVC	falmouth wharf	*Serratia liquefaciens*	*Serratia*	100.00	0.45	0.56	5,131,311	33	531,913	2

436

437

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438 **Table 2** Antimicrobial resistance genes present in 20 clones isolated from plastics using
439 the *Galleria* enrichment method.

isolate	AMR gene
1	multidrug efflux RND transporter permease subunit SdeB
	class C beta-lactamase
2	multidrug efflux RND transporter permease subunit SdeY
	multidrug efflux RND transporter permease subunit SdeB
	class C beta-lactamase SRT-3
	tetracycline efflux MFS transporter Tet(41)
	multidrug efflux MFS transporter SmfY
	aminoglycoside 6'-N-acetyltransferase
3	class A beta-lactamase BlaP
4	multidrug efflux MFS transporter SmfY
	tetracycline efflux MFS transporter Tet(41)
	class C beta-lactamase SRT-3
	multidrug efflux RND transporter permease subunit SdeB
	multidrug efflux RND transporter permease subunit SdeY
	aminoglycoside 6'-N-acetyltransferase
5	multidrug efflux RND transporter permease subunit SdeB
	class C beta-lactamase
6	class A beta-lactamase BlaP
7	cephalosporin-hydrolyzing class C beta-lactamase MIR-16
	multidrug efflux RND transporter permease subunit OqxB9
	fosfomycin resistance glutathione transferase FosA
8	class A beta-lactamase BlaP
9	cephalosporin-hydrolyzing class C beta-lactamase MIR-16
	multidrug efflux RND transporter permease subunit OqxB9
	fosfomycin resistance glutathione transferase FosA
10	multidrug efflux MFS transporter SmfY
	tetracycline efflux MFS transporter Tet(41)
	class C beta-lactamase SRT-3
	multidrug efflux RND transporter permease subunit SdeB
	multidrug efflux RND transporter permease subunit SdeY
	aminoglycoside 6'-N-acetyltransferase
11	class A beta-lactamase FONA-4
12	trimethoprim-resistant dihydrofolate reductase DfrE
	ABC-F type ribosomal protection protein Lsa(A)
13	multidrug efflux MFS transporter SmfY
	tetracycline efflux MFS transporter Tet(41)
	class C beta-lactamase SRT-3
	multidrug efflux RND transporter permease subunit SdeB
	multidrug efflux RND transporter permease subunit SdeY
	aminoglycoside 6'-N-acetyltransferase
14	multidrug efflux RND transporter permease subunit SdeY
	multidrug efflux RND transporter permease subunit SdeB
	class C beta-lactamase SRT-3
	tetracycline efflux MFS transporter Tet(41)
	multidrug efflux MFS transporter SmfY
	aminoglycoside 6'-N-acetyltransferase
15	class A beta-lactamase BlaP
16	multidrug efflux RND transporter permease subunit SdeB
	multidrug efflux RND transporter permease subunit SdeY
	multidrug efflux RND transporter permease subunit OqxB
	phosphoethanolamine--lipid A transferase MCR-10.1
	multidrug efflux MFS transporter SmfY
	tetracycline efflux MFS transporter Tet(41)
	class C beta-lactamase SRT-3
	class A extended-spectrum beta-lactamase OXY-6-2
	cephalosporin-hydrolyzing class C beta-lactamase MIR-12
	aminoglycoside 6'-N-acetyltransferase
17	multidrug efflux MFS transporter SmfY
	tetracycline efflux MFS transporter Tet(41)
	class C beta-lactamase SRT-3
	multidrug efflux RND transporter permease subunit SdeB
	multidrug efflux RND transporter permease subunit SdeY
	aminoglycoside 6'-N-acetyltransferase
	class A beta-lactamase FONA-6
18	multidrug efflux MFS transporter SmfY
	tetracycline efflux MFS transporter Tet(41)
	class C beta-lactamase SRT-3
	multidrug efflux RND transporter permease subunit SdeB
	multidrug efflux RND transporter permease subunit SdeY
	aminoglycoside 6'-N-acetyltransferase
19	class C beta-lactamase
	multidrug efflux RND transporter permease subunit SdeB
20	class C beta-lactamase
	multidrug efflux RND transporter permease subunit SdeB

440

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441 **Discussion**

442 There has been a recent flurry of research into the colonisation of marine plastics by bacteria, but
443 our understanding of this process is still incomplete, especially regarding pathogenic bacteria. To
444 help resolve ongoing debate, we submerged household plastics in the Fal Estuary (UK) and
445 tracked bacterial colonisation as a function of plastic type, location, and time. Our results indicate
446 that plastic colonisation in the coastal environment is largely independent of plastic type (LDPE,
447 PE, PP, PVC and PET) with no or little effect on the number of culturable bacteria or 16S-based
448 community composition across locations or time. The exception is LDPE, which showed lower
449 colonisation rates in the location and colonisation experiments, although these differences were
450 only significant compared to HDPE in the former and PET and PVC in the latter experiment (and
451 only in the case of *E. coli* in the colonisation experiment). However, as LDPE-derived biofilms
452 exhibited lower virulence in the *Galleria* model, it is possible that LDPE poses lower risks in terms
453 of pathogen colonisation.

454 Bacterial colonisation of plastics occurred rapidly, with bacterial diversity reaching peak
455 diversity after two weeks. Bacterial density increased with time and was highest at our final
456 timepoint of five weeks - the only timepoint at which significant *Galleria* mortality indicated the
457 presence of pathogenic bacteria. Our data thus suggest that total bacterial density, rather than
458 community composition, is a better predictor of the virulence of plastic-associated biofilms.
459 Bacterial plastic colonisation, including by pathogens, might be more rapid in summer months
460 when higher water temperatures favour bacterial growth and persistence (63), although reduced
461 rainfall in summer may result in lower input of terrestrial bacteria into the sea. Colonisation by
462 bacteria likely to be of terrestrial origin (particularly those that are gut-associated and/or able to
463 grow at 37°C on nutrient-rich medium) was more pronounced at the two locations closer to the
464 shore and habitation, as expected. Future studies employing replicated transects could shed more
465 light on the effect of coastal proximity on plastic colonisation.

466 Approaches to determine the presence of pathogenic bacteria on plastics are usually
467 based on the ability to detect specific taxa that are selected a priori, typically via agar-based

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468 isolation (e.g. 19)). By using selective enrichment in a proven virulence model, we were able to
469 isolate virulent bacteria, including known human pathogens such as *Enterococcus faecalis*. This
470 species is present in marine environments (64) and has been shown to successfully colonise LDPE
471 in lab experiments (65), but to our knowledge this is the first time that this important nosocomial
472 pathogen has been isolated from marine plastics. The genomes recovered from plastic biofilms
473 harboured a wide variety of antimicrobial resistance genes, highlighting the importance of
474 plastics as a reservoir for resistance.

475 Plastic pollution is unabating and human exposure to micro-plastics and their associated
476 microbial communities is likely to increase even more rapidly with a higher incidence of flooding
477 due to climate change. Our results show that different plastic types accumulate similar bacterial
478 communities that include a range of known human pathogens, posing a potential risk to human
479 health.

480

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489

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650 **Supplementary Information for: Bacterial colonisation dynamics of household plastics in**
651 **a coastal environment**
652
653 **Table S1. Tukey pairwise comparisons of cultural abundance of bacteria on LB**
654 **media, coliform media, and *E. coli* from the location experiment**. Results of multiple
655 comparisons from the best model looking at differences between location and polymer
656 type. Significant p values are highlighted in bold.
657

type	contrast	estimate	SE	*d.f.*	t ratio	p value
LB media	dockyard - falmouth wharf	0.613	0.125	83	4.911	**<0.001**
	dockyard - mylor bank	1.464	0.125	83	11.723	**<0.001**
	falmouth wharf - mylor bank	0.851	0.125	83	6.812	**<0.001**
	HDPE - LDPE	0.623	0.161	83	3.864	**0.002**
	HDPE - PET	0.553	0.161	83	3.43	**0.008**
	HDPE - PP	0.499	0.161	83	3.097	**0.022**
	HDPE - PVC	0.432	0.161	83	2.679	0.066
	LDPE - PET	-0.07	0.161	83	-0.434	0.992
	LDPE - PP	-0.124	0.161	83	-0.767	0.939
	LDPE - PVC	-0.191	0.161	83	-1.185	0.76
	PET - PP	-0.054	0.161	83	-0.333	0.997
	PET - PVC	-0.121	0.161	83	-0.751	0.944
	PP - PVC	-0.067	0.161	83	-0.418	0.993
coliform	dockyard - falmouth wharf	0.549	0.157	83	3.5	**0.002**
	dockyard - mylor bank	1.608	0.157	83	10.254	**<0.001**
	falmouth wharf - mylor bank	1.059	0.157	83	6.755	**<0.001**
	HDPE - LDPE	0.787	0.202	83	3.886	**0.002**
	HDPE - PET	0.518	0.202	83	2.56	0.087
	HDPE - PP	0.549	0.202	83	2.71	0.061
	HDPE - PVC	0.411	0.202	83	2.028	0.262
	LDPE - PET	-0.268	0.202	83	-1.325	0.676
	LDPE - PP	-0.238	0.202	83	-1.175	0.765
	LDPE - PVC	-0.376	0.202	83	-1.858	0.348
	PET - PP	0.03	0.202	83	0.15	1
	PET - PVC	-0.108	0.202	83	-0.532	0.984
	PP - PVC	-0.138	0.202	83	-0.682	0.96
E.coli	dockyard - falmouth wharf	0.549	0.157	83	3.5	**0.002**
	dockyard - mylor bank	1.608	0.157	83	10.254	**<0.001**
	falmouth wharf - mylor bank	1.059	0.157	83	6.755	**<0.001**
	HDPE - LDPE	0.787	0.202	83	3.886	**0.002**
	HDPE - PET	0.518	0.202	83	2.56	0.087
	HDPE - PP	0.549	0.202	83	2.71	0.061
	HDPE - PVC	0.411	0.202	83	2.028	0.262
	LDPE - PET	-0.268	0.202	83	-1.325	0.676
	LDPE - PP	-0.238	0.202	83	-1.175	0.765
	LDPE - PVC	-0.376	0.202	83	-1.858	0.348
	PET - PP	0.03	0.202	83	0.15	1
	PET - PVC	-0.108	0.202	83	-0.532	0.984
	PP - PVC	-0.138	0.202	83	-0.682	0.96

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660 **Table S2. Results of the analysis of beta-diversity of community composition between**
661 **weeks and plastic type.**

	model	*d.f.*	AIC	Log Lik	ANOVA comparison	*F*	*P*
1	distance to centroid ~ week * plastic	19	-360.93	201.47			
2	distance to centroid ~ week + plastic	7	-371.21	194.60	1,2	1.00	0.451
3	distance to centroid ~ week	3	-378.96	194.48	2,3	0.06	0.994
4	distance to centroid ~ plastic	4	-369.41	190.71	2,4	2.50	0.063
5	distance to centroid ~ 1		-377.10	190.55	3,5	2.62	0.055
5	distance to centroid ~ 1		-377.10	190.55	4,5	0.07	0.990

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664 **Table S3. Hazard ratios between locations and plastics for both experiments.**
665 Probability above 1 is the probability that the first location/polymer type is more virulent
666 than the baseline location/polymer type.

contrast	experiment	Hazard ratio			Probability above 1
		Median	2.5%	97.5%	
dockyard vs. falmouth wharf	location experiment	1.00	0.28	3.76	0.50
dockyard vs. mylor bank	location experiment	2.57	0.51	12.98	0.87
falmouth wharf vs. mylor bank	location experiment	2.55	0.49	13.35	0.87
HDPE vs. LDPE	location experiment	4.57	0.88	23.79	0.97
	colonisation experiment	1.83	0.46	6.70	0.80
HDPE vs. PET	location experiment	2.29	0.44	11.12	0.85
	colonisation experiment	0.37	0.10	1.35	0.06
HDPE vs. PP	location experiment	1.99	0.41	9.04	0.81
	colonisation experiment	0.87	0.23	3.27	0.41
HDPE vs. PVC	location experiment	1.37	0.19	9.09	0.63
	colonisation experiment	0.35	0.10	1.29	0.06
PET vs. LDPE	location experiment	1.97	0.33	13.64	0.78
	colonisation experiment	5.02	1.22	19.15	0.99
PP vs. LDPE	location experiment	2.31	0.39	13.73	0.83
	colonisation experiment	2.05	0.56	8.27	0.86
PP vs. PET	location experiment	1.17	0.29	4.43	0.58
	colonisation experiment	0.42	0.11	1.61	0.11
PVC vs. LDPE	location experiment	3.34	0.43	30.79	0.88
	colonisation experiment	5.06	1.30	19.74	0.99
PVC vs. PET	location experiment	1.68	0.28	10.34	0.72
	colonisation experiment	1.05	0.28	3.78	0.53
PVC vs. PP	location experiment	1.46	0.21	11.35	0.65
	colonisation experiment	2.47	0.60	9.17	0.91

667