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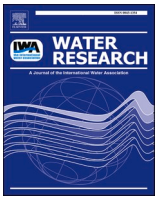

Exploiting microplastics and the plastisphere for the surveillance of human pathogenic bacteria discharged into surface waters in wastewater effluent

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ABSTRACT

Discharge from wastewater treatment plants (WWTPs) is a well-characterised source of human pathogens and antimicrobial resistance genes entering the environment. However, determining whether pathogens released from effluent into surface waters are viable, and consequently pose a risk to human health, is hindered by the use of transient grab-sampling monitoring approaches. Here we present a novel surveillance system using low-cost microparticles (polyethylene, cork and rubber) deployed upstream and downstream of a WWTP effluent pipe, that exploits the ability of bacterial pathogens to form biofilms. Using quantitative culture-based and molecular methods, viable *E. coli, Klebsiella* spp., *Citrobacter* spp., and *Enterococcus* spp. were identified after only 24-hour of deployment. Moreover, these pathogens were continually present at each timepoint (2, 4, 6, 8, 10, 14 and 23 days) as biofilm communities matured, with all pathogens detected at higher concentrations downstream of the WWTP effluent pipe. Long-read whole genome sequencing revealed a suite of plasmids, virulence genes and antimicrobial resistance genes in bacterial pathogens isolated from biofilms formed downstream of the effluent pipe. Furthermore, recognising that pathogens are typically present at proportionally low concentrations within mixed biofilm communities, total biofilm pathogenicity was confirmed using a *Galleria mellonella* infection model. Full-length 16S rRNA gene sequencing revealed that human pathogens present in microplastic biofilms (the 'plastisphere') dominated the microbial community of infected *G. mellonella* larvae within 24 hr, suggesting these bacteria remained highly virulent. Overall, this study demonstrated the efficacy of an easy-to-deploy system for the surveillance and rapid detection of pathogenic bacteria being discharged from point-source pollution. We envisage that if used as part of an integrated environmental management approach, this approach could help to reduce the public and environmental health risks of human pathogens and antimicrobial resistance genes, by monitoring viable human pathogens entering surface waters.

1. Introduction

Wastewater treatment plants (WWTPs) play a vital role in reducing the release of harmful human pathogens and associated antibiotic resistance genes (ARGs) into the environment. Although WWTPs are meant to capture and prevent the release of most faecally derived microorganisms, many potentially harmful pathogens and associated ARGs still escape this process and are introduced into the environment where they can then accumulate (Dai et al., 2022; Raza et al., 2022). In urban areas, pathogenic bacteria and ARGs are often more prevalent in receiving water downstream of WWTPs, particularly after combined sewage overflow (CSO) events (Honda et al., 2020; Mukherjee et al., 2021). Once discharged, pathogens can be transported through freshwater and coastal systems, increasing the potential for human exposure, e.g., at bathing water beaches (Numberger et al., 2019; Urban et al., 2021).

The combined pressures of rising urbanisation, inadequate infrastructure and climate change, increases the impetus for developing a low-cost monitoring approach that can be deployed independently from the water company managing the WWTP. Such a system could improve our understanding of the frequency and magnitude of human pathogenic bacteria being released through wastewater discharge and provide data on the actual pathogenic and antimicrobial resistance (AMR) potential of the microorganisms being discharged into the environment.

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Traditional monitoring methods for measuring microbial concentrations in receiving waters involve sampling water over specific areas and periods of time, and screening for pathogens using culture-based methods or, adapting a 16S rRNA gene sequencing approach (Urban et al., 2021). Wastewater-based epidemiology (WWBE) has also become a useful tool for monitoring disease outbreaks in communities, e.g., through genomic surveillance of influenza and coronaviruses, with detectable viral RNA load in wastewater directly correlating with disease cases in the community (Fitzgerald et al., 2021). However, these grab samples will only ever give a snapshot of potential pathogens at any one time, and as many effluent releases have no defined schedule it is difficult to capture such transient events. In marine environments, sentinels, such as the bivalve *Mytilus*, have been successfully used for the biomonitoring of marine pollutants, including *E. coli* and norovirus (Desdouits et al., 2023; Winterbourn et al., 2016). However, the feeding behaviour of bivalves mean they quickly ingest organic material and any attached pathogens, or excrete them in pseudofaeces, meaning they are less reliable for retaining and monitoring the flux of pathogens being released in wastewater (Burge et al., 2016).

During the last decade, research has focused on the increasing volumes of microplastic pollution in freshwater and marine environments. Notably, the surfaces of environmental plastic pollution are rapidly colonised by a microbial biofilm (referred to as the 'plastisphere'), which often contains human pathogens (Metcalf et al., 2022; Zettler et al., 2013). Evidence has demonstrated that pathogenic bacteria in the plastisphere can harbour virulence traits, and contain plasmids with AMR genes acquired from other bacteria, suggesting that the plastisphere could be a hotspot for interspecific plasmid exchange (Metcalf et al., 2024). Importantly, the rapid colonisation and subsequent successional stages of the plastisphere could offer the potential for the development of a novel tool that exploits the ability of microplastics to capture pathogens being discharged in wastewater that would otherwise be missed or under-reported by single timepoint grab-sampling methods.

Therefore, the aim of this study was to quantify the potential for microplastics to act as sentinels for the surveillance and rapid detection of human pathogenic bacteria released into receiving surface waters via a point-source discharge effluent pipe from a WWTP. We quantify the concentrations of target pathogens on microplastics situated upstream and downstream of the discharge pipe, and determine the pathogenicity of the total plastisphere community using a *Galleria mellonella* model of infection; in addition, we determine which plastisphere pathogens are still detectable in *Galleria* larvae 24 h post-injection, using full-length Nanopore 16S rRNA gene sequencing. Finally, we used whole genome sequencing of single colony pathogen isolates from the plastisphere to assemble bacterial genomes, and annotated plasmids, virulence genes and antibiotic resistance genes, to determine the potential of this approach for identifying the potential public health risk of wastewater discharge.

2. Materials and methods

2.1. Experimental approach

Ultra-high molecular weight polyethylene (PE) beads (ca. 2 mm diameter; Goodfellow, UK), cork pieces (SPD, UK) and rubber granules (Big Atom, UK) were passed through a 2 mm metal sieve to give uniform sized materials. The PE beads were selected due to their uniform size and

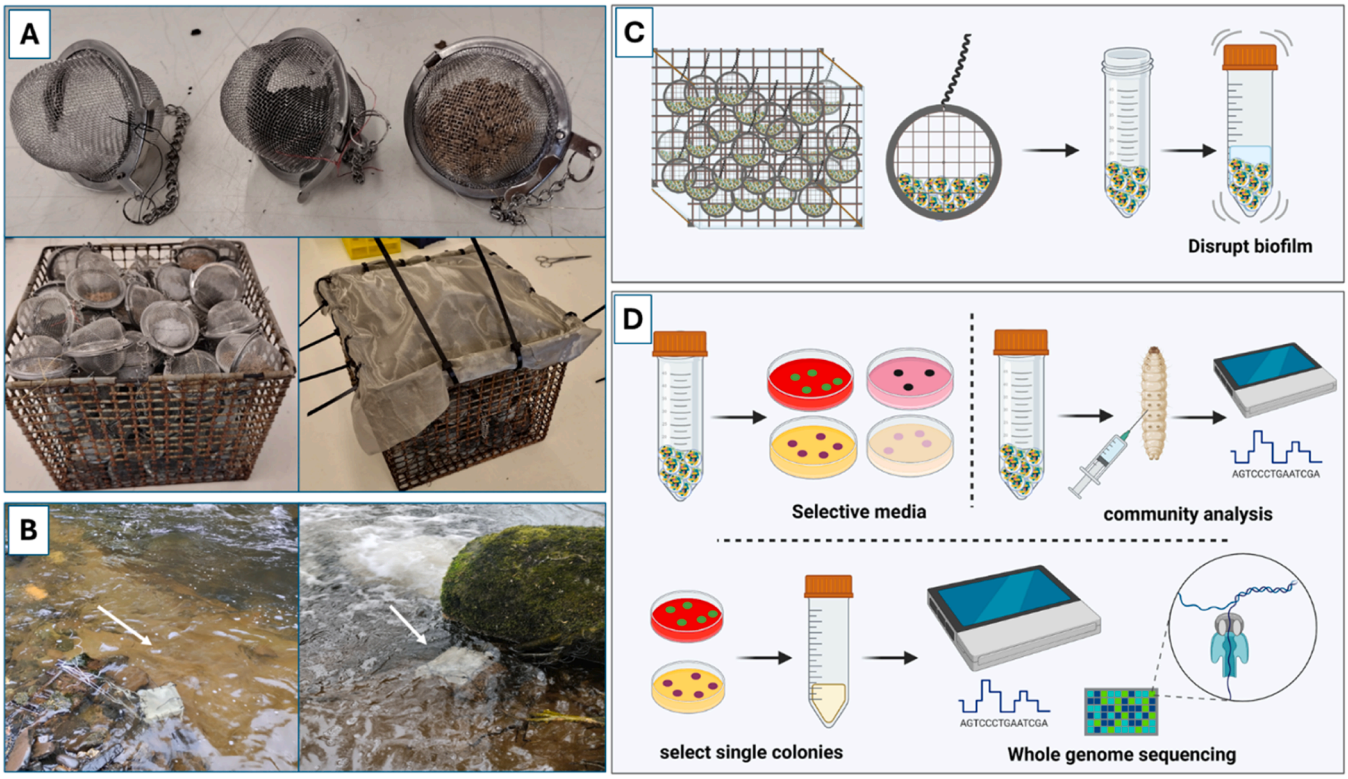

Fig. 1. Cage construction and deployment upstream and downstream of the effluent pipe; and schematic of sample processing. (A) Small metal cages were filled with 200 pieces of either polyethylene, cork, or rubber, and added to large metal baskets; (B) Baskets were positioned upstream (left image) and beside the effluent pipe in an area exposed to continuous backflow from the pipe. White arrows indicate the direction of waterflow; (C) At each time point, four replicate cages of each material were removed from the baskets, rinsed three times in molecular grade water and the material added to glass universal tubes with PBS and glass beads, and vortexed to disrupt the biofilm; (D) Resuspended biofilm was (i) spread on selective media (for target pathogens), and (ii) injected into *G. mellonella* to examine the pathogenicity of the total biofilm community (and to compare the biofilm microbial communities with those injected into *G. mellonella* by sequencing). Single pathogen colonies from selective media were isolated and used for Nanopore longread sequencing analysis. Panels C and D were created in BioRender (2024).

the ease of consistent recovery of the biofilm, the rubber granules were selected as they can enter the environment via vehicle tyre wear, and the cork was used as a natural 'control' material. Two hundred pieces of each material were placed into individual autoclaved replicate spherical stainless-steel metal cages (45 mm high, 38 mm diameter, 1 mm pore size, Amazon, UK), which were added to two large metal baskets (20 cm^3) (Fig. 1A) securely fixed in the river upstream and downstream of a WWTP discharge pipe, located at a site which carries out secondary treatment of wastewater before discharge to surface waters. At each sampling time point, four randomly selected replicate cages containing each material (4 x plastic, 4 x cork and 4 x rubber) were removed from the upstream and downstream baskets. Enough cages were placed in each basket to allow for eight sampling time points. A metal mesh sheet was fastened across the top of each basket using cable-ties to stop the cages being washed away. The metal baskets were suspended in the river using fishing line (Fig. 1B) approximately 15 m upstream of, and directly next to, the WWTP effluent pipe.

Water samples were collected (in triplicate) upstream and downstream at each sample time point and the turbidity, electrical conductivity (EC), and pH were measured using an HI88703 turbidimeter and an HI2550 EC/pH meter (Hanna Instruments, UK) respectively (Table S1). The depth of the river was recorded from a specific location north of the sampling site and the daily rainfall and average temperature data was provided from the nearest meteorological station (data provided by the Met Office, UK). Meteorological data for ten days prior to deployment of the cages, and for the duration of the sampling period, is included (Figure S1).

2.2. Sample processing and bacterial screening

At each sampling time point (day 1, 2, 4, 6, 8, 10, 14 and 23) four randomly selected replicate metal cages containing each of the three materials were removed from the upstream and downstream baskets (12 cages per basket). Each cage was rinsed by submerging in 100 ml MilliQ water (Millipore) three times for five seconds each. One hundred pieces of plastic, cork or rubber were randomly selected and removed using sterile forceps and placed in sterile 30 ml glass vials with 2 ml PBS and five 4 mm glass beads. Each glass vial was vortexed at 1500 rpm for 30 s to dislodge the biofilm from the particles (Fig. 1C).

Following vortexing, the suspension was used for serial dilutions on selective media for target pathogenic bacteria, i.e., *Klebsiella* selective media (Merck, Germany) with selective supplement (Merck, Germany) for detection of *Klebsiella* spp.; Membrane Lactose Glucuronide Agar (MLGA, Oxoid, UK) for detection of *E. coli* and *Pseudomonas* spp.; *Salmonella Shigella* Agar (SSA, Oxoid, UK) for detection of *Salmonella* spp.; and Slanetz and Bartley media (Oxoid, UK) for detection of *Enterococcus faecium* and *E. faecalis* (intestinal enterococci). For each media, 100 μl of the extracted biofilm solution was spread on the agar and all plates were incubated at 37 °C overnight. After enumeration of colony forming units (CFU), single colonies were picked from each media type and mixed 1:1 in LB broth and 80 % glycerol and stored at −80 °C for further analyses.

To confirm species identification, DNA from between 8–12 colonies for each phenotypically presumptive pathogen were amplified using 16S rRNA gene primers (Frank et al., 2008), followed by Sanger sequencing. Briefly, selected colonies were mixed in molecular grade water and heat shocked at 95 °C to disrupt the cells, and the solution added to a PCR reaction mix made with: 2x master mix (Qiagen, Germany), 0.4 μM forward and reverse primer (forward primer - 5′-AGAGTTTGATCMTGGCTCAG- 3′, reverse primer - 5′-TACCTTGTTACGACTT-3′), 5 μl of single colony solution and made up to 25 μl using molecular grade water. Cyclic conditions of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 2 min were run for 35 cycles, with an initial 3 min at 95 °C and final extension of 72 °C for 5 min. Amplification was confirmed on a 1 % agarose gel and each PCR product purified using a Promega Wizard PCR purification kit (Promega, UK). Samples were subsequently Sanger sequenced using the forward primer, and NCBI BLAST used to confirm species ID (Table S3).

2.3. Challenging Galleria mellonella with total biofilm communities

To determine the pathogenicity of the total biofilm extracted from particles of plastic, cork and rubber placed upstream and downstream of the WWTP effluent pipe, a *G. mellonella* infection model was used (Harding et al., 2013). Biofilm was collected from replicate particles at days 2, 6, 10, 14 and 23, suspended in PBS, and 10 μl injected using a sterile Hamilton syringe and a 0.45 mm gauge needle directly into the hemocoel, via the last right pro-limb (Fig. 1D), of ten individual *Galleria* larvae (Livefood, Axbridge, UK). Following injection with the biofilm, *G. mellonella* larvae were placed in an incubator at 37 °C for 72 h and recorded as dead when they were non-responsive to touch. To test whether *G. mellonella* mortality was caused by viable pathogens present in the biofilm, rather than other compounds present in the biofilm, an equal volume of biofilm for each sample at one time point was heat shocked at 95 °C for 1 h, then cooled to room temperature prior to *G. mellonella* injection. Injections of sterile PBS were used as a negative control and a serial dilution of *E. coli* DH5-Alpha competent cells were used as a microbial non-pathogenic control. Additionally, to act as a positive control, known concentrations of a pathogenic strain of a modified *E. coli* O157 (*stx*$^-$) strain (Riedel et al., 2007) were injected into replicate *G. mellonella* larvae. All three control treatments were carried out at the same time as the larval injections with biofilm, and subsequently treated in the same way.

2.4. Sequence analysis of bacterial plastisphere communities before and after injection into G. mellonella

G. mellonella larvae were injected with plastisphere biofilm collected from the plastic particles on days 6, 10, 14 and 23 for subsequent full length 16S rRNA sequence-based community analysis. Larvae were snap-frozen in liquid nitrogen 24 h after being injected and stored at −80 °C until further processing. A modified *E. coli* O157 (*stx*$^-$) strain was used as a positive control at each time point and all larvae processed in the same way.

To examine changes in the bacterial population following injection of plastisphere biofilm into *G. mellonella*, larvae injected with biofilm from just one timepoint (from plastic particles in cages collected from the river at day 14) were processed for further analysis. Total biofilm DNA was extracted from the plastic particles (from 3 replicate cages upstream, 3 replicate cages downstream) and from the *G. mellonella* larvae that had been injected with the corresponding biofilms, using a Zymo DNA/RNA prep kit (Zymo, USA). For the *G. mellonella*, DNA was extracted from a pool of five larvae injected with each replicate biofilm, by crushing the larvae with a sterile pipette tip and processing the homogenate using a Zymo DNA/RNA prep kit. This gave a total of 12 samples (biofilm from plastic particles in three upstream replicate cages, and the three corresponding *G. mellonella* larvae injected with that biofilm; and biofilm from plastic particles in three downstream replicate cages, and the three corresponding *G. mellonella* larvae injected with that biofilm) (Table S2). A negative extraction/sequencing control was included throughout sample processing. Samples were normalised to the same concentration for all samples to give a total concentration of 10 ng DNA and prepared for amplicon sequencing using the Oxford Nanopore Technologies (ONT) 16S Barcoding Kit 24 (SQK-16S114.24) following the manufacturer's instructions. The library was analysed using the EPI2ME Labs 16S workflow to classify all sequences by species and genus, with the read count data presented as a proportional representation of each sample and a 0.5 % abundance cut-off applied to species classification (everything below this threshold is presented as 'other species'). The negative extraction and sequencing control contained <40 reads, which were likely base-calling errors only. The *E. coli* O157 positive control sample produced a 91 % match for *Escherichia* species, with the remaining 9 % of reads composed of matches below the 0.5 %

threshold applied here (data not shown).

2.5. Single colony DNA extractions and long-read library preparation

To investigate the presence of virulence genes, AMR genes and plasmids, genomes were constructed from a selection of single colony isolates. Based on the Sanger sequence analysis of 16S rRNA regions of selected isolates (Section 2.2), samples were selected for whole genome sequence analysis for each pathogen type from one time point only (extracted from plastic particles in cages collected from the river at day 14). Where possible, samples were selected from each material type (PE, cork and rubber) both upstream and downstream (Table S3). An aliquot from each single colony glycerol stock was spread on an LB agar plate and left overnight at 37 °C. Single colonies were placed in 5 ml of LB broth and grown overnight at 37 °C. Genomic DNA was extracted from each sample using a Monarch Genomic DNA Purification kit (NEB, UK) according to the manufacturer's instructions. DNA was eluted in 100 µl and concentrated using streptavidin beads, where an equal volume of beads to eluate were mixed, placed on a magnet, washed twice with 80 % ethanol and resuspended in 10 µl nuclease-free water. The samples were incubated for five min at room temperature, placed back on the magnet, and the supernatant placed in a new tube. Samples were quantified using Qubit fluorometer quantification (Fisher, UK).

DNA was prepared for long-read sequencing using an Oxford Nanopore Technologies (ONT) Native barcoding kit (SQK-NBD112.24, Oxford Nanopore Technologies, UK), following the manufacturer's protocol. Each library was sequenced for 72 h using a MinION MK1C with Basecalling performed in fast mode, with only reads displaying quality scores >8 and read length >200 bp being used for downstream analysis.

2.6. Bioinformatics pipeline

Base-called reads for each barcoded sample were merged into a single fastq file for analysis (see Table S3). Genomes were assembled into contigs using Flye (Kolmogorov et al., 2019), genome completeness and contamination was assessed using CheckM (1.4.0 (Parks et al., 2015)) and species were determined using GTDB tk (v2.3.2, (Chaumeil et al., 2022)), leveraging KBase (Department of Energy Systems Biology Knowledgebase, USA). The Centre for Genomic Epidemiology research database (DTU, National Food Institute, Denmark) was used to determine the presence of resistance genes (ResFinder V4 (Bortolaia et al., 2020; Camacho et al., 2009; Zankari et al., 2017)), plasmids (PlasmidFinder V2.1, (Carattoli et al., 2014; Clausen et al., 2018)), virulence genes (VirulenceFinder V2.0, (Camacho et al., 2009; Clausen et al., 2018; Joensen et al., 2014; Tetzschner et al., 2020)), and to predict pathogenicity (PathogenFinder V1.1, (Camacho et al., 2009)). Each sample was analysed individually before the results were combined where possible for each material type (i.e., upstream plastic, upstream cork, upstream rubber, downstream plastic, downstream cork and downstream rubber). All 16S rRNA and genome sequence data files generated in this project are publicly available under the NCBI BioProject ID: PRJNA1195730.

2.7. Statistical analyses

Statistical analyses were conducted using GraphPad Prism Software (V10.2.1). To compare bacterial pathogen CFU on materials placed upstream and downstream of the discharge pipe, and differences in *G. mellonella* survival at each time point, paired T-tests were carried out with correction for multiple comparisons using the Holm-Šídák's method, with P values < 0.05 considered significant.

3. Results

3.1. Culturable bacterial pathogens in biofilms upstream and downstream of a WWTP effluent pipe

The four targeted bacterial pathogens were detected after 24 h on all three of the substrates placed in the river downstream of the discharge pipe, and at every sampling time point thereafter (Fig. 2). Sanger sequencing of the 16S rRNA from selected isolates of *E. coli, Enterococcus faecium, Enterococcus faecalis*, and *Klebsiella* spp. grown on selective media, provided confirmation for the majority of colonies (Table S3). However, all putatively identified *Salmonella* growing on SS agar were confirmed to be *Citrobacter* spp., (9/9 samples), which can also form black colonies on SS agar, similar in appearance to *Salmonella* spp. (Antonara and Ardura, 2017; Pławińska-Czarnak et al., 2021).

Concentrations of cultured *Klebsiella* and *Enterococcus* recovered from biofilms on materials positioned downstream of the discharge pipe were significantly higher than those placed upstream in most cases (significant in 21 out of 24 pair-wise comparisons, $P < 0.05$). Comparatively, there were fewer significant differences between the upstream and downstream materials for concentrations of *E. coli* (14 out of 24) and *Citrobacter* (9 out of 24), although concentrations of these potential pathogens remained higher downstream of the pipe throughout the sampling period. The concentration of *Pseudomonas* was not significantly different between the upstream and downstream sites for any of the materials (with the exception of PE on day 23 where the concentration was significantly higher upstream ($P < 0.05$)). All five bacterial targets were detectable during the whole 23-day duration of the study both upstream and downstream of the effluent pipe on at least one of the PE, rubber or cork.

3.2. Pathogenicity of biofilm communities extracted from plastic, rubber, and cork

Biofilms recovered from plastic and rubber downstream of the WWTP induced a significantly higher mortality rate in *G. mellonella* larvae than the corresponding upstream biofilms ($P < 0.05$), although a similar effect was not seen for the biofilm recovered from the cork particles (Fig. 3). There were no significant differences in larval survival rates following biofilm injection from the three different material types either upstream or downstream of the pipe. The PBS and *E. coli* DH5-α negative controls did not cause a higher mortality rate in the *G. mellonella* than the biofilms isolated from the materials (Figure S2A/C), but the *E. coli* O157 positive control had an LD50 of 5×10^7 CFU/*G. mellonella* (Figure S2B). Following heat shock treatment, the biofilm from the downstream materials lead to a significantly lower larval mortality rate than the equivalent untreated biofilm ($P < 0.05$), but there was no significant difference in mortality rate between the upstream biofilm and the equivalent heat shocked samples (Figure S2E).

3.3. Diversity and relative abundance of target plastisphere pathogens following injection into G. mellonella larvae

To investigate the relative pathogenicity of the bacterial plastisphere community upstream and downstream of the WWTP, Nanopore 16S rRNA sequence analysis was used to compare changes in the bacterial community on the plastisphere with the community 24 h after its injection into *G. mellonella*. In larvae injected with the upstream plastisphere (100 % survival rate after 24 h) all three larval samples were dominated by the *G. mellonella* commensal bacterium, *Enterococcus innessi* with a relative abundance of 84, 87 and 85 % (Fig. 4A, B and C, respectively). In *G. mellonella* larvae challenged with the downstream plastisphere (~33 % survival rate after 24 h), *E. innessi* was only detected in one out of three larval samples, constituting ~0.5 % relative abundance; in contrast, these larvae were dominated by known species of human enteric pathogens, including *Serratia marcescens* (Fig. 4D),

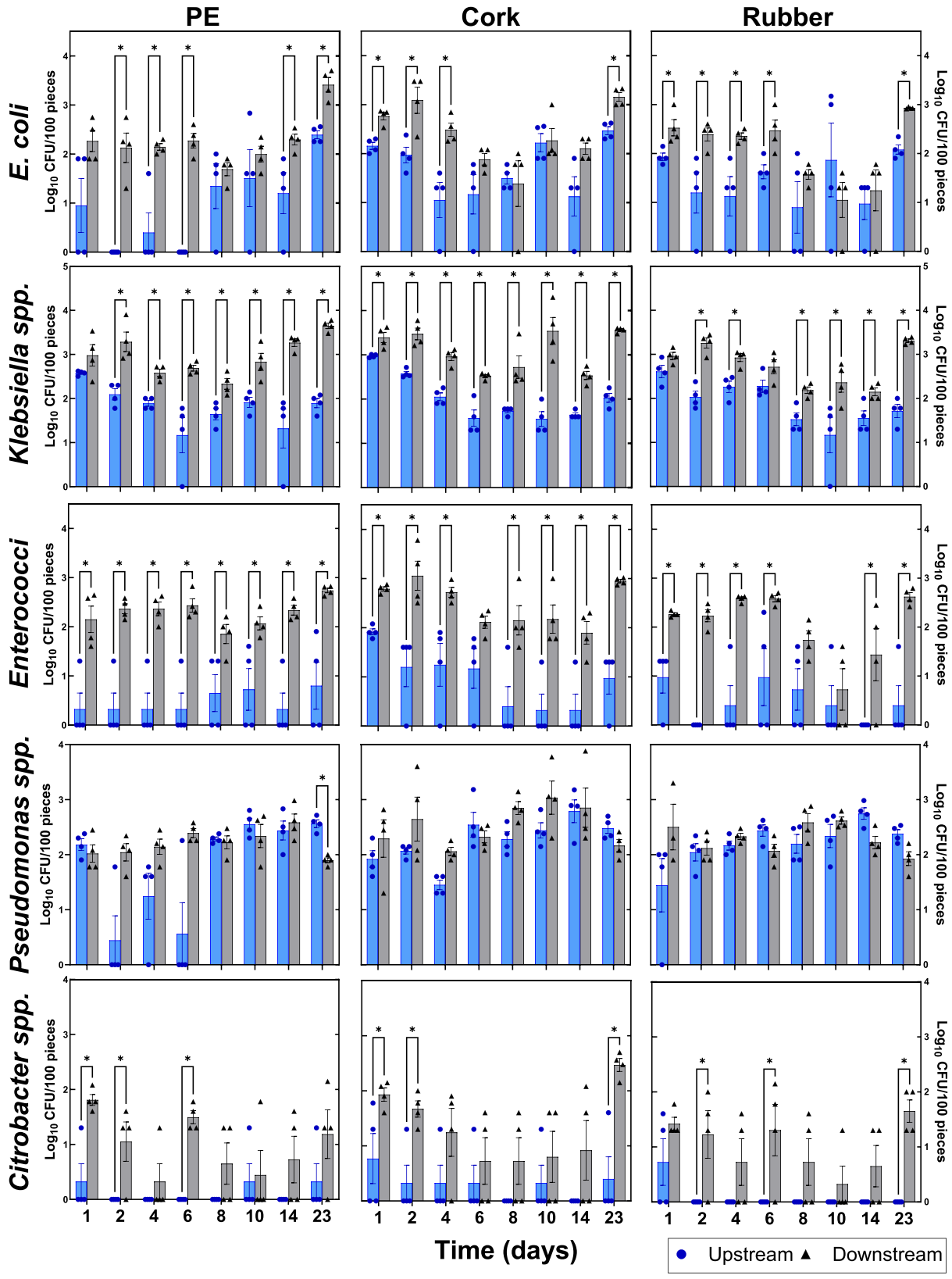

Fig. 2. Concentrations of target bacteria recovered from biofilm colonising plastic, cork or rubber particles, placed upstream or downstream of a WWTP effluent discharge pipe. Each bar represents the log transformed mean (± SE) of four biological replicates (indicated by the individual data points) per time point. Asterisks show significant differences ($P < 0.05$) between upstream and downstream bacterial concentrations (paired T-tests).

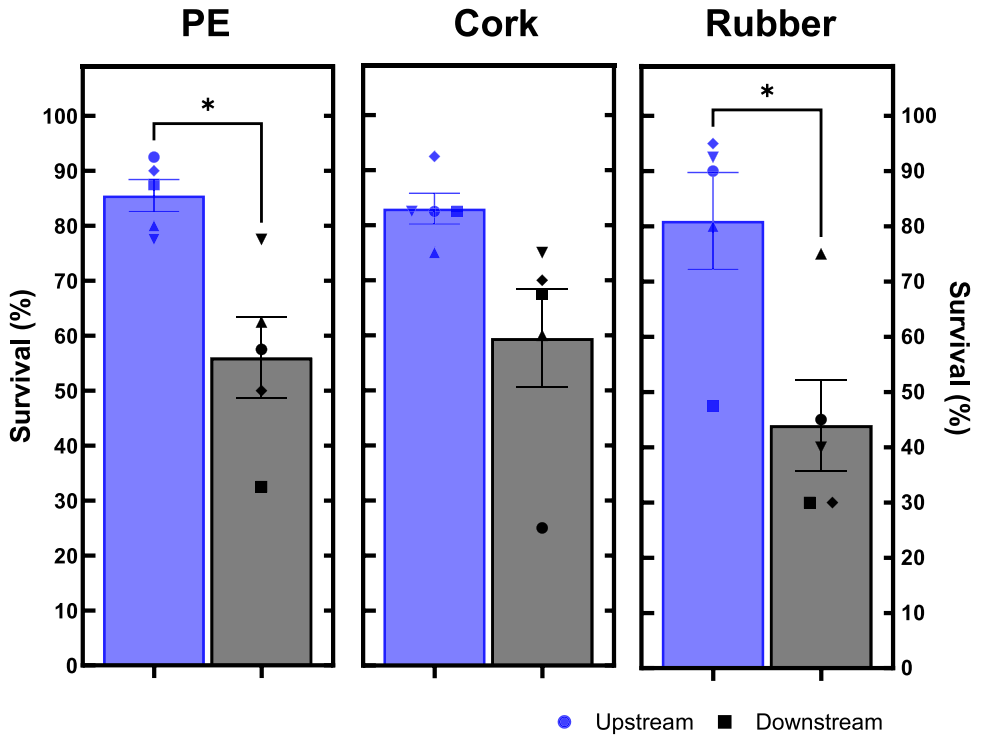

Fig. 3. Comparison of *G. mellonella* survival 72 h after injection with biofilm from plastic, rubber or cork placed either upstream or downstream of a WWTP effluent pipe. Each data point represents the mean of four replicates from each of the five time points (days 2(●), 6(■), 10(▲), 14(◆) and 23(▼)), with the bar representing the mean of all five time points (± SE). Asterisks indicate a significant difference between survival following injection of biofilm from upstream and downstream ($P < 0.05$).

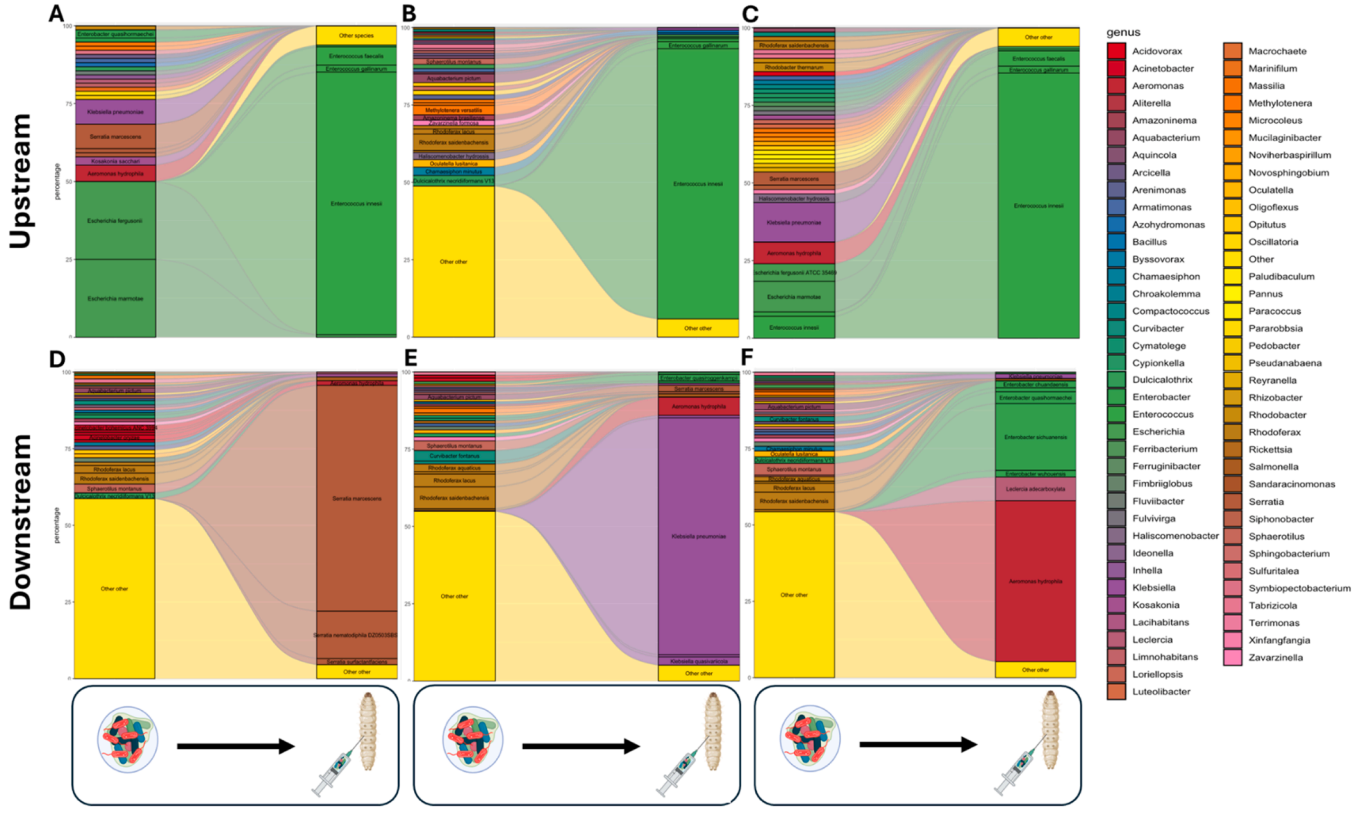

Fig. 4. Bacterial community composition of three replicate plastispheres from plastic particles upstream (A-C) and downstream (D-F) of an eluent pipe, and 24 h after this plastisphere community has been injected into *G. mellonella* larvae. Each block within each column represents a single bacterial species, with the colours determined by genus (identified as being >0.5 % in at least one of the twelve samples), which are shown as a percentage of the population for each sample. All species which produced <0.5 % sequences were classed as 'other' (yellow). Figure was generated using ggplot (Darrin and Bryan, 2021).

Klebsiella pnuemoniae (Fig. 4E), *Aeromonas hydrophila, Leclercia adecarboxylata* and *Enterobacter sichuanensis* (Fig. 4F), with the *Serratia* and *Klebsiella* species comprising up to 73 % and 76 % relative abundance within their respective communities (Fig. 4D and E). Although these pathogens were all detected in the native plastisphere community on plastic particles downstream of the effluent pipe, they only represented a negligible proportion of the total plastisphere community (i.e., < 0.5 % relative abundance), demonstrating a clear increase in relative abundance once introduced into *Galleria* larvae.

In all six larval samples, the diversity of the bacterial community (species identified above the 0.5 % threshold) decreased considerably following injection into *G. mellonella* (between 25–41 species identified in the biofilm and between 5–12 species identified in the injected *G. mellonella*). A large proportion of most of the biofilm reads were below the 0.5 % threshold (~50 % of all reads), indicating a highly diverse community, whereas in reads from corresponding *G. mellonella* larvae following injection of the total biofilm the proportion below this threshold was <5 %.

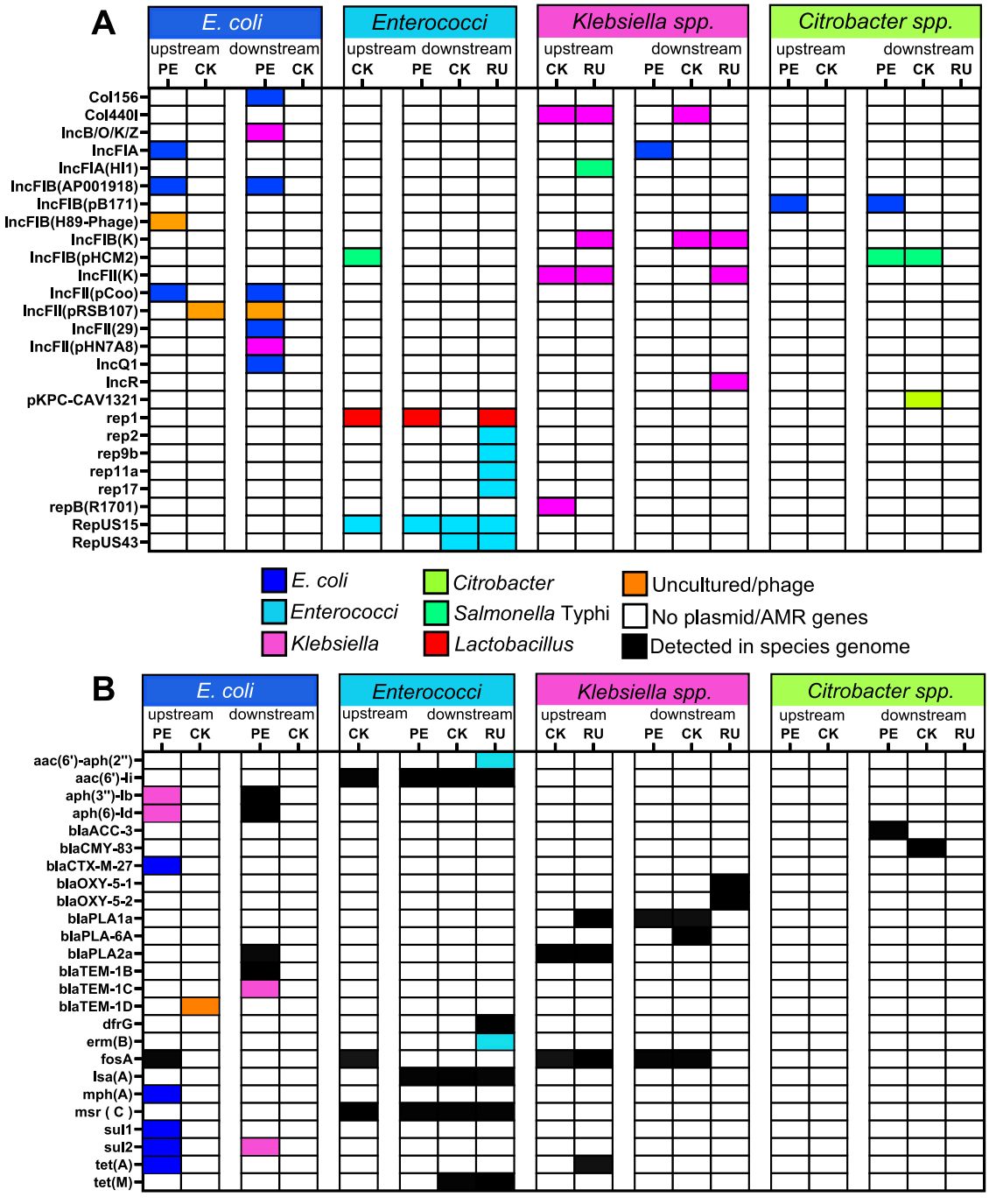

Fig. 5. Presence and absence of plasmids and antimicrobial resistance genes in bacterial strains isolated from biofilms from PE (polyethylene), CK (cork) and RU (rubber) placed upstream and downstream of a WWTP discharge pipe. Where a material is not listed it indicates that no genomes were sequenced. (A) plasmids identified in genomes; (B) AMR genes identified in different genomes, whereby colours indicate AMR genes which were isolated on a plasmid sequence, rather than the host genome (shown as black). Each column represents the combination of all genomes isolated from that particular material (Table S3 for full list of sequences).

3.4. Variation in plasmids, virulence factors and antimicrobial resistance genes in bacterial isolates

Isolates of *E. coli, Klebsiella* spp., *Enterococcus* spp., and *Citrobacter* spp. from the upstream and downstream biofilms, were selected for whole genome sequencing based on the Sanger sequencing results (Table S3), to identify plasmids and genes associated with pathogenicity and virulence. *In silico* analysis revealed a high number of plasmids in all target pathogens (Fig. 5A), with those in biofilms from the downstream materials containing more plasmids compared to those from upstream materials. Although many plasmids were intra-specific, several plasmids were inter-specific, with *E. coli* plasmids identified in *Klebsiella* and *Citrobacter; Klebsiella* plasmids identified in *E. coli*; and two *Salmonella typhi* plasmids observed in *Enterococcus, Klebsiella* and *Citrobacter*. Moreover, AMR genes conferring resistance to a range of clinically relevant antibiotics were present, some of which were encoded on the intra- and inter-specific plasmids (Fig. 5B). Specifically, genes such as *Tet(A), aph(6)-ld, Bla-tem* and *Sul1*, conferring resistance to antibiotics such as Tetracycline, Aminoglycoside, Beta-lactam and Sulfonamide, were present in a number of genomes and plasmids.

All target bacteria, on all three materials (plastic, rubber and cork) encoded virulence mechanisms. *E. coli* isolated from downstream plastic particles, and all *Enterococci* isolated from downstream plastic, cork and rubber contained a larger number of virulence factors compared to bacteria isolated from the same materials upstream of the effluent pipe (Figure S3). Isolates of *Klebsiella* and *E. coli* had virulence factors such as *nlpl* which encodes for motility, *fimH* which encodes an adhesion factor, *fyuA* which encodes fitness mechanisms and several other genes (including *irp2*), and *iutA,* which encodes the synthetases for siderophores, which are used to acquire iron.

4. Discussion

Monitoring effluent discharge from WWTPs is important for understanding the viability, potential virulence, and pathogenicity of human enteric pathogens entering surface waters. This information can be used to inform management practices for controlling the risks associated with exposure to wastewater. This study has demonstrated that microbial biofilms on microplastics can act as a viable sentinel surveillance system for the rapid detection and recovery of targeted human pathogenic bacteria released from WWTPs into aquatic systems. Although there were no significant differences in biofilm communities between the materials used (i.e., polyethylene, cork, or rubber), polyethylene microbeads were easier to deploy and recover subsequent biofilms from. Viable cells of *E. coli, Enterococci* and *Klebsiella* spp that were detected in biofilms on all three materials downstream of the WWTP effluent discharge continued to be present for the full 23-day duration of the study. This demonstrates that this method of surveillance can provide information on pathogen discharge over a 24 h period or provide information on pathogen discharge occurring at least once over a longer period.

4.1. Using microplastics as an in-situ sentinel system to detect major enteric bacterial pathogens

Rapid detection of pathogens in wastewater systems and effluent discharge is an important tool for forecasting emerging diseases in a community and was effectively used as an efficient surveillance tool for the monitoring of SARS-Cov-2 (Fitzgerald et al., 2021; Levy et al., 2023). However, water quality monitoring tools typically involve collecting grab samples from an inherently transient body of water, e.g., a river, meaning samples are liable to miss a significant proportion of any potential pollutants. More recently, meta-genomic tools have been deployed successfully (Osunmakinde et al., 2019; Urban et al., 2021), but these can be expensive, require specialist knowledge and equipment, and do not necessarily provide data on viable pathogens.

The simple sentinel system presented in this study has demonstrated that viable *E. coli, Klebsiella* spp., *Enterococci* and *Citrobacter* spp., were all detectable on microplastics within 24 h of deployment downstream of the WWTP discharge pipe. As drug resistance is common in all four of these target pathogens (Moy and Sharma, 2017; Rice, 2008), monitoring their release into the environment is fundamental for reducing human exposure and infection risk. Importantly, these pathogens remained detectable and viable for at least 23-days, indicating that biofilms of all three materials could be used as either a short-term or longer-term sentinel surveillance tool depending on the type of testing required. However, due to ecological succession in the plastisphere, long-term surveillance could become impractical, as human pathogens start to get outcompeted by environmental communities (Tagg et al., 2022).

Although concentrations of pathogens in biofilms of materials upstream of the WWTP were low, it is likely that they would also have been exposed to those pathogens entering the water via more diffuse sources, e.g., agricultural run-off, from wildlife faeces, or septic tank soakaway (Black et al., 2021; Rosen, 2000). However, the downstream plastic particles would also be exposed to these same background concentrations of pathogens in addition to those being discharged in the wastewater effluent; although it is possible that pathogens were binding more readily to the plastics downstream of the pipe due to the additional nutrients captured in the biofilm on those microparticles.

4.2. G. mellonella bacterial communities were dominated by human pathogens following downstream biofilm injections

Human pathogenic bacteria are commonly isolated from the plastisphere (Junaid et al., 2022), with a few studies demonstrating that populations of single species of human pathogens recovered from the plastisphere retain their virulence when introduced into a *G. mellonella* model (Metcalf et al., 2024; Ormsby et al., 2024b, 2024c). However, the pathogenic potential of the whole plastisphere (where the proportion and concentration of human pathogens will be low) has never before been determined, despite exposure to plastic pollution being the most likely way for humans to come into potential contact with pathogens in the plastisphere. Here, we have demonstrated that the whole plastisphere extracted from plastics downstream of the WWTP effluent pipe caused a higher mortality rate in *G. mellonella* larvae compared to the plastisphere upstream of the pipe. Despite species of known human enteric pathogens making up <0.5 % of the original plastisphere community, after injection into *G. mellonella* larvae, the microbiome was dominated by species of human pathogens. This demonstrates that highly pathogenic bacteria binding to these microplastic sentinels, remain highly competitive and infectious when they encounter a viable host. Although the three replicate groups of *G. mellonella* larvae injected with the downstream plastisphere had similar mortality rates, each group was dominated by different enteric pathogens including *Serratia marcescens, K. pneumoniae* and in one sample a mix of *Aeromonas hydrophila* together with rarer human enteric pathogens like *Leclercia adecarboxylata,* an emerging pathogen found in aquatic environments (Keyes et al., 2020) and *Enterobacter sichuanensis,* which was first recovered in human urine (Wu et al., 2018).

Following injection with the whole plastisphere from plastics placed upstream of the WWTP effluent pipe, the microbiome of all *G. mellonella* larvae were dominated by *Enterococcus innesii*, which is a common gut commensal bacterial species in *Galleria* and not knowingly pathogenic to humans (Gooch et al., 2021). Importantly, this bacterial species was not detected in any of the plastisphere communities, indicating that the human enteric pathogens in the downstream plastisphere can rapidly outcompete commensal gut communities in *G. mellonella*. Additionally, as all samples were processed by DNA extraction, the presence of and effect of RNA viruses was not examined in this study. Whether mammalian viruses infect *G. mellonella* is unknown; however, enteric viral pathogens such as norovirus and hepatitis E have been detected in WWTP effluent and represent a threat to human health (Fumian et al.,

2019; Rau et al., 2024; Wang et al., 2020). It is possible that these viruses would bind to the plastisphere of sentinels positioned downstream of WWTP effluent pipes, where they could persist for several days (Moresco et al., 2022).

4.3. Pathogens in the plastisphere downstream of effluent discharge contain a high number of plasmids and AMR genes

Whilst culture-based screening is a useful indication of which pathogens are present and viable in an environmental sample, understanding how pathogens are evolving and co-existing is important for determining the risks these pathogens pose. Genomes assembled from single colony isolates in this study revealed a wide range of ARGs and plasmids, which were often acquired from other species (particularly in the *E. coli* isolates). The frequency of antibiotic discharge into surface water facilitates the evolution of highly drug resistance bacteria (Raza et al., 2022), and compared to other bacteria in the community, the genomes of *E. coli* in riverine plastispheres have been shown to contain a high number of ARGs and virulence genes (Zadjelovic et al., 2023). Here, we identified a number of ARGs conferring resistance to aminoglycosides, tetracycline and beta-lactams, particularly on plasmids, suggesting resistance is being conferred via plasmid transfer rather than a chromosomal contig in isolates from WWTP effluent (Raza et al., 2022; Silva et al., 2023) and could be a significant resistance mechanism for plastisphere communities. The intrinsic conditions of a WWTP can also promote the emergence of ARGs and pathogenic bacteria, whilst operational conditions, including the use of UV or chlorine-based treatments, can induce stress mechanisms that further promote horizontal gene transfer (HGT) (Conco et al., 2022; Nguyen et al., 2021).

Single colony isolates of *Klebsiella* spp. and *Enterococcus* spp. contained plasmids originally isolated in *Salmonella* Typhi (Sherburne et al., 2000), and these plasmids contained several virulence and antimicrobial resistance genes. Mobile genetic elements can assemble in close proximity to one another, generating complex resistance regions (Nguyen et al., 2021), and when this occurs the acquisition of a single plasmid can result in enhanced drug resistance, especially if these plasmids contain virulence genes (Mangat et al., 2017). This was seen in some of the plasmids in this study, which were originally identified from genomic sequences of *E. coli, Klebsiella* and *S.* Typhi. In enabling environments, such as wastewater effluent, these bacteria can obtain genetic material through a range of HGT mechanisms, potentially leading to the emergence of pathogens with novel combinations of virulence and AMR genes (Ormsby et al., 2024a). Understanding where pathogens are being released and with what frequency is therefore of fundamental importance for monitoring risks to human health and for understanding how potentially novel pathogens may evolve in the environment.

5. Conclusion

Developing appropriate tools to monitor and control the dissemination of human enteric pathogens from WWTPs into freshwater systems is a high priority for human health, particularly within the context of a One-health framework. In this study, we propose the use of microplastics as environmental sentinels for capturing human pathogenic bacteria being released into surface waters from wastewater discharge. Highly pathogenic and viable bacterial species were detected on all three materials within 24 h and were still detectable by day 23; sample screening revealed these pathogens contained a suite of virulence genes and plasmids conferring antibiotic resistance that could easily cause infections via human contact, as shown by the dynamic changes in the *G. mellonella* model. The sentinel surveillance system described here provides a simple, easy to use, highly sensitive and user-friendly approach that can be easily deployed by environmental regulators independently of the water industry. Furthermore, the ability of microplastics to strongly sorb a range of other contaminants offers the potential for further optimising this approach to quantify a wider range of pollutants being released in wastewater discharge (e.g., heavy metals and pharmaceuticals).

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CRediT authorship contribution statement

Luke Woodford: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lauren F. Messer:** Writing – review & editing, Methodology. **Michael J. Ormsby:** Writing – review & editing, Investigation. **Hannah L. White:** Investigation. **Rosie Fellows:** Investigation. **Richard S. Quilliam:** Writing – review & editing, Supervision, Funding acquisition, 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.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.123563.

Data availability

data links are provided in the manuscript for all the sequencing data generated in this manuscript

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