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Sewers to Seas: exploring pathogens and antimicrobial resistance on microplastics from hospital wastewater to marine environments

Emily M. Stevenson [a,b,c,*], Angus Buckling [c], Matthew Cole [b], April Hayes [a], Penelope K. Lindeque [b,c], Aimee K. Murray [a]

[a] European Centre for Environment and Human Health, Environment and Sustainability Institute, University of Exeter Medical School, Faculty of Health and Life Sciences, Penryn Campus, Cornwall, UK
[b] Marine Ecology & Society, Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth PL1 3DH, UK
[c] Faculty of Environment, Science and Economy, University of Exeter, Penryn Campus, Cornwall TR10 9FE, UK

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ABSTRACT

Microplastic particles are extremely prevalent environmental pollutants which support microbial biofilms known as the 'plastisphere'. Antimicrobial resistant (AMR) and pathogenic bacteria have been detected in these communities, but it is currently unknown whether microplastics pose a unique risk in terms of AMR or pathogen enrichment. In addition, previous work has been largely lab-based, so it is difficult to understand the role of different substrates in supporting AMR pathogens within the environment, and how this varies as a function of levels of pollution from wastewater. This study investigated *in situ* bacterial colonisation dynamics on microplastics alongside natural, inert and free-living controls. Samples were incubated along a transect predicted to decrease in anthropogenic pollution, and taxonomy, AMR gene and pathogen presence were assessed using whole metagenome sequencing. Several AMR gene (e.g. aminoglycosides, oxazolidinones and tetracyclines) and pathogen classes (e.g. Flavobacteriia, Chlamydiia and Sphingobacteriia) of concern were detected, and increased in relative abundance in biofilms moving downstream, with polystyrene and HDPE nurdle communities posing a particular risk by supporting AMR bacteria. This work contributes to our understanding of how microplastics may support AMR development, persistence and dispersal in natural systems. In addition, these findings highlight the importance of considering the combined impacts of co-contaminants in wastewater settings, especially following spills into surface water.

1. Introduction

Microplastics are plastic particles less than 5 mm in size (Thompson, 2004) and are extremely widespread pollutants, with up to 125 trillion particles predicted to have accumulated across global sea surfaces (Lindeque, 2020). These particles have also been detected in soils (Yang, 2021), rivers (Guo, 2025), lakes (Chen, 2024) and the human body (Leonard, 2024). A recent concern associated with these substrates are the microbial communities which rapidly form biofilms on the particle surface, known as the plastisphere (Zettler et al., 2013). Concerns are exacerbated by the discovery of pathogenic and antimicrobial resistant (AMR) bacteria within these communities (Zadjelovic, 2023; Silva, 2023).

Concurrently, anthropogenic pollution has been highlighted to drive selection for AMR within environmental compartments (Swift, 2019; Rzymski, 2024; Murray, 2024). Typically, in areas of higher anthropogenic pollutants, such as wastewater treatment plants (WWTPs) or solid waste landfill sites (Pärnänen, 2019; Uluseker, 2021; Wu, 2017; Song, 2023), AMR genes (ARGs) become enriched in environmental communities (Murray, et al., 2018; Murray, 2024; Maurya et al., 2021; Sharma, 2025). Crucially, antimicrobials, heavy metals, human or animal pathogens, plastics and microplastics also exist in these environments (Habib et al., 2020), yet the interactions between these co-contaminants remain relatively unexplored.

One of the key AMR risks posed by microplastics may simply be the provision of a substrate for biofilm formation and horizontal gene transfer (HGT) of ARGs (Stevenson, 2024; Arias-Andres, 2018). However, microplastics may also impose additional risks, including the

* Corresponding author at: European Centre for Environment and Human Health, Environment and Sustainability Institute, University of Exeter Medical School, Faculty of Health and Life Sciences, Penryn Campus, Cornwall, UK.
E-mail addresses: es718@exeter.ac.uk (E.M. Stevenson), a.k.murray@exeter.ac.uk (A.K. Murray).

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incorporation or adsorption of compounds within the plastic matrix (Vlaanderen, 2023), which could select for AMR. Regardless of whether microplastics pose a unique risk in AMR development in comparison to natural substrates, evidence suggests that there is variation between types of microplastics in terms of the communities they can support (Luo, 2025; Zhu, 2022; Silva et al., 2024; Ormsby, 2023). In addition, plastics are widely acknowledged as highly persistent and prevalent pollutants which – due to hydrophobicity and recalcitrance – can be transported vast distances and be highly resistant to degradation (Priya, 2022). Therefore, it could be proposed that microplastics may pose a greater risk in terms of spread and persistence of AMR pathogens than some natural counterparts which degrade over shorter timescales.

Here, we assessed colonisation of microplastics in comparison to natural and inert substrates. To ensure full exposure to the environment, we suspended substates on free-floating, novel structures. To create a natural pollution gradient, we set up these structures along a riverine-estuarine transect, starting with highly contaminated hospital wastewater; hypothesising that a greater abundance of AMR and pathogens would be found nearer to source. We also collected water/wastewater samples from each site to provide free-living comparisons to our study biofilms and environmental paraments (pH and temperature) were also recorded. After 2 months, samples were retrieved, DNA extracted and whole metagenome sequencing was performed to analyse both taxonomy and resistome. From this work, we can start to prioritise high risk particles for improved monitoring, or develop necessary mitigation efforts, including changes to waste management practices or improving water treatment infrastructure. In the face of increasing anthropogenic pollution and the rising One Health threat of AMR (Larsson, 2023), it is critical to identify the substrates which pose the greatest AMR burden, to reduce the synergistic impacts of plastic pollution, environmental contaminants and AMR.

2. Materials and methods

2.1. Study location

Four locations were chosen as incubation sites and permissions obtained by relevant landowners (Fig. 1). These locations represent a transect expected to decrease in anthropogenic pollution (sewage related bacteria and micropollutants), temperature, and pH; but increase in distance downstream of a WWTP which receives both clinical and domestic sewage. The WWTP in this study (i) serves a catchment of over 30,000 people, (ii) receives the untreated wastewater from the hospital study site and, (iii) prior to an accidental spill of 5 billion bio-beads into the Truro River system in 2010 (Turner et al., 2019), included a microplastic bio-media treatment step as part of the treatment processes.

Our study sites (Fig. 1) include: the raw sewage tank for a 750-bed hospital serving a population of 430,000 people (Hearsey, 2023) ('Hospital'; 50.265483, −5.092122); an inner city section of the river ~1 mile upstream of the WWTP main effluent point ('Upstream'; 50.261304, −5.046133); a small maritime docking site ~3.5 miles downstream of the WWTP ('Downstream'; 50.216166, −5.028009); and a marker buoy at the opening of Falmouth Harbour ('Marine'; 50.161274, −5.059232). Whilst not marked on the map to conceal sensitive information, there is also a commercial aquaculture farm neighbouring our downstream site.

2.2. Incubation structures

One major challenge of investigating environmental plastisphere communities is containing microplastics, given their small size; with existing works opting for mesh bags, plastic boxes or metal cages. However, bacteria will also form biofilms on these containment materials (Qian, 2022), potentially leading to transfer of genes or immigration of taxa (Vincent, 2024; Liu, 2016). The influences of these external

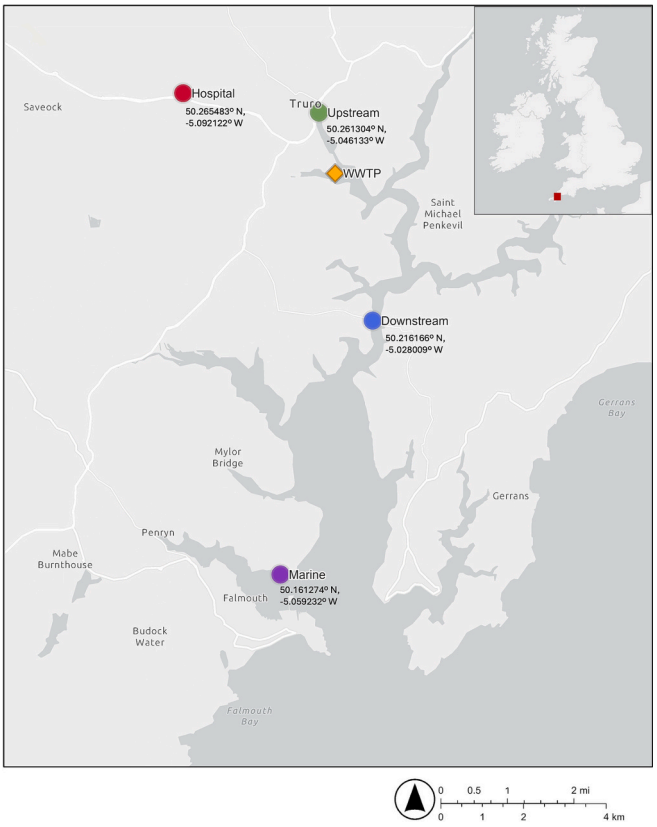

Fig. 1. Map showing study area with all incubation sites marked, southwest England. Red: hospital; Green: upstream; Blue: downstream; Purple: marine. WWTP is also marked (Orange). Sites were plotted using ArcGIS Online based on field-collected GPS coordinates. The basemap used is "Light Grey Canvas" provided by Esri. Map data ©2025 Esri, HERE, Garmin, FAO, NOAA, USGS, and © OpenStreetMap contributors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

biofilms may result in formation of study biofilms not wholly representative of environmental colonisation dynamics. Therefore, building on previous lab-based work (Stevenson, 2024), we developed a novel incubation method, allowing us to submerge five different particle types across four different environments. Our low-cost structure grants the ability for samples to be anchored in flowing water, with completely exposed surfaces. The structures are made and transported in sterile conditions and are small so can be concealed in sensitive areas, like wastewater tanks.

Five different particles were sourced prior to the generation of incubation structures. Bio-beads (WWTP derived polyethylene microplastic/bio-media) and polystyrene were sampled, sterilised and analysed (polymer identification) as previously described (Stevenson, 2024; Stevenson, 2023). Virgin, high-density polyethylene (HDPE) 'nurdles' (pellets) were purchased (Sigma Aldrich, 427985–1 KG). Threadable, top-drilled holes were created in both bio-beads and nurdles using a pillar drill. Untreated, threadable wooden beads and clear, threadable glass beads were purchased from a local craft store (Cornwall, UK), which came pre-constructed with top-drilled holes. All particles were ~4 mm in size.

All particles were threaded onto 0.5 mm 316 Grade (marine) stainless steel wire (SS0500-316-500, Wires, UK), except polystyrene. Particles were arranged onto 4 separate wires (biological replicates), with individual particle types threaded in duplicate (technical replicates), leaving gaps for polystyrene. Particles were arranged systematically, ensuring each biological replicate (N = 4) per structure had different particle types at varied heights, which ensured no bias in sunlight exposure when submerged in the environment (Fig. 2). Stainless steel

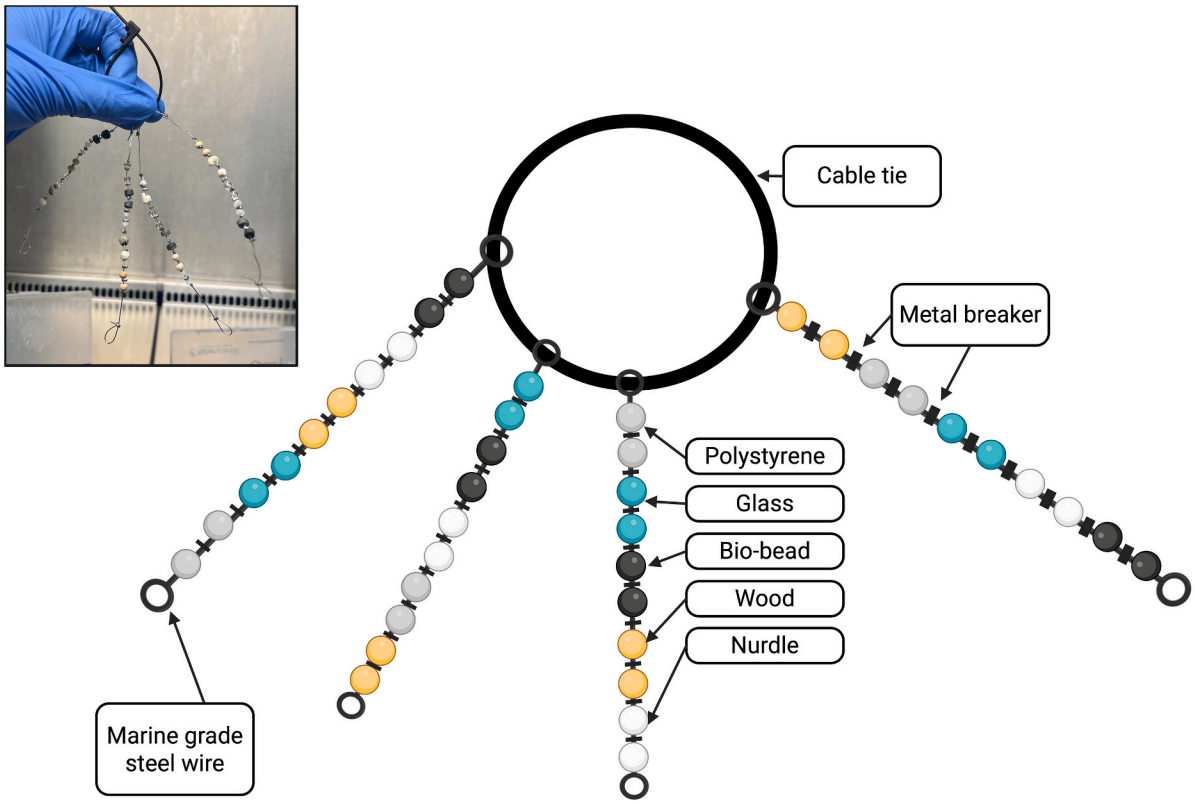

Fig. 2. Schematic diagram of incubation structure alongside image of finalised structure (top left). Created in BioRender. Stevenson, E. (2025) https://BioRender.com/70yhrp8.

'crimp beads' (2.5 mm) were secured between each particle to keep each particle separated.

At this stage, all 4 biological replicates (wires) per structure (8 total, 2 structures per site to account for accidental losses) were submerged in deionised water in 500 mL Duran bottles and autoclaved at 121 °C for 15 min. Submersion in water prevented delamination of plastic particles and accidental burning of wooden particles. Following autoclave sterilisation of structures, they were removed from water and left to air dry in sterile conditions (CAT-II cabinet). Polystyrene particles were not autoclaved given the volatility of polystyrene at high temperatures and were instead Gamma-irradiated for sterilisation. These were then added to structures under sterile conditions by pushing the stainless-steel wire directly through the centre of the particle using sterile forceps. Finally, all 4 wires (biological replicates) were threaded onto one plastic cable tie and enclosed in separate, sealed, sterile boxes ready for deployment.

2.3. Environmental incubation

2.3.1. Deployment

Structures were deployed in early February 2023. All structures were transported in sterile boxes to each location. At the hospital, structures were attached to a weighted rope and submerged by hospital staff directly into the untreated wastewater tank below the hospital. Structures were also attached to a weighted rope which was secured to a privately owned location (with permission) at the upstream site, placing the samples at the edge of the river. At the downstream location, structures were secured via cable tie to the docking pontoon, ensuring that the samples were free moving and would not be disturbed during pontoon use. Finally, a marker buoy at the marine site (~300 m offshore, tidal) was accessed via boat provided by the local harbour and structures were secured using plastic cable ties to the buoy's anchoring chain. All structures were secured in a way that would allow movements of samples with the tide and complete, constant submersion. A submersion period of 2 months was chosen as this timescale has been previously shown to be long enough to support complex biofilm development in the environment (Pinto, 2019). Furthermore, whilst transit times of microplastics in wastewater may be relatively short (< 24 h (Wu, 2021), various treatment methods may result in the long-term retention of microplastics within WWTPs (Huang, 2024), in addition to the intentional long-term use of microplastic bio-media in WWTPs worldwide (Muliyadi, 2023).

2.3.2. Collection

After 2 months (early April 2023) samples were detached using sterile scissors, placed into sterile boxes and transported on ice to the laboratory. Water/wastewater samples (1 L) were also collected from all locations in sterile 1 L Duran bottles. At each location, environmental parameters were recorded upon sample collection using a multi-probe (Hanna; HI 98127). All environmental parameters were collected in triplicate and an average value was calculated.

In the laboratory, biological replicates (wires) were separated from cable ties using sterile scissors in a CAT-II cabinet, washed thoroughly (twice) with 40 mL sterile 0.85 % NaCl (Sigma-Aldrich, PCode: 1003326144) to remove any loosely attached bacteria and left to air dry under sterile conditions (Fig. 3). Then, using sterile wire cutters and forceps, particles were removed from the wire and placed in duplicate (technical replicates) directly into PowerBiofilm Bead Tubes containing 350 µL of lysis buffer obtained from a DNeasy PowerBiofilm kit (Qiagen, LOT: 175013851), which was later used during downstream DNA extraction processes for biofilm samples. These were then stored at −70 °C until use (Fig. 3).

Water/wastewater samples were mixed by hand (Duran bottle inverted thrice), aliquoted into 4 × 250 mL, sequentially spun down and concentrated (3500 rpm, 10 min) with supernatant removed until all liquid was processed and a pellet was formed. Pellets were resuspended

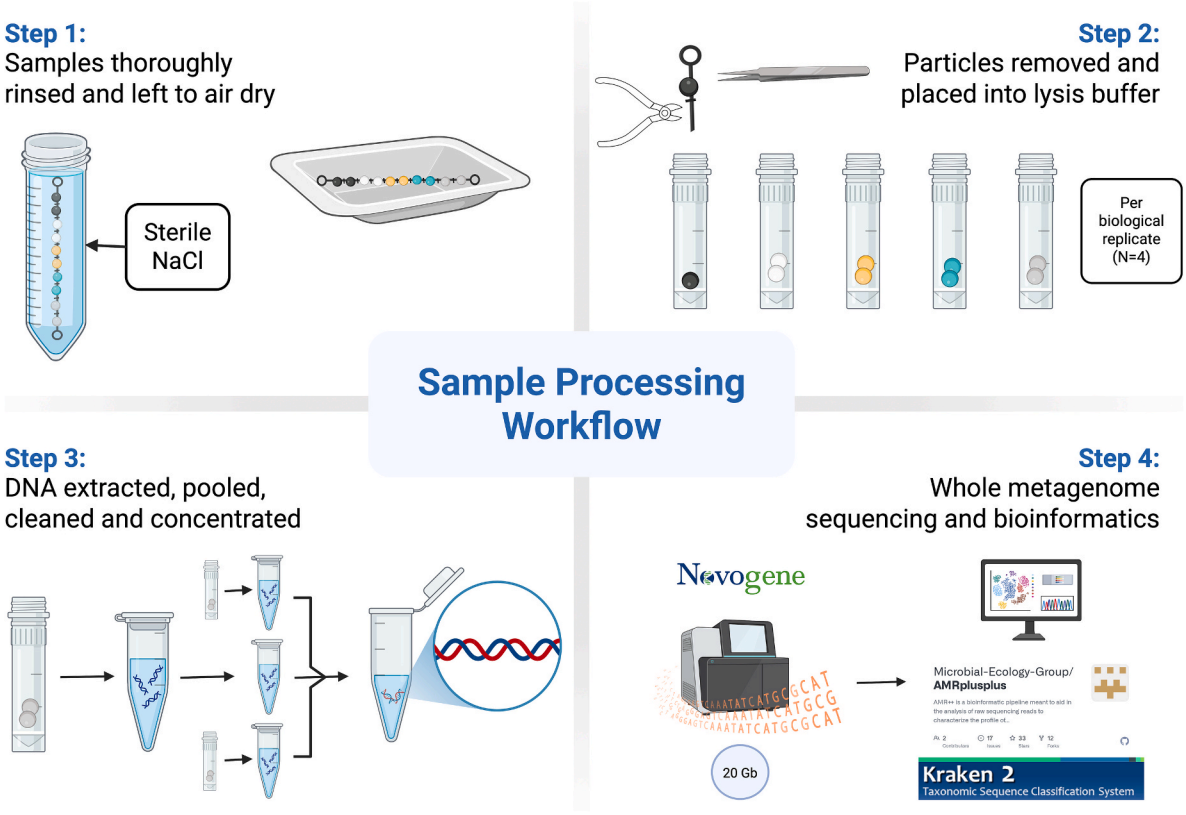

Fig. 3. Workflow detailing the processing of biofilm samples. Created in BioRender. Stevenson, E. (2025) https://BioRender.com/rdnr5sz.

in 300 µL of Powerbead Solution and transferred into PowerBead Tubes provided by a DNeasy Ultraclean Microbial Kit (Qiagen, LOT: 172043975), which was used to extract DNA from water/wastewater samples during downstream DNA extraction processes. These were also stored at −70 °C until use.

All samples were processed on the day of their respective collection.

2.4. Metagenome sequencing

Cryogenic stores of particles and bacterial pellets from water/wastewater samples were thawed and DNA was extracted following manufacturer instructions using the DNeasy PowerBiofilm kit and DNeasy Ultra-Clean Microbial kit, respectively. An aliquot (35 µL) from 3 biological replicates of each particle type, per location, were then pooled, cleaned and concentrated using a DNA Clean and Concentrator kit (Zymo Research, LOT: 215675), resulting in samples of required quantity and quality eluted in TE buffer ready for sequencing (Fig. 3). Prior to pooling, DNA concentrations (ng/µL) were measured using a NanoDrop Spectrophotometer (Thermo Scientific) to ensure comparability across replicates. Library preparation (NEB Next® Ultra™ DNA Library Prep Kit (Cat No. E7370L)) was performed prior to shotgun-based metagenomic sequencing conducted by Novogene (Europe) using a Novaseq 6000 (Illumina PE150) to a sequencing depth of up to 20 GB per sample.

2.5. Metagenomic analyses

Analyses were conducted using trimmed reads provided by Novogene, with all reads processed using the AMR ++ pipeline (Bonin, 2023). Quality assessments were carried out using FastQC and MultiQC (Ewels, 2016). For taxonomic classification, *kraken2* (Wood et al., 2019) was used within the AMR ++ workflow, using the minikraken database (O'Leary, 2016; Pruitt et al., 2007). Resistome analyses was performed by aligning reads to the MEGARES 3.0 database (Bonin, 2023). This database integrates several resistome databases, including BacMet (Pal, 2014); ResFinder (Florensa, 2022) and CARD (Alcock, 2023). Rarefaction curves were also generated for resistome analyses using the AMR++ pipeline (Supplementary Material, Fig. 1), where the flattening curves across all samples indicate sufficient and representative sequencing data at our chosen sequencing depth (Wang, 2023).

2.6. Statistics

All statistical analyses were conducted using R Studio (using R version 4.4.3 (Team and R.d.c., r, 2010), with figures created using *ggplot2* 3.5.1 (Wickham, 2016).

2.6.1. Taxonomy analyses

Taxonomy outputs from the AMR ++ pipeline were combined into one data frame, and any bacteria not identified to species level were removed. Any species with an unclassified class were allocated as 'not described' (n.d.). All fungal, viral, archaeal and eukaryote species were removed from the data frame. Relative abundance of bacterial species by class were calculated as a proportion of the total community per pooled sample and the top 15 classes (based on relative abundance) were visualised. All other classes outside of the top 15 were grouped as 'Other' and visualised. Alpha diversity of taxa was estimated using Shannon's Index, which was tested for normality using a Shapiro-Wilk test. The Shannon's Index and absolute abundance across location ($N = 6$) or sample ($N = 4$) were then compared using either an ANOVA test followed by Tukey's post hoc test (normal data) or Kruskal-Wallis followed by a Dunn's post hoc test (non-parametric data). All analyses were adjusted for multiple comparisons using the false discovery rate (FDR) adjustment. Free-living community data were not included in absolute abundance analyses due to the complexities in comparing a volume of liquid to the surface area of solid matter.

Using the *vegan* 2.6.10 package (Dixon, 2003), differences in community composition between location and sample were also assessed via Bray-Curtis dissimilarity matrices using absolute read counts for each species. Principal Coordinates Analysis (PCoA) was then performed using the resulting distance matrix to visualise patterns of dissimilarity in microbial communities across samples. Statistical comparisons were made using a PERMANOVA test, with pairwise comparisons (FDR adjusted) calculated using the *RVAideMemoire* 0.9.83.7 package (Hervé and Hervé, 2020).

2.6.2. Pathogen analyses

Species identified via taxonomy analyses were matched with a database of bacterial pathogens known to infect humans (Vos et al., 2025). All non-matched species were deemed human commensals or environmental taxa and removed for downstream analyses. Relative abundance of human bacterial pathogens across locations and samples was explored using descriptive statistics on pathogen reads standardised to total bacterial reads, per sample. Average relative abundance across location (N = 6) or sample (N = 4) were then compared using a Kruskal-Wallis followed by a Dunn's post hoc test, following normality testing. All analyses were adjusted for multiple comparisons using FDR adjustment.

2.6.3. Resistome analyses

Absolute abundance was compared following normality assessments using either an ANOVA test followed by Tukey's post hoc test (normal data) or Kruskal-Wallis followed by a Dunn's post hoc test (non-parametric data), with FDR adjustments. Free-living community data were not included in absolute abundance analyses due to the complexities in comparing a volume of liquid to the surface area of solid matter. Unique classifications at the gene level were calculated using the *ggvenn* 0.1.10 package. Diversity metrics were also explored as described above.

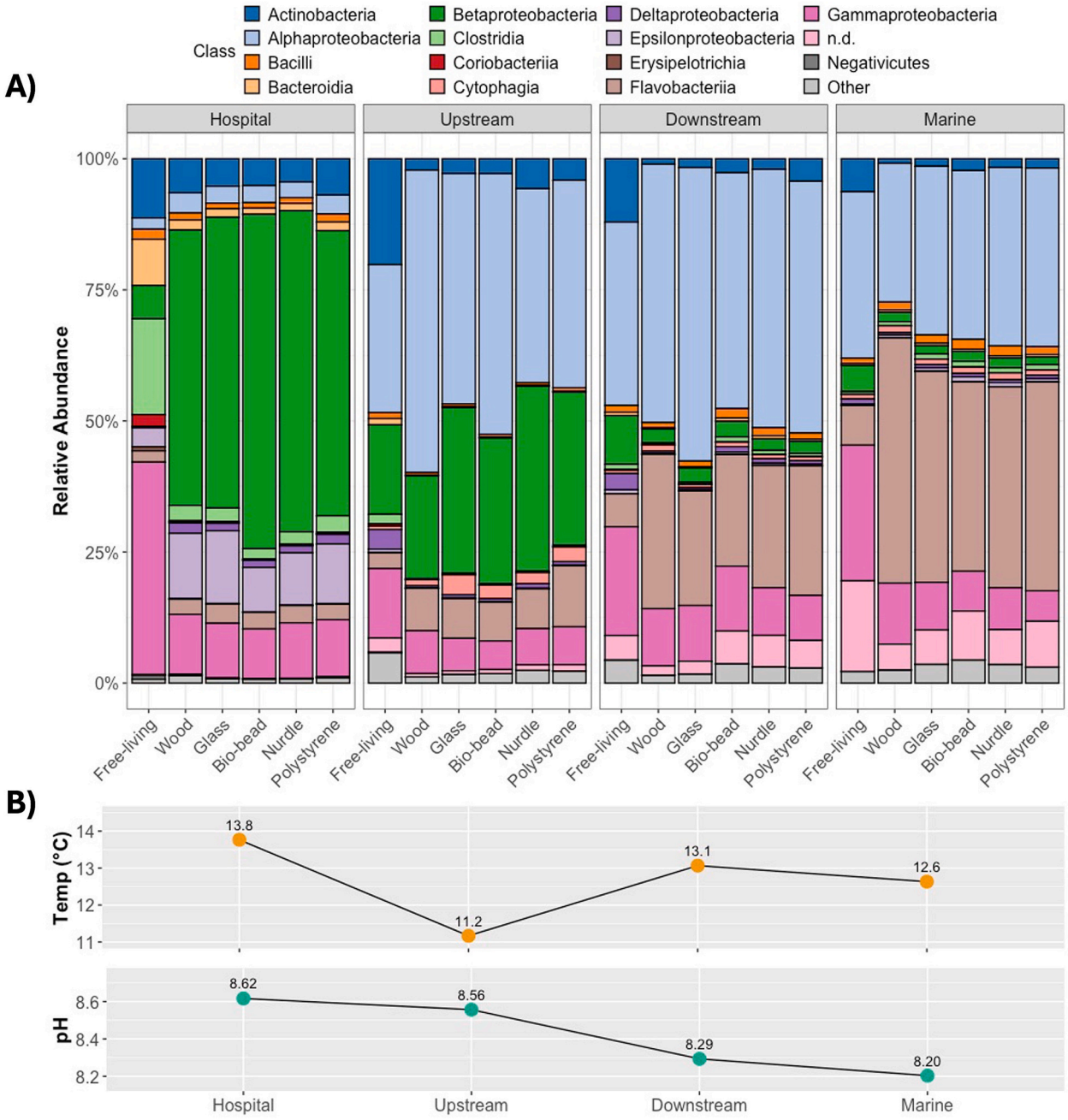

Fig. 4. Relative abundance of bacteria by class (A) with environmental parameter data (B).

Absolute read counts for all antibiotic resistance genes were standardised to total bacterial reads identified in the taxonomy analyses, per pooled sample. These standardised read counts were used to compare relative abundance of genes within each sample, accounting for differentiations in biomass. In most cases, AMR genes were grouped by class when describing differences in relative abundance between location and sample. Average relative abundance across location (N = 6) or sample (N = 4) were then compared using a Kruskal-Wallis followed by a Dunn's post hoc test, following normality testing. All analyses were adjusted for multiple comparisons using FDR adjustment.

3. Results

3.1. Taxonomy analyses

Metagenome data was analysed alongside environmental parameters to determine whether the environment or substrate specific determinants were drivers of community composition. Overall, we detected 41 distinct phyla, 74 classes, 166 orders, 371 families, 1325 different genera and 5427 unique species.

Each site was found to vary by temperature and pH (Fig. 4), creating potential for environmental selection on bacterial density and diversity. Absolute (Fig. 5A–B) and relative (Fig. 4) abundance were found to vary by site, but not by sample type. Specifically, average absolute abundance of taxa significantly varied by site (χ^2 (3) = 16.41, p = 0.0009), where abundances of taxa were significantly greater in the hospital site than both the downstream (Z_3 = -3.05, p = 0.007) and marine environments (Z_3 = -3.63, p = 0.002). Abundance of taxa was also found to be significantly greater in the upstream site than marine (Z_3 = −2.3, p = 0.04).

Beta diversity analyses based on Bray-Curtis dissimilarities found taxonomy variance significantly varied by site (PERMANOVA: pseudo-F = 16.28, R^2 = 0.71, p < 0.001, 999 permutations), but not by sample (PERMANOVA: pseudo-F = 0.43, R^2 = 0.11, p > 0.05, 999 permutations) (Fig. 5E–F). Specifically, pairwise testing found that all sites were significantly different to each other (p < 0.05), except downstream and marine (p > 0.05).

Shannon diversity metrics were also calculated and compared based

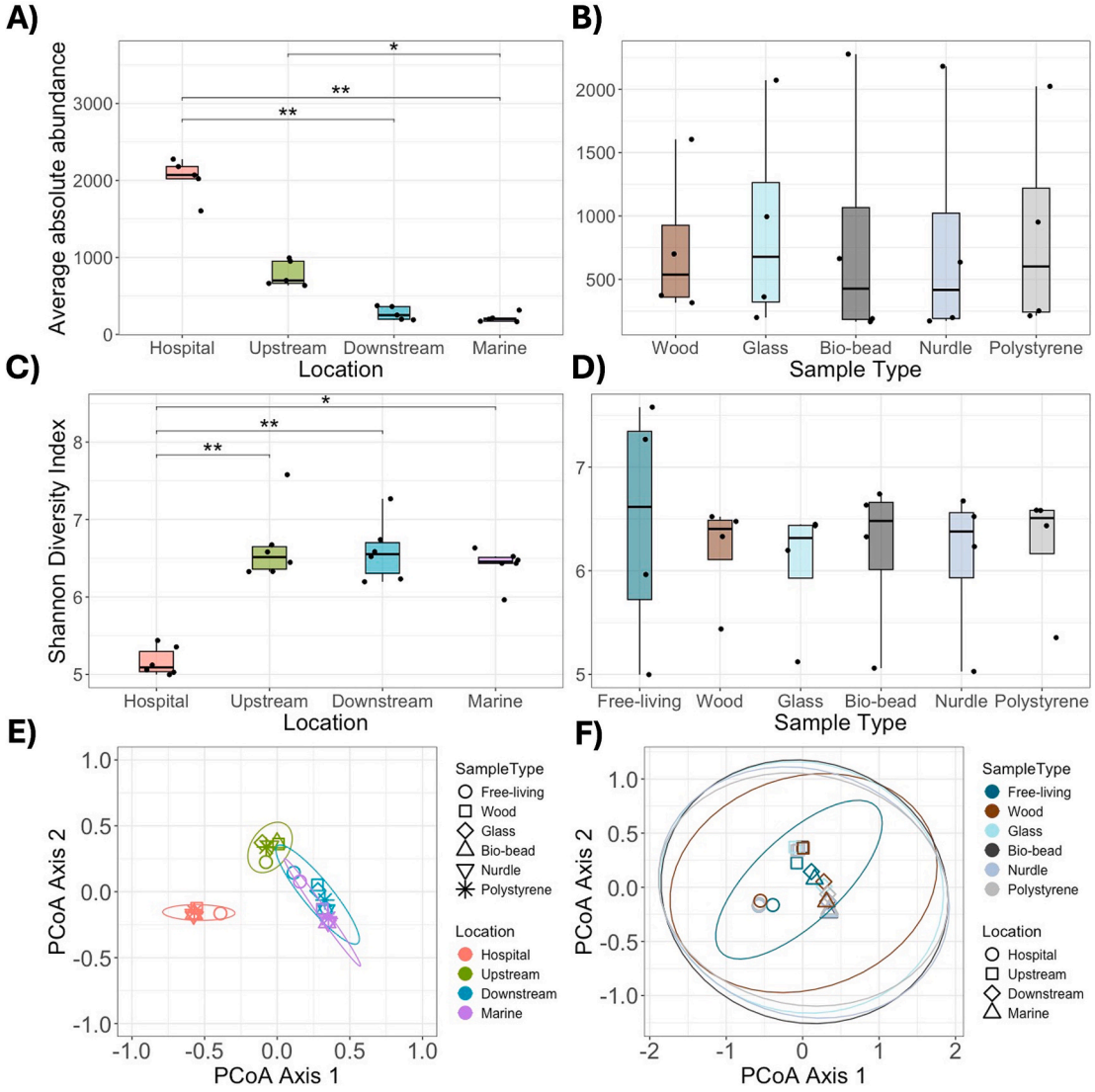

Fig. 5. Absolute abundance and diversity metrics for taxonomy. A–B: average absolute abundance (read counts) by location (A; biological replicate (samples) = 6) and Sample Type (B; biological replicate (locations) = 4). *P < 0.05, **P < 0.01 pairwise test (Kruskal Wallis followed by Dunn's post hoc test and FDR adjusted for multiple comparisons). C–D: Shannon Diversity Index of taxonomy by Location (C; biological replicate (samples) = 6) and Sample Type (D; biological replicate (locations) = 4). *P < 0.05, **P < 0.01 and ****P < 0.0001 pairwise test (Kruskal–Wallis followed by Dunn's post hoc test and FDR adjusted for multiple comparisons). E–F: Principal Coordinates Analysis (PCoA) plots showing Bray-Curtis taxonomy dissimilarities at species level for: E, between locations (N = 6) and F, between sample types (N = 4). Ellipses represent 95 % confidence intervals.

on species evenness and richness by site and sample (Fig. 5C–D). Overall, Shannon diversity significantly varied by site (χ^2 (3) = 13.37, p = 0.004), but not by sample (χ^2 (5) = 0.96, p = 0.97. Specifically, the Shannon diversity was significantly greater in the upstream, downstream and marine sites than the hospital (Z_3 = 3.14, 3.1, 2.58), but these three sites were not significantly different from each other (p > 0.05).

3.1.1. Pathogens

Taxonomy data was matched with a list of bacterial pathogens known to have caused infection in humans (Vos, 2025). From this, we were able to calculate a relative abundance of pathogen species by standardising raw read counts per species to total bacterial reads per pooled sample. When combining all classes together, pathogen prevalence significantly varied by site (χ^2 (3) = 13.25, p = 0.004) but not sample type (p > 0.05). Expectedly, pathogens were at a greater prevalence in the hospital than the upstream (Z_3 = −2.65, p = 0.016), downstream (Z_3 = −2.98, p = 0.009) and marine sites (Z_3 = −3.18, p = 0.009).

Furthermore, distinct patterns emerged at the class level (Fig. 6). In several cases, classes of pathogens were at high prevalence in the hospital, but had reduced abundance downstream, including Actinobacteria, Bacteroidia, Betaproteobacteria, Coriobacteriia, Deltaproteobacteria, Epsilonproteobacteria and Gammaproteobacteria (Fig. 6).

However, several classes of pathogens appeared to increase in prevalence from the hospital to marine environment. These included Chlamydiia, Flavobacteriia, Fusobacteriia, Mollicutes, Sphingobacteriia and Spirochaetia (Fig. 6). Notably, the classes of pathogens which tended to decrease in prevalence downstream remained at relatively high abundance in the free-living community, compared to the particle-associated samples. However, where pathogen abundance increased moving downstream, this was primarily in the particle biofilms and not in the free-living communities. A particularly stark example of this can be seen in Flavobacteriia (Fig. 7), but this was also observed in Fusobacteriia, Mollicutes and Sphingobacteriia (Supplementary Material, Figs. 3-5).

Finally, we looked specifically at species belonging to the Vibrio genus, which have been of particular interest in previous plastisphere research because of their threat to shellfisheries worldwide (Lacerda, 2024; Kimura, 2023). Based on absolute abundance, some of the most commonly detected *Vibrio* spp. across all of our samples were *Vibrio rumolensis, V. parahaemolyticus, V. vulnificus* and *V. chlorerae*. Specifically, *V. rumolensis* absolute abundance was greatest within the hospital environment, but the other species were found on substrates at similar densities or even greater abundance in downstream sites than the hospital.

3.2. Resistome analyses

Absolute abundance and diversity of AMR genes followed the same qualitative patterns as taxonomy, except for Shannon diversity, which was significantly lower in the upstream, downstream and marine sites than the hospital (p < 0.05) (Supplementary Material, Fig. 2). We also determined the number of unique ARG sequences across sites. Overall, the greatest number of unique ARG sequences were found within plastic biofilms (110), followed by natural substrates (30), the planktonic community (27), and lastly, inert substrates (17), with 18.1 % of ARGs shared across sample types (Fig. 8).

We next considered relative abundance of ARGs by standardising raw ARG reads to total bacterial reads, allowing interpretations as a proportion of the total biomass of the community and accounting for variations in size of community. Overall, average ARG prevalence significantly varied by site (χ^2 (3) = 16.36, p = 0.001) but not sample type, with a significantly greater average ARG prevalence in the downstream and marine sites than hospital and upstream (p = 0.008; Fig. 9).

However, as with pathogen data, distinct trends were observed when describing ARG relative abundance across different classes. As predicted, several ARG classes decreased in prevalence moving

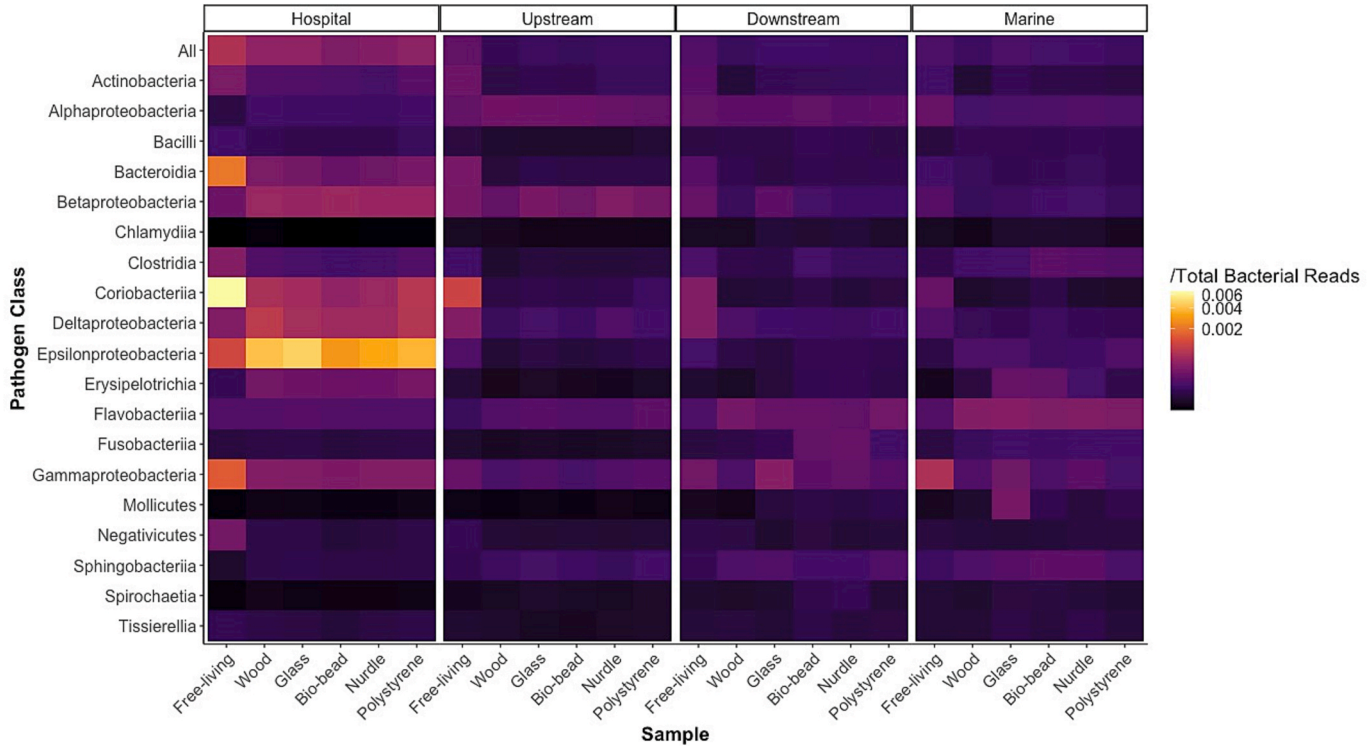

Fig. 6. Heat map showing relative abundance of human pathogen species by class, standardised to total bacterial reads. 'All' represents the average relative abundance across all pathogen classes.

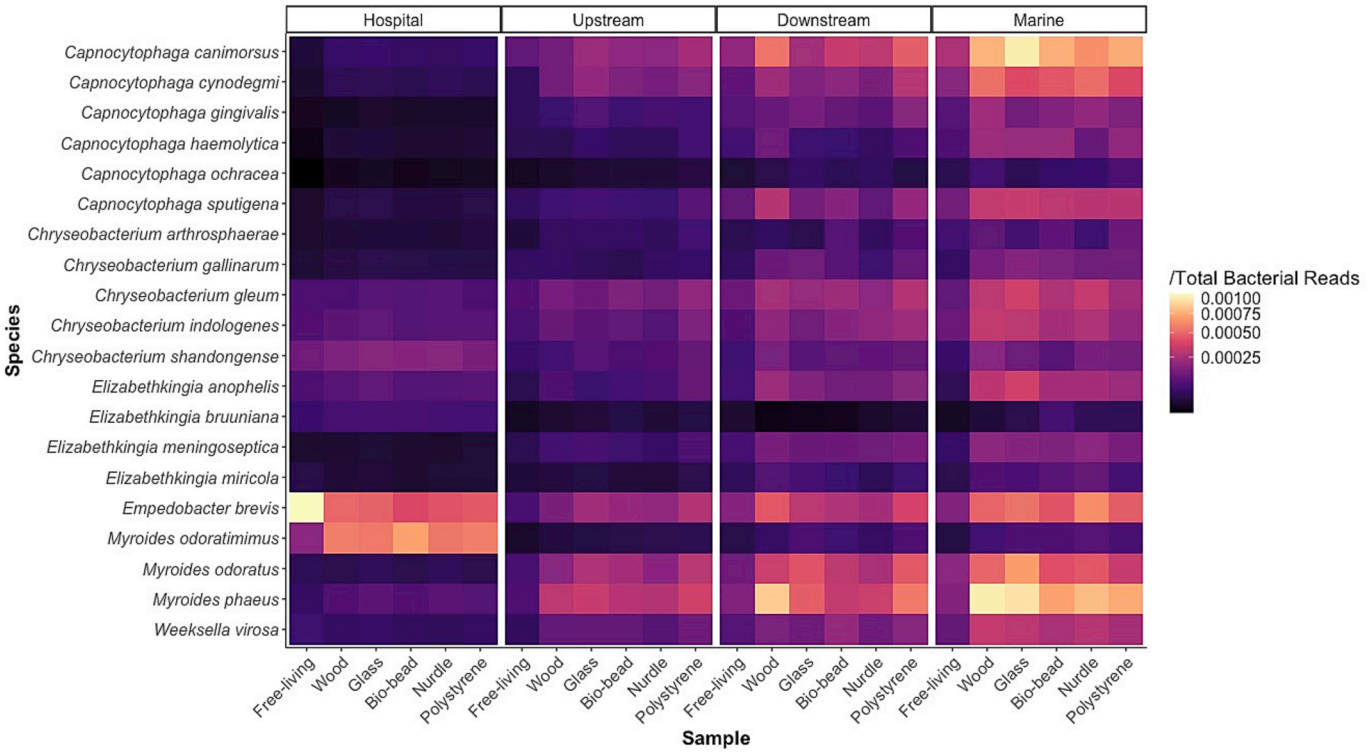

Fig. 7. Heat map showing relative abundance of Flavobacteriia species. Raw species reads were standardised to total bacterial reads.

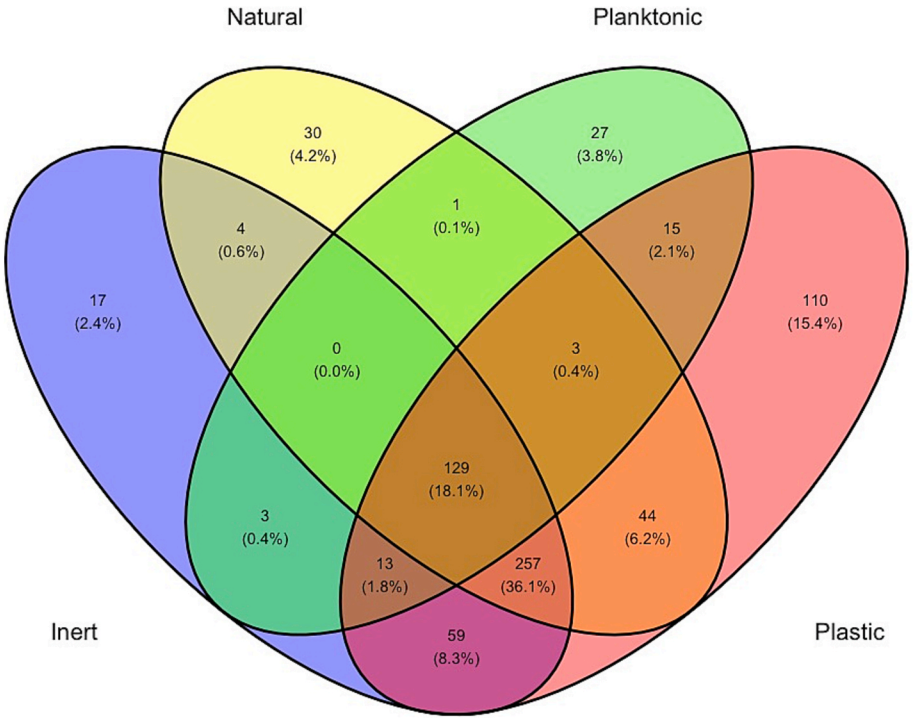

Fig. 8. Venn diagram showing unique ARG sequences according to substrate type across sites. For the purpose of these analyses, all microplastics have been grouped under 'Plastic'. 'Natural' refers to wood samples, 'Inert' refers to glass and 'Planktonic' refers to the free-living community.

downstream, including aminocoumarins, fluoroquinolones, rifampin and sulfonamides (Fig. 10). On the other hand, several ARG classes increased in prevalence moving downstream, including aminoglycosides, cationic antimicrobial peptides, elfamycins and tetracyclines (Fig. 10).

Of particular interest are the classes where there appeared to be a potential influence of substrate on relative abundance between sites (Fig. 10). The clearest demonstration of this is the oxazolidinone genes, which are the most prevalent for all sample types in the hospital and upstream sites, but notably, increase only in prevalence for glass, HDPE nurdle and polystyrene communities in downstream sites and only HDPE nurdle and polystyrene communities in marine sites.

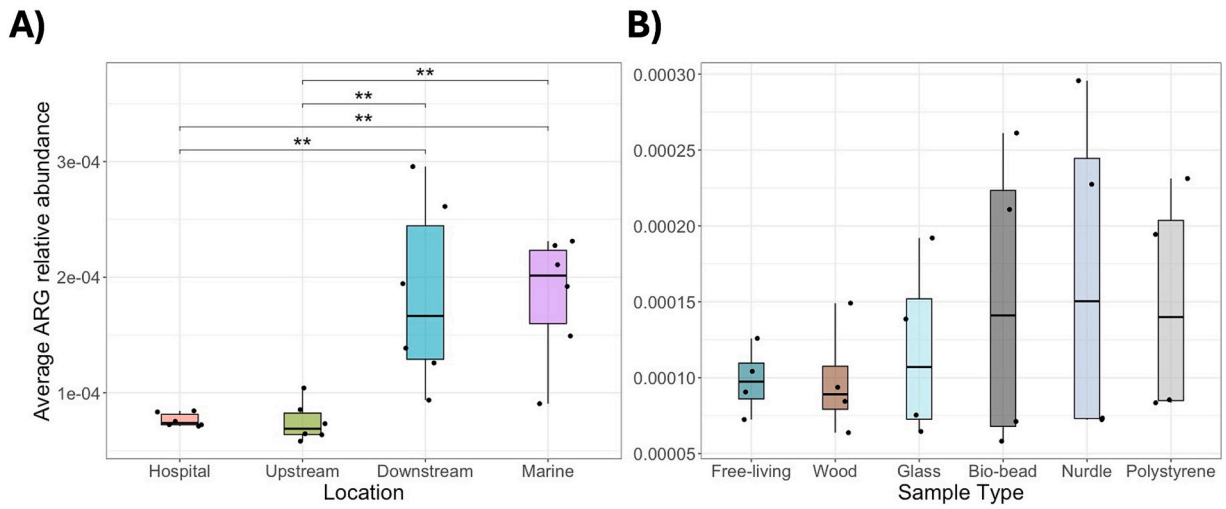

Fig. 9. Average ARG prevalence by Location (A; biological replicate (samples) = 6) and Sample Type (B; biological replicate (locations) = 4). *$P < 0.05$, **$P < 0.01$ pairwise test (Kruskal Wallis followed by Dunn's post hoc test and FDR adjusted for multiple comparisons).

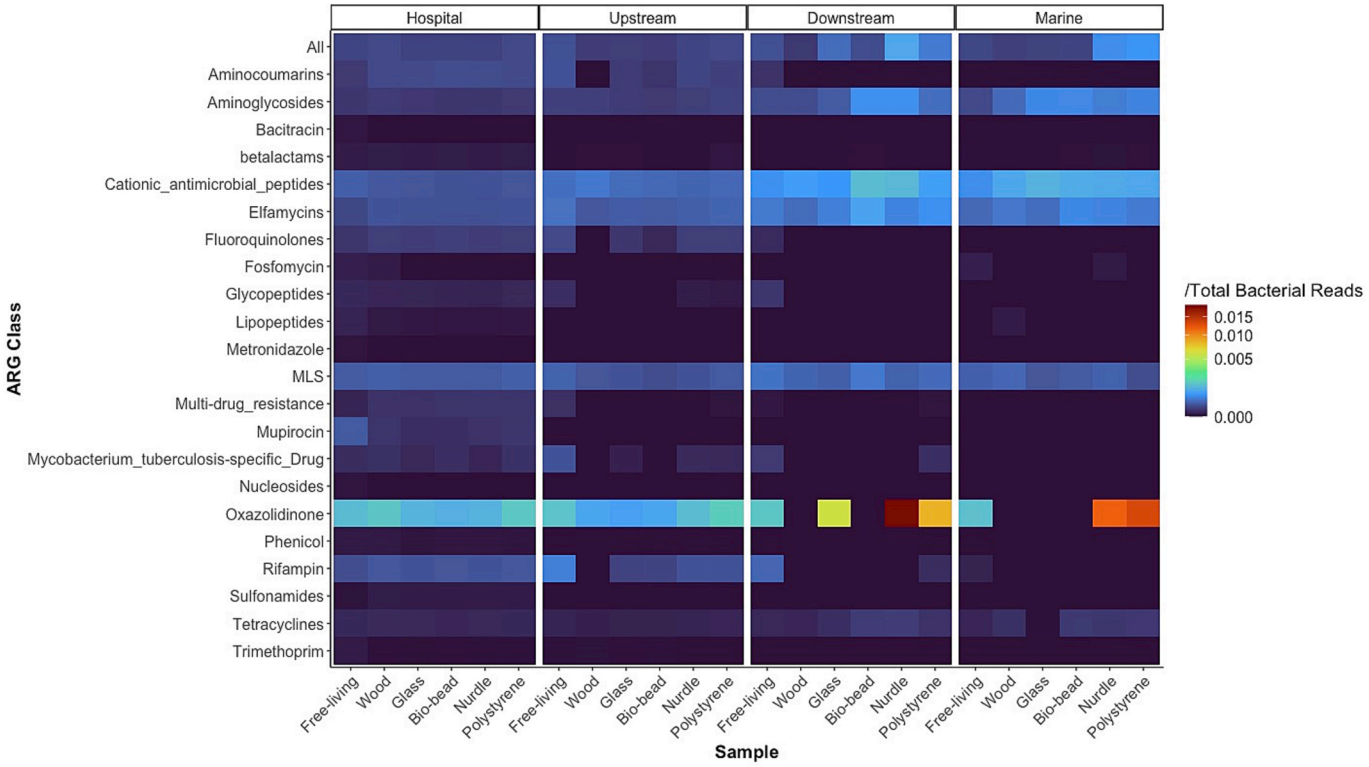

Fig. 10. Heat map showing relative abundance of antibiotic resistance genes (ARGs) by class, standardised to total bacterial reads. 'All' represents the average relative abundance across all gene classes. MLS: macrolides, lincosamides, streptogramines.

4. Discussion

This study aimed to explore bacterial colonisation dynamics on different substrates along an environmental transect, from a highly polluted hospital environment to marine surface waters. Overall, we found that site was more selective than substrate for both taxonomy and resistome, with the particles at the hospital site containing the greatest absolute abundance of both taxa and AMR genes. However, we also found that relative abundance of both AMR genes and pathogens were likely also influenced by substrate specific drivers. We suggest that polystyrene and HDPE nurdles may be higher risk particles by supporting the attachment and persistence of AMR bacteria, with surfaces in general found to support some pathogens in downstream environments. This work builds a foundational understanding of environmental colonisation of microplastics, and we highlight areas for future research.

4.1. The environment selects

Our results show that community composition varied significantly by site, but not by substrate (Figs. 4 and 5). The importance of environmental pressures in colonisation dynamics have been previously found in other environmental plastisphere studies (Miao, 2021; Wang, 2022). Given that temperature and pH also varied across these sites (Fig. 4), it could be proposed that changes in these parameters were important

drivers for colonisation. Previous work has also found a significant effect of these determinants (Pinnell and Turner, 2020; Li, 2019; Dong, 2018; Xu, 2019). However, additional environmental factors have also been shown to be important in community composition, including nutrient availability (Li, 2019; De Tender, 2015), salinity, dissolved organic matter and hydrodynamic forces (Yan, 2024). Given that these metrics were not considered in this work, future work should prioritise a more thorough estimation of environmental parameters.

When looking at particular sites, samples incubated in both the hospital wastewater tank and upstream site were found to be highly distinct from all other locations. This is the first study (to our knowledge) to analyse and compare biofilms from incubated substrates along a transect from a clinical to marine setting. We predicted that the hospital communities would be distinct to the other sites, as it was pre-wastewater-treatment, and it could be expected that WWTPs would significantly reduce the microbial load in treated wastewater. Notably, whilst the hospital had the greatest absolute abundance of bacteria, this site also had the lowest alpha diversity (Shannon Diversity Index). This may be interpreted as the hospital having several abundant taxa, whereas the other sites have significantly more taxa (richness) but at lower abundance. This may be because only a small number of taxa are able to survive in the hospital's conditions, given the high chemical pollution and abundance of human waste (Azuma, 2019).

4.2. Some pathogens increased in relative abundance downstream when associated with particles

A wide variety of both human and animal pathogens have already been detected within environmental plastisphere communities, with many studies detecting a greater abundance of pathogens associated with microplastic biofilms than control substrates (Sun, 2020; Wu, 2019; Metcalf, 2022). Our data demonstrated that average pathogen prevalence was greatest in the hospital, with several classes decreasing in relative abundance moving downstream such as Epsilonproteobacteria and Gammaproteobacteria (Fig. 6). On the other hand, there were several pathogen classes which appeared to increase in relative abundance moving downstream. Of particular interest were bacteria belonging to Flavobacteriia (Fig. 7), Fusobacteria, Mollicutes and Sphingobacteriia (Fig. 6). Furthermore, not only was an increase in prevalence observed moving downstream, but also a greater relative abundance of bacteria across biofilm samples in comparison to free-living communities, when moving downstream.

The general role of biofilms in supporting pathogenic bacteria is widely known, with research suggesting that particle attachment itself can induce certain virulence factors (Wang, 2024; Schulze, 2021). Quorum sensing is especially important in the communication systems within complex biofilm networks and has also been found to correlate with pathogenesis (Brindhadevi, 2020). A previous study found that bacteria exposed to microplastics developed the ability to form stronger biofilms, with mechanisms associated with changes in cell motility (Gross, 2025). From these findings, it was proposed that microplastics may select for species which are better at forming biofilms, which is particularly concerning given the link between biofilms and pathogenicity of bacteria.

Of the classes of bacteria which demonstrated an increased prevalence within biofilms moving downstream, one of the most interesting was Flavobacteriia (Fig. 7). These bacteria have previously been found to be prevalent within microplastic biofilms (Wu, 2019; Feng, 2020), and have been noted to be particularly prevalent in the late colonisation period (Pinto, 2019). Previously, Zhou (2023) performed an exposure assay, where the toxicological effects of polystyrene microplastics on the white leg shrimp (*Litopenaeus vannamei*) were explored. This work found that exposure to polystyrene particles led to an enrichment of various bacterial pathogens within the shrimp gut microbiome, including Flavobacteriia. Many species within this class can cause infection in marine species, mostly within freshwater and marine fish (Bernardet, 1998). Specifically, *Chryseobacterium* spp. were widely detected within our data, and not only are these prevalent fish pathogens, but they are also intrinsically resistant to a wide spectrum of antibiotics, including tetracyclines and aminoglycosides (Loch and Faisal, 2015). This highlights a significant concern raised by these findings, where pathogens specifically enriched within the plastisphere may threaten biosecurity, due to the ingestion of colonised particles by species of commercial or dietary importance (Bowley, 2021). Particular concern is raised where high relative abundances of these pathogens are present within biofilms in the downstream environment, as structures were incubated neighbouring a shellfish-based aquaculture facility, where filter feeding bivalves are known to act as reservoirs for several bacterial pathogens (including Flavobacterium (Hariharan and Amadi, 2016) and ingest microplastics (Cole, 2023).

4.3. Over 100 unique ARG sequences were identified within microplastic biofilms

Overall, the greatest number of unique ARG sequences were identified within biofilms associated with plastics (110), in comparison to the 30 associated with wood, 27 associated with the planktonic community and 17 unique to glass (Fig. 8). This supports the hypothesis that microplastics within aquatic systems form a novel niche which can support attachment of bacteria harbouring ARGs (Yang, 2025). We propose two key reasons for this: increased rate of horizontal gene transfer (HGT) associated with microplastics and the adsorption of ARGs, antibiotics and other AMR selective entities (Stevenson, 2023).

In general, HGT is associated with biofilm formation (Madsen, 2012), where the proximity of biofilm cells grants greater opportunity for the transfer of genetic information (Flemming, 2016). Whilst this is a widespread phenomenon, microplastics have been suggested to present a uniquely high propensity for HGT of ARGs. A well-cited study found that transfer of a mobile genetic element (MGE) harbouring an ARG was at a significantly greater frequency within a microplastic biofilm than in a free-living community and between naturally aggregated bacteria (Arias-Andres, 2018). A more recent study also compared conjugative transfer of an ARG between microplastic-associated biofilms and free-living communities, finding enhanced HGT within the microplastic biofilm at nearly 20 times that of the planktonic community (Zhou, 2024).

Besides conjugation, transformation of ARGs is also found to occur at an increased rate within microplastic associated biofilms (Wang, 2023). This is likely a result of the abundance of extracellular ARGs sequestered in the biofilms associated with microplastics (Nielsen et al., 1997). Extracellular DNA (eDNA) can be incorporated into the three-dimensional structure of the biofilm and protected, suggesting the EPS matrix may act as a repository for ARGs, increasing transformation in biofilms compared to bacterioplankton (Wang, 2023; Merod and Wuertz, 2014).

Indeed, the very formation of biofilms on microplastic surfaces increases the number of oxygen-containing functional groups (Yan, 2024). This enhances both the hydrophobicity of the polymer and the number of available adsorption sites, resulting in a stronger affinity for hydrophilic antibiotics present in the environment, such as oxazolidinones (Guo et al., 2019). This may be a further reason for the presence of unique ARG sequences, as antibiotics which are not typically found at high concentrations in the liquid phase of these systems are adsorbing to the microplastics, creating concentrated micro-hotspots for selection of ARGs within the biofilm community.

4.4. Polystyrene and nurdles may be higher risk substrates for AMR

When looking at average relative abundance across all ARG classes, HDPE nurdles and polystyrene were found to have a comparatively higher relative abundance in both the downstream and marine sites (Fig. 10). When looking at specific classes, it is highly likely that this is

driven by the notably high abundance of oxazolidinone ARGs within these communities and, to a lesser extent, ARGs conferring resistance to aminoglycosides, tetracyclines, cationic antimicrobial peptides and elfamycins.

Oxazolidinone antibiotics inhibit protein synthesis (Wilson, 2008) and are recommended as last resort antimicrobials for severe infections which have survived treatment by other antibiotics, including pneumonia, bloodstream infections and MRSA (Hashemian et al., 2018). Furthermore, oxazolidinone ARGs are typically associated with MGEs and can be transferred via HGT (D'Andrea, 2019; Fioriti, 2020), which may be why these resistance genes are present at high abundances within these biofilm communities and not free-living communities. Another reason may relate to the use of phenicol antibiotics (including florfenicol) in livestock, which readily select for the *optrA* ARG, conferring resistance to both phenicol and oxazolidinone antimicrobials (Yang, 2020). Whilst we do not have data on specific antibiotic usage, the areas surrounding the river system are largely agricultural with aquaculture facilities also present, and the use of florfenicol is licenced in the UK for cattle, pigs and aquaculture (Faulkner, 2016). It could therefore be proposed that residues from these industries are leaching into the environment, selecting for oxazolidinone ARGs or even becoming selectively adsorbed to substrates like microplastics, facilitating the establishment of ARGs in environmentally exposed bacteria.

4.5. Relative abundance of ARGs increased downstream

Based on the knowledge that the hospital wastewater would likely be more contaminated than our downstream sites, we expected that all ARG classes would decrease in prevalence moving downstream. However, we found average relative abundance of ARGs was greatest in the downstream sites (Fig. 9). When relative abundance by class was visualised by location and substrate, we began to observe specific trends. Many ARG classes, including aminocoumarins, fluoroquinolones, rifampin and sulfonamides, did decrease moving downstream (Fig. 10) which may have resulted from the expected decrease in antimicrobial micropollutant concentrations, changes in environmental parameters, and/or the shift in community composition.

However, many ARG classes increased in relative abundance moving downstream, including aminoglycosides, cationic antimicrobial peptides, oxazolidinones, elfamycins and tetracyclines (Fig. 6). Aminoglycoside ARGs remained at a high absolute abundance throughout all sites, which may be a result of the use of these antimicrobials as plant protection products and run-off from surrounding agricultural land. Some of the most commonly used aminoglycoside antibiotics used in agriculture include streptomycin, kasugamycin and gentamicin, which have all been documented to result in the development of AMR in exposed bacteria (Haynes, 2020). Antimicrobials applied to crops are of particular concern in terms of AMR development, given that they are applied to soils at effect concentrations, resulting in high concentrations entering aquatic systems as run-off (Stevenson, 2022). Though we have not quantified antibiotic concentrations in the present study, we highlight this as a potential area of concern for this geographical region, and others where high levels of agriculture may impact the watershed.

Tetracycline ARGs were also found to increase in relative abundance moving downstream. Tetracycline antibiotics (e.g. oxytetracycline) are also used in agriculture (Haynes, 2020) and have been widely documented to adsorb to microplastics within aquatic settings (Yu, 2020; Chen, 2022; Zahmatkesh Anbarani, 2023). Development of tetracycline resistance within microplastic-associated biofilms following adsorption has been previously documented (Tian, 2023). Practically, it could be proposed that these pollutants would typically dissipate within surface waters or drastically reduce in concentration due to the dilution effect of high flow waters. However, with the presence of microplastics, these compounds become concentrated, driving these high prevalences of corresponding ARGs under an elevated selection pressure.

Finally, ARGs which were consistently present across all environments irrespective of sample type are also interesting to note. Included in these are ARGs conferring resistance to aminoglycosides, macrolides, oxazolidinones, tetracyclines and elfamycins. From this, it could be proposed that future monitoring of these sites should be conducted to quantify environmental concentrations, sources and hotspots of these antimicrobial micropollutants.

4.6. Limitations and future research

Whilst we endeavoured to conduct this research in a robust manner, limitations remain. Namely, given the financial constraints of the project, we opted to pool our biological replicates per particle, per site. Though this is something commonly observed in metagenomic analyses, the reduced number of replicates significantly reduced our statistical power and the complexity of models we could perform. As a result, we restricted our statistical analyses to the limitations that were presented and have relied on descriptive commentaries in areas. This is incredibly beneficial data, and is crucial in developing our foundational knowledge, though, future works should prioritise greater replication. We also note the advantages of our novel sampling approach using the purposely designed structures. These present the unique benefit of capturing data on communities present on naturally free-floating substrates, without the typical bias and influence of containment vesicles used in other studies.

Also, as previously discussed, research is already revealing that the plastisphere may represent a novel niche where elevated HGT frequencies, and therefore selection for AMR, occurs (Arias-Andres, 2018; Zhang et al., 2022). The present study did not incorporate any assessment of genetic transfer frequencies within these environmental biofilms, nor were we able to evidence any evolutionary pathways exploited by our study particles. This represents a remaining gap in the current research, and further investigation is required to elucidate the evolutionary potential of plastisphere communities within environmental settings.

Finally, seasonal variations in environmental parameters have been found to influence colonisation dynamics (Pinnell and Turner, 2020). Given that this experiment was conducted across the months of February – April, only Winter to Spring seasonal conditions were explored. Therefore, additional experimentation should be conducted to further unravel how colonisation may vary across the year.

5. Conclusion

Microplastics are extremely prevalent and persistent pollutants, which are not only increasing in quantity, but potentially enriching harmful human/animal pathogens and drug-resistant bacteria. This study found that both AMR and pathogenic species were present on microplastic samples across all tested environments. We also found that over 100 unique ARG sequences were present in microplastic samples, markedly more than on control substrates. We suggest that HDPE nurdles and polystyrene are particles of potential concern in terms of supporting AMR bacteria, due to comparatively higher ARG relative abundances, conferring resistance to several important antimicrobial classes. Assessing the full risk of microplastic particles in terms of AMR enrichment, evolution and dissemination is crucial in preserving human or animal health, biosecurity and maintaining sustainable waste management practices. Whilst the true impact on human health as a result of exposure to these colonised particles is not yet empirically determined, this research indicates there is a potential threat posed by the co-occurrence of microplastics, AMR, pathogens and antimicrobials in aquatic environments, which warrants further investigation. Finally, these findings also indicate that risks posed by co-contaminants and their interactions should be prioritised in aquatic systems typically presumed to be less polluted (i.e. surface waters), as well as highly polluted settings (i.e. wastewater).

CRediT authorship contribution statement

Emily M. Stevenson: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Angus Buckling:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Matthew Cole:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **April Hayes:** Writing – review & editing, Validation, Formal analysis. **Penelope K. Lindeque:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Aimee K. Murray:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2025.109944.

Data availability

The datasets generated and analyses code used in this study are available at Zenodo DOI: 10.5281/zenodo.15694662. The raw metagenome sequence files generated as part of this study are available at ENA with Accession PRJEB90275.

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