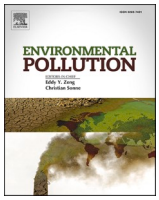
Environmental Pollution 319 (2023) 120983

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol



Detection of faecal bacteria and antibiotic resistance genes in biofilms attached to plastics from human-impacted coastal areas☆

Hongxia Liang [a,b,1], William P. de Haan [c,1], Marc Cerdà-Domènech [c], Javier Méndez [a], Francisco Lucena [a], Cristina García-Aljaro [a], Anna Sanchez-Vidal [c], Elisenda Ballesté [a,*]

[a] Departament de Genètica, Microbiologia I Estadística, Facultat de Biologia, Universitat de Barcelona, Diagonal 643, E-08028, Barcelona, Spain
[b] College of Resources and Environment, University of Chinese Academy of Sciences, 100049, Beijing, China
[c] GRC Geociències Marines, Departament de Dinàmica de La Terra I de L'Oceà, Facultat de Ciències de La Terra, Universitat de Barcelona, E-08028, Barcelona, Spain

ARTICLE INFO

Keywords:
Microplastics
Faecal pollution
Faecal indicators
ARGs
Plastic biofilms

ABSTRACT

Plastics have been proposed as vectors of bacteria as they act as a substrate for biofilms. In this study, we evaluated the abundance of faecal and marine bacteria and antibiotic resistance genes (ARGs) from biofilms adhered to marine plastics. Floating plastics and plastics from sediments were collected in coastal areas impacted by human faecal pollution in the northwestern Mediterranean Sea. Culture and/or molecular methods were used to quantify faecal indicators (*E. coli*, Enterococci and crAssphage), and the ARGs *sul*I, *tet*W and bla_{TEM} and the 16S rRNA were detected by qPCR assays. *Pseudomonas* and *Vibrio* species and heterotrophic marine bacteria were also analysed via culture-based methods. Results showed that, plastic particles covered by bacterial biofilms, primarily consisted of marine bacteria including *Vibrio* spp. Some floating plastics had a low concentration of viable *E. coli* and Enterococci (42% and 67% of the plastics respectively). Considering the median area of the plastics, we detected an average of 68 cfu *E. coli* per item, while a higher concentration of *E. coli* was detected on individual plastic items, when compared with 100 ml of the surrounding water. Using qPCR, we quantified higher values of faecal indicators which included inactive and dead microorganisms, detecting up to 2.6×10^2 gc mm^{-2}. The ARGs were detected in 67–88% of the floating plastics and in 29–57% of the sediment plastics with a concentration of up to 6.7×10^2 gc mm^{-2}. Furthermore, enrichment of these genes was observed in biofilms compared with the surrounding water. These results show that floating plastics act as a conduit for both the attachment and transport of faecal microorganisms. In contrast, low presence of faecal indicators was detected in plastic from seafloor sediments. Therefore, although in low concentrations, faecal bacteria, and potential pathogens, were identified in marine plastics, further suggesting plastics act as a reservoir of pathogens and ARGs.

1. Introduction

Humans extensive use and improper disposal of plastic constitute a serious threat to the environment. Plastics are ubiquitous in all natural and anthropogenic aquatic systems including the sea surface and the water column (Cózar et al., 2014; Law and Thompson, 2014), freshwater (Koelmans et al., 2019; Li et al., 2020), rain (Brahney et al., 2020; Enyoh et al., 2019), sea ice (Obbard, 2018), wastewater (Sun et al., 2019) and drinking water (Koelmans et al., 2019; WHO, 2019). It has been estimated that in 2016, 11% of globally generated plastic waste entered aquatic systems (Borrelle et al., 2020). Furthermore, according to Borrelle et al. predictive model, even if the most ambitious global pledges to reduce contamination are fulfilled, by 2030 between 20 and 53 Mt of plastics per annum will be discharged into aquatic ecosystems.

Land-based activities is one of the main pollution sources for coastal waters, either coming from rivers, inland run-off, or sewage discharges (Borrelle et al., 2020; Lebreton et al., 2017; Lu et al., 2018). In fact, rivers have been identified as major contributors to marine plastic

Abbreviations: qPCR, quantitative PCR; ARG, antibiotic resistance genes.

☆ This paper has been recommended for acceptance by Eddy Y. Zeng.

* Corresponding author.
E-mail address: eballeste@ub.edu (E. Ballesté).

[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.envpol.2022.120983
Received 29 October 2022; Received in revised form 28 December 2022; Accepted 29 December 2022
Available online 31 December 2022

pollution (estimated 1.15 to 2.41 Mt) (Lebreton et al., 2017; McCormick et al., 2014). On the other hand, wastewater treatment plants (WWTPs) are essential infrastructures that help to reduce human pollution in the environment and improve water quality. In fact, they remove a large part of plastics from wastewater, however around 0.1–14% of microplastics can by-pass the plant and reach aquatic ecosystems (Sun et al., 2019). In general, 0–447 microplastics per litre have been detected in the effluent of WWTPs, which amounts to a discharge of 2×10^2 to 1×10^{10} microplastics day^{-1} (Sun et al., 2019). Several studies have reported an increase of microplastic concentrations in river catchments downstream of WWTPs compared with upstream (Kay et al., 2018; McCormick et al., 2014). Thus, WWTP effluent may represent a significant source of plastic pollutants in the aquatic environment (Koelmans et al., 2019). In addition, wastewater treatment also reduces faecal bacteria load, but abundances of around 10^4 cfu 100 ml^{-1} of *E. coli* and Enterococci are still detected in WWTP effluents (Balleste et al., 2019).

Plastic surfaces exposed to water rapidly adsorb nutrients and attract microbial colonizers, which generate an early biofilm (Zettler et al., 2013). Communal life in a biofilm offers microbes many advantages, including easy access to organic matter, protection from environmental stressors, and dispersal opportunities. Within these complex communities, which behave like functioning ecosystems, organisms interact symbiotically, and horizontal transfer of genes is facilitated, including pathogenicity islands and antibiotic resistance genes (ARGs).

Although there is an alarm about the risk of plastics to life, the dangers they pose are still being debated and remain largely unknown (Andrady, 2011; Hermsen et al., 2018; Koelmans et al., 2022; WHO, 2019). However, the rapidity with which bacteria colonise and form biofilms on plastic surfaces has alerted researchers about the risk that these particles may shelter and disperse pathogens (Bowley et al., 2021; Law and Thompson, 2014; Yang et al., 2019) and act as a source of ARGs (Balcázar et al., 2015).

Bacterial clades with pathogenic members, including species of *Vibrio* and *Aeromonas* that affect fish, animals, and humans, have been detected on plastics (McCormick et al., 2014; Wu et al., 2019; Zettler et al., 2013). Besides, a higher concentration of ARGs has been observed on plastic particles than in surrounding water (Bowley et al., 2021; Yang et al., 2019). In an incubation experiment performed in a WWTP, plastics were colonised by potential pathogens such as *Pseudomonas*, *Arcobacter,* and *Mycobacterium* and bacteria carrying ARGs *sul*I and *tet*M (Martínez-Campos et al., 2021). On the other hand, extended-spectrum beta-lactamase-producing *E. coli* were detected in the water by culture methods, but the bacteria were scarcely attached to the introduced plastic polymers incubated in the estuary (Song et al., 2020). Mainly, these studies rely on molecular techniques sequencing a fragment of the 16S rRNA gene so, they barely can identify the bacterial community at genus level, and they do not provide information about viability.

In the present study, we used molecular and culture techniques to quantify the concentration of bacteria in plastic biofilms collected in two coastal areas adjacent to the Barcelona Metropolitan Area, which with >5 million inhabitants, is one of the most densely populated urban conglomerations along the Mediterranean coast. We evaluated if plastics collected in these human-impacted areas are colonised by faecal bacteria (*E. coli*, and Enterococci) becoming a potential reservoir of faecal pathogens and the human-associated faecal indicator crAssphage to identify their potential source. In addition, we monitored the presence of three ARGs (*sul*I, *tet*W and bla_{TEM}) to evaluate if they can become enriched from their surrounding environment.

2. Materials and methods

2.1. Study area

Two coastal areas adjacent to the Barcelona Metropolitan Area in the northwestern Mediterranean were investigated. Both areas are strongly affected by multiple human pressures, including the discharge of water, sediments, and pollutants from the Besòs and Llobregat Rivers, water exchange from recreational and industrial harbours, and the presence of two large metropolitan WWTPs, which have sub-marine outfalls (Fig. S1). Besòs and Llobregat river basins cover 1035 km^2 and 4938 km^2 respectively, equating to over 19% and 10% of their respective drainage areas being urban (Liquete et al., 2009); both rivers fall mainly within the Barcelona Metropolitan Area, a densely populated industrial and commercial zone. The Besòs WWTP, which has a treatment capacity of 3,000,000 population equivalents (PE), discharges 368,243 m^3 of treated sewage daily 2.9 km from shore at a depth of 50 m. The Llobregat WWTP, with a treatment capacity of 2,000,000 PE, discharges 247,187 m^3 of treated sewage daily 3.2 km from shore at a depth of 60 m. Both plants treat sewage water with biological processes using activated sludge, whereas the Llobregat WWTP also provides nutrient reduction of nitrogen and phosphorus.

2.2. Sample collection

Water, sediments, and plastics were collected from both study areas on two separate cruises in 2021. The first cruise (BACT1) took place on June 22 on board the research vessel (RV) Garcia del Cid and the second (BACT2) on October 12 on the RV SOCIB. During each cruise, three transects were sampled adjacent to the mouths of the Besòs River (B1, B2 and B3) and Llobregat River (L1, L2 and L3) parallel to the coast at an increasing offshore distance of 1–2.5 km (Fig. S1).

A total of 12 floating plastic samples were collected using a manta trawl net with a 0.61 × 0.25 m mouth opening and 200 μm mesh, which was deployed for 20–30 min at an average cruising speed of 3 knots at each transect (1.5 m s^{-1}). Furthermore, 3 sediment samples were collected using a KC Denmark multicore during the BACT1 cruise and 5 samples using a Van Veen grab (VV) during the BACT2 cruise. At each sampling station, the top 10 cm of 4 sediment cores and the whole content of the Van Veen grab (i.e., up to 10 cm in depth) were washed through a 0.5 mm sieve. A rapid assessment of the surface and sediment samples was performed on board the vessel, and the visible plastics in each sample were extracted using fine forceps. All plastics were transferred to a clean tray for visual inspection and stored in filtered and autoclaved seawater at 4 °C for less than 24 h to preserve any biofilm conditions.

Furthermore, water samples at three different depths were collected to detect potential sewage plumes from the outfalls and evaluate the sewage impact in the sampled areas. Water was collected using Niskin bottles on a CTD rosette (SBE-911 plus) with dual sensors for oxygen, fluorescence, turbidity, and irradiance. Water samples were stored at 4 °C for <24 h for further analysis in the laboratory.

In the laboratory, plastics kept in sterile seawater were immediately sonicated twice for 1 min using an ultrasonic bath (J.P. Selecta, Barcelona, Spain) to detach the cells. The two resulting fractions (plastics and bacterial suspension in sterile seawater) were separated using Corning cell strainers with a 100 μm mesh (Corning Inc., NY, USA). The suspension was used to enumerate bacteria with culture and molecular methods, whereas plastics were isolated, counted, measured, characterized, and chemically identified.

2.3. Enumeration of marine bacteria and faecal indicators by culture media

Bacteria from plastics were analysed using different culture media to measure marine bacteria and faecal indicators. The abundance of heterotrophic marine bacteria was quantified using Marine Agar 2216 (Difco, Madrid, Spain) after incubation for 48 h at 20 °C. *Vibrio* spp. were enumerated using Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar CM0333 (Oxoid, Thermo Scientific, Madrid, Spain) incubated for 24 h at 20 °C. *Pseudomonas* spp. were enumerated using *Pseudomonas* Agar Base (Conda, Madrid, Spain) and incubated for 48 h at 20 °C. *E. coli* was measured using Chromocult® Coliform Agar (Merck, Darmstadt,

Germany) including the *E. coli*/Coliform selective-supplement (Merk) (2.5 mg of cefsulodine and vancomycin per 500 ml of Chromocult) and incubated at 37 °C for 24 h (ISO, 2000a). Enterococci were enumerated in accordance with ISO standard 7899–2:2000 (ISO, 2000b) in Enterococcus Agar medium (Difco) and then confirmed by incubation on Bile Esculin Agar (Difco). A volume of 0.1 ml was inoculated in the different media; when a high concentration of bacteria was expected, different dilutions were assayed. Results were expressed as cfu 100 mm^{-2}.

Water samples (100, 10 and 1 ml) were filtered through a 0.45 μm pore size filter (EZ-PAK, Millipore, Darmstadt, Germany) to measure the faecal indicators *E. coli* and Enterococci. Results were expressed as cfu 100 ml^{-1}.

Faecal indicators were also analysed from seafloor sediments. Sediment samples were mixed at a 1:10 ratio in phosphate buffered saline (pH 7.0), homogenised by shaking for 30 min at room temperature and centrifuged at 300×*g* (Garcia-Aljaro et al., 2017). The supernatant was collected and used for the subsequent analyses. *E. coli* was enumerated using the pour plate method in Chromocult agar, and Enterococci in accordance with ISO standard 7899–2:2000 (ISO, 2000b). Spores of sulphite-reducing clostridia (SRC) were enumerated by mass inoculation in SPS agar (Scharlab, Barcelona, Spain) and incubated for 24 h at 44 °C, as previously described (Garcia-Aljaro et al., 2017). Results were expressed as cfu 100 mg^{-1}.

2.4. Enumeration of microorganisms by molecular methods

2.4.1. DNA extraction

DNA was extracted from 0.5 ml of the sonicated solution of plastic samples, from 1 L of seawater concentrated by filtration with a 0.22 mm pore size cellulose ester membrane (SO-PAK, Millipore, Darmstadt, Germany) and from 200 mg of sediments. The DNeasy PowerBiofilm Extraction Kit (Qiagen) was used following the manufacturer's instructions. Filtration and DNA extraction controls were run together with the samples.

2.4.2. Quantification of faecal indicators, antibiotic resistance genes and total 16S rRNA gene by real-time quantitative PCR

Total *E. coli* was quantified targeting a fragment of the 16S rRNA gene by qPCR, as previously described (Huijsdens et al., 2002). Total Enterococci was enumerated using the EPA Method 1611 (U.S. EPA, 2012). The human-specific microbial source tracking marker crAssphage was quantified using the primers, probes and protocols previously described (García-Aljaro et al., 2017). Additionally, three ARGs (bla_{TEM}, *tet*W, and *sul*I) were screened using previously described protocols (Calero-Cáceres et al., 2014; Fernández-Orth et al., 2019; Lachmayr et al., 2009). The total 16S rRNA gene was quantified using the primers 341F and 534R using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) (Muyzer et al., 1996, 1995). Specific information about the qPCR assays including reaction mixture, cycling conditions, limits of detection, and information related to the standard curves are shown in supplementary material (Tables S1 and S2). Molecular results of microorganisms from plastics, water and sediments were expressed as gc mm^{-2}, gc 100 ml^{-1} and gc 100 mg^{-1}, respectively.

2.5. Plastics characterization

After the bacteria were detached, all plastics were separated from the bacterial suspension, placed on glass Petri dishes and scanned with an HP G4050 flatbed scanner at high resolution (1200 dpi; 47.2 pixels mm^{-1}). The resulting images were processed with ImageJ v1.53 software (Schneider et al., 2012). Particles were counted, and the area was measured following the methodology described by de Haan et al. (2022). The chemical composition of 12% of the plastics was identified using a PerkinElmer Frontier FT-IR Spectrometer in "Centres Científics i Tecnològics" from Universitat de Barcelona (CCiTUB) (supplementary material).

2.6. Scanning electron microscopy

A few surface plastics were fixed with 2.5% glutaraldehyde in phosphate buffer at pH 7.4 at 4 °C. Samples were successively washed with phosphate buffer at pH 7.4 (4 × 10 min), fixed with 1% of osmium tetroxide, washed with Milli-Q water (4 × 10 min), and dehydrated with different EtOH solutions in water: 50% (1 × 10 min), 70% (ON), 80% (1 × 10 min), 90% (3 × 10 min), 96% (3 × 10 min), and 100% EtOH (3 × 10 min). Finally, the samples were dried using Emitech K850 critical point dryer, mounted on double-coated carbon conductive tape and carbon coated to improve their conductivity. Scanning electron microscope (SEM) observation was done with a JEOL JSM 7001 F at the CCiTUB.

2.7. Data analyses

Microbial abundances were analysed by descriptive statistics and plotted using the statistical software R version 4.0.3 (R Core Team, 2016) through the RStudio interface (Cahn et al., 2011) including the packages "ggplot2" v. 3.0.1 (Wickham, 2016) and "reshape 2" (Wickham, 2007). One-way or two-way ANOVA tests were used to evaluate differences in faecal indicators from water samples between coastal areas and/or cruises. The Wilcoxon signed-rank test was used to evaluate differences in abundance of plastic-attached bacteria between cruises and between sampling areas. Statistical significance of the results was set at $p \leq 0.05$.

3. Results

3.1. Characterization of the study areas

3.1.1. Microbiological quality

The presence of the faecal indicators *E. coli* and Enterococci in water columns of both study areas show that these coastal waters received inputs of faecal pollution (Table S3, Fig. S2). The detection of the human-associated faecal marker crAssphage in 88% of the water samples (29 of the 33), points out human as an important faecal source in these areas, either from the WWTP outfalls or from the rivers. In Besòs coastal water, faecal indicators were more abundant in water collected at the bottom and medium points of the water column and in transects B2 and B3, which are closer to the outfall of Besòs WWTP, showing the impact of the outfall plume (Table S3, Fig. S2). On the other hand, *E. coli* was detected in 20% (1 out of 5) of the surface water of the Llobregat area by culture methods, whereas 40% (2 out of 5) of the samples were positive for Enterococci (Table S3). The levels of the three indicators were higher in the Besòs coastal area than that of the Llobregat coastal area, and significant differences were observed for all the faecal indicators except for culturable Enterococci ($p > 0.05$). No significant differences were observed between cruises. Two ARGs, *sul*I and *tet*W, were detected in all the water samples analysed, whereas bla_{TEM} was identified in 19 of the 33 samples (Table S3). The three ARGs were also more abundant in the Besòs than in the Llobregat coastal areas (Table S3), with statistically significant differences for *tet*W and bla_{TEM} ($p = 0.004$ and 0.018) but not for *sul*I ($p > 0.05$).

Culture methods detected low abundance of both *E. coli* and Enterococci in sediments: 3 of 8 samples, were positive for *E. coli* and 5 for Enterococci with very low abundances (Table S4). When using molecular techniques, all sediment samples were positive for Enterococci and 5 of 8 for *E. coli* (Table S4). In contrast, SRC spores, more persistent in the environment, were detected in all samples, indicating old pollution. Sediments from B2 and B3 located close to the WWTP outfall showed a higher concentration of the human-associated crAssphage. The ARGs *sul*I and *tet*W were detected in all the samples from both coastal areas, whereas bla_{TEM} was detected in 5 of the 8 samples (Table S4).

3.1.2. Plastic abundance and characterization

A total of 875 plastic particles were extracted from all 12 surface water samples and 7 sediment samples. Most of the plastics were retrieved from surface waters (71.8%) rather than sediments (28.2%), and similar amounts were extracted from the Besòs (56.2%) and Llobregat (43.8%) areas (Table S5). Average plastic concentrations were estimated to be 0.05 ± 0.05 items m^{-2} (max. 0.15 items m^{-2}) in the Besòs area and 0.06 ± 0.02 items m^{-2} (max. 0.10 items m^{-2}) in the Llobregat area, which resulted in an overall average plastic concentration of 0.05 ± 0.04 items m^{-2}.

Among all the plastics, 79.9% were classified as microplastics, followed by mesoplastics (19.3%) and macroplastics (0.8%). Higher proportions of mesoplastics (26.7% vs. 16.4%) and macroplastics (1.6% vs. 0.5%) were extracted from the sediment compared to the sea surface ($\chi^2 = 15.8$, d. f. = 2, $p < 0.001$). The surface area of the plastic particles was within the range of 0.1–656 mm^2 (median = 2.84 mm^2) in sediments and 0.1–324 mm^2 (median = 2.83 mm^2) (Table S5). Data about plastic composition are shown in the supplementary material.

3.2. Marine bacteria on plastics

The abundance of bacteria in the plastic biofilms was 1.9×10^6 ($\pm 2.4 \times 10^6$) 16S rRNA gc mm^{-2} with similar values detected in floating plastics of both coastal areas and slightly higher in plastics collected in the BACT2 compared to the BACT1 cruise (Figs. S3 and S4 and Table 1). Lower concentration was observed in plastics from sediments with values of 9.1×10^4 ($\pm 4.8 \times 10^4$) 16S rRNA gc mm^{-2} (Table 1). The abundance of culturable marine heterotrophic bacteria quantified using marine agar was 1.1×10^4 ($\pm 1.3 \times 10^4$) cfu mm^{-2} in floating plastics and 3.0×10^3 ($\pm 4.9 \times 10^3$) cfu mm^{-2} for sediment plastics. Species of the *Vibrio* genus were detected in all the floating plastics of the Besòs (6.9×10^2 ($\pm 8.4 \times 10^2$) cfu mm^{-2}) and Llobregat (9.1×10^1 ($\pm 1.1 \times 10^2$) cfu mm^{-2}) areas. *Vibrio* spp. were also detected in 4 of the 7 plastic samples from sediments, but with a very low concentration (a maximum value of 2.6 cfu mm^{-2}) (Figs. S3 and S4 and Table 1). Low levels of *Pseudomonas* spp. were detected in 9 of the 12 floating plastics (a maximum value of 5.9 cfu mm^{-2}) and 3 of the 7 sediment plastics (a maximum value of 0.7 cfu mm^{-2}) (Figs. S3 and S4 and Table 1). The presence of bacteria attached to plastics was confirmed by SEM analyses (Fig. S5).

3.3. Faecal bacteria on plastics

Faecal microorganisms were analysed as a proxy of potential faecal pathogens, using culture to evaluate viability and molecular methods to detect DNA. Similar levels of total coliforms were detected in 10 of the 12 plastic samples collected from the Besòs and Llobregat coastal surface waters (mean = $1.7 \times 10^1 \pm 9.4$ cfu mm^{-2}) (Table 2, Fig. S3). Fewer samples harbouring culturable *E. coli* were collected: 3 from the Besòs and 2 from the Llobregat area (Table 2). Its abundance was higher in Besòs samples ($1.2 \times 10^1 \pm 1.1 \times 10^1$ cfu mm^{-2}), with low levels found in Llobregat samples (0.1 ± 0.1 cfu mm^{-2}) (Table S6 and Fig. S4). During both cruises, the two samples with the highest abundance were collected in transect B1 close to the shoreline. However, water from this area showed lower levels of faecal indicators than those from B2 and B3, suggesting that these plastics may have been colonised by the bacteria elsewhere and drifted to this area. The abundance of *E. coli* by qPCR was 1 logarithm higher (Figs. S3 and S4). Although, due to the higher detection limit of this technique, two samples, which were positive by culture methods, were negative by qPCR (Table S6).

More samples were positive for Enterococci (8/12) than for *E. coli* (5/12) by culture methods (Table 2), with lower concentrations, as these bacteria are more persistent in seawater. Four samples were also positive for both indicators by qPCR, with similar abundance between areas, the highest values were detected in BACT2-B1 (2.6×10^2 gc mm^{-2}) (Table S6).

The 50% of the samples were positive for crAssphage, indicating potential colonisation of bacteria associated with human faecal pollution, including potential pathogens. The abundance of crAssphage was similar in samples from the two coastal areas (Table 2, Figs. S3 and S4). The highest abundance of crAssphage was also detected in BACT2-B1 (2.5×10^2 gc mm^{-2}) (Table S6). In general, the abundance of faecal indicators was higher in floating plastics from Besòs area than in the water column.

Biofilms on plastic from sediments did not show culturable faecal indicators (Table 2). However, using molecular methods, 2 plastic samples from the Besòs area (B2 and B3) were positive for the 3 indicators. A high concentration of these indicators was also detected in sediments of these points (Fig. S4).

The ratio between the gene copies of *E. coli*, Enterococci and crAssphage with the 16S rRNA gene was used to compare the concentration of faecal indicators in contrast with the whole bacterial community in biofilms from plastics and with water and sediments (Fig. 1). When detected, a major proportion of faecal indicators was observed in biofilms than in the surrounding matrix, especially in floating plastics from Besòs area. Just the proportion of Enterococci in sediments was higher than that observed in plastics.

3.4. Antibiotic resistance genes on plastics

Among the floating plastics from 12 surface water transects, 10 were positive for *sul*I and 8 were positive for *tet*W and bla_{TEM} (Table 2) (means of 1.9×10^2 ($\pm 1.5 \times 10^2$), 6.2×10^1 ($\pm 1.7 \times 10^1$), and $1.8 \cdot 10^1$ ($\pm 1.7 \times 10^1$) gc mm^{-2}, respectively). The abundance of these genes was similar between samples from both areas, although slightly higher in the Besòs samples (Fig. S3, Table S6).

Table 1
Number of positive samples, mean abundance and standard deviation (sd) of marine bacteria measured targeting the 16S rRNA gene by quantitative PCR (gc mm^{-2}), culturable marine heterotrophic bacteria in Marine Agar, culturable *Vibrio* spp. with TBCS and culturable *Pseudomonas* spp. with *Pseudomonas* agar (cfu mm^{-2}) in plastics collected from surface water and sediments in Besòs and Llobregat area.

Source of the plastic	Area		16S rRNA gene	Marine heterotroph	*Vibrio* spp.	*Pseudomonas* spp.
Water	Besòs	n	6/6	6/6	6/6	4/6
		Mean	2.5×10^6	1.6×10^4	6.9×10^2	6.5
		sd	3.1×10^6	1.7×10^4	8.4×10^2	6.8
	Llobregat	n	6/6	6/6	6/6	5/6
		Mean	1.5×10^6	4.9×10^3	9.1×10^1	1.7
		sd	1.7×10^6	4.5×10^3	1.1×10^2	3.5
Sediments	Besòs	n	3/3	3/3	3/3	2/3
		Mean	6.6×10^4	5.4×10^3	1.7	0.4
		sd	2.3×10^4	7.0×10^3	1.4	0.5
	Llobregat	n	4/4	4/4	1/4	1/4
		Mean	1.1×10^5	1.2×10^3	0.8	0.1
		sd	5.6×10^4	2.3×10^3	NA	NA

Table 2
Number of positive samples and mean abundance and standard deviation (sd) of faecal indicators: Total coliforms (TC), *E. coli* measured by culture (EC c) and by qPCR (EC m), Enterococci measured by culture (ENT c) and by qPCR (ENT m) and crAssphage and the antibiotic resistance genes: *sul*I, *tet*W, bla_{TEM} in plastics collected from surface water and sediments in Besòs and Llobregat area. Results are expressed as cfu mm^{-2} for culture bacteria and as gc mm^{-2} for qPCR.

Source of the plastic	Area		TC	EC c	EC m	ENT c	ENT m	crAssphage	*sulI*	*tetW*	bla_{TEM}
Water	Besòs	n	4/6	3/6	2/6	4/6	2/6	4/6	5/6	4/6	5/6
		Mean	2.4×10^1	1.2×10^1	2.2×10^2	1.0	1.3×10^2	7.2×10^1	3.0×10^2	5.1×10^1	2.9×10^1
		sd	3.7×10^1	1.0×10^1	2.4×10^1	1.1	1.8×10^2	1.2×10^2	3.0×10^2	3.3×10^1	3.3×10^1
	Llobregat	n	6/6	2/6	2/6	4/6	2/6	2/6	5/6	4/6	3/6
		Mean	1.1×10^1	0.1	3.7	1.4	2.0	1.4×10^1	8.5×10^1	7.5×10^1	8.1
		sd	2.0×10^1	0.1	1.1	1.9	0.1	1.6×10^1	1.3×10^2	1.3×10^2	6.0
Sediments	Besòs	n	0/3	0/3	2/3	0/3	2/3	2/3	3/3	2/3	2/3
		Mean	NA	NA	1.9	NA	1.3	4.6×10^1	3.2×10^1	1.8×10^1	1.3
		sd	NA	NA	2.5	NA	0.5	1.7×10^1	2.3×10^1	7.3	0.2
	Llobregat	n	0/4	0/4	0/4	0/4	0/4	0/4	1/4	0/4	0/4
		Mean	NA	NA	NA	NA	NA	NA	3.5×10^0	NA	NA
		sd	NA	NA	NA	NA	NA	NA	NA	NA	NA

NA. Not applied.

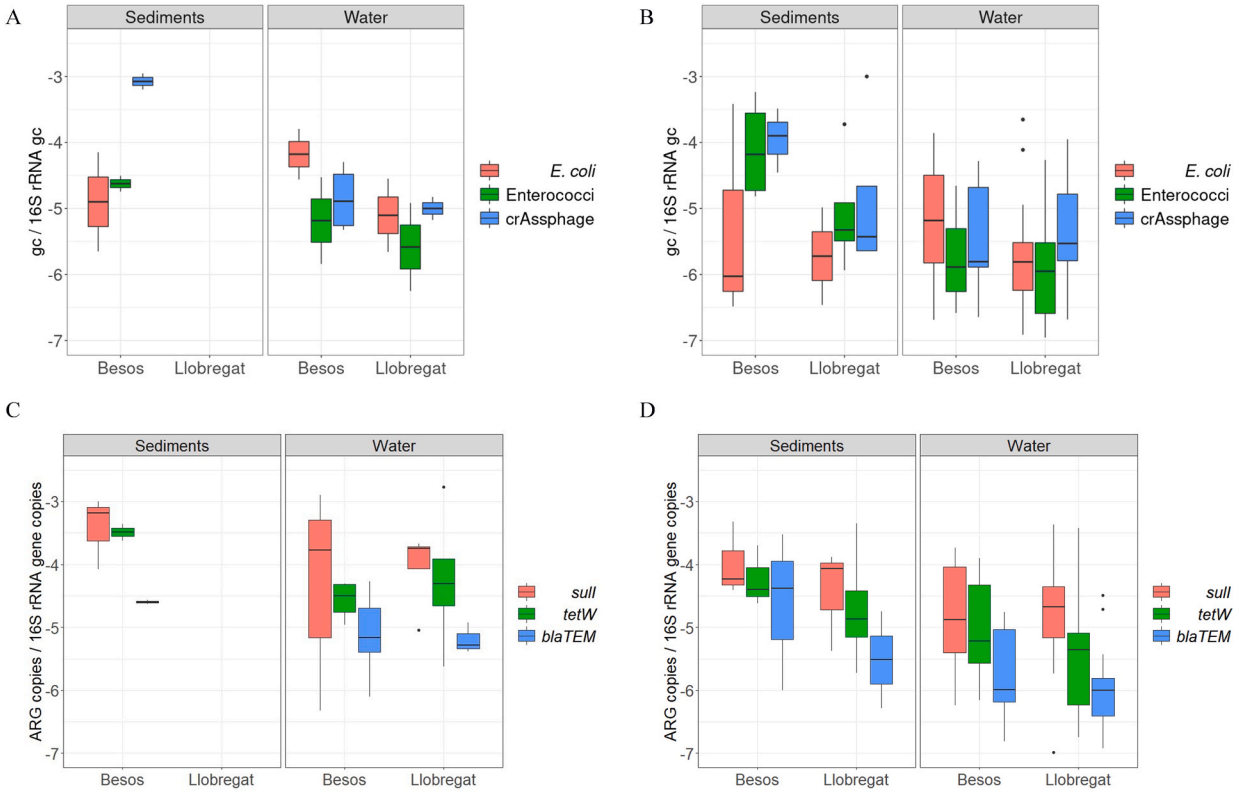


Fig. 1. Distribution of the ratio between the gene copies of the faecal indicators: *E. coli*, Enterococci and crAssphage and the gene copies of the 16S rRNA (A, B) and the antibiotic resistance genes: *sul*I, *tet*W and bla_{TEM} and the gene copies of the 16S rRNA (C, D) of plastics collected in sediments and water of the coastal areas of Besòs and Llobregat (A, C) and of sediment and water samples of both areas (B, D).

Two of the three plastic samples collected from sediments in the Besòs coastal area carried all the three ARGs, whereas only one of four samples from the Llobregat area had a very low concentration of *sul*I (Table 2, Fig. S3). These results can be explained by differences in human impacts, in addition to the substantially higher number of plastic particles collected from the Besòs (233) than the Llobregat area (22).

We also used the ratio between the gene copies of the ARGs and the 16S rRNA gene to compare the concentrations of ARGs in biofilms from plastics and with the surrounding matrices (water or sediments) (Fig. 1). We observed a major proportion of the ARGs mainly *sul*I and *tet*W per 16S rRNA gene in biofilms than in water and sediments.

4. Discussion

Due to their ubiquity and persistence in aquatic environments, plastics have been proposed as vectors of bacteria. High-throughput sequence analysis of the "plastisphere" (Zettler et al., 2013) has shown the presence of sewage-associated human pathogens such as *Pseudomonas, Campylobacteraceae, Aeromonas, Arcobacter* and *Mycobacterium* (Jiang et al., 2018; Martínez-Campos et al., 2021; McCormick et al., 2014; Wu et al., 2019). However, microbial community amplicon sequencing does not give information about viability and pathogenicity (Wright et al., 2021). Despite this emerging data, the association of faecal bacteria and ARGs with microplastics in the marine environment has been poorly studied to date. This study assessed the abundance of

bacteria on plastics from two coastal areas which are strongly impacted by anthropogenic activity, and we provide evidence of the presence of faecal bacteria and ARGs adhering to buoyant and benthic plastics. This information is the first step to estimate the bacterial and viral risk associated with plastic particles.

In biofilms of floating plastics, we measured concentrations from 1.5 $\times$ 10^5 to 8.7 $\times$ 10^6 gc of the 16S rRNA mm^{-2}, whereas the abundance of culturable bacteria was more variable, ranging from 7.4 $\times$ 10^1 to 4.3 $\times$ 10^4 cfu mm^{-2}. The ratio of viable marine bacteria able to grow in marine agar to total bacteria varied from 1:14 to 1:15,000, depending on the sample. Results from this study show higher bacterial abundances than other similar studies, which normally refer to individual fragments of plastics, and use direct observation with microscopy instead of qPCR of the 16S rRNA gene. For example, Dussud et al. reported bacterial counts of between 1.1 $\times$ 10^3 and 1.9 $\times$ 10^5 cells mm^{-2} using DAPI staining of independent pieces of plastic (Dussud et al., 2018). In Pacific Gyre samples, about 10^2–10^3 cells mm^{-2} were counted with scanning electronic microscopy (Carson et al., 2013). A colonisation experiment found 4.0 $\times$ 10^4 cells mm^{-2} after one week of incubation and 1.1 $\times$ 10^5 cells mm^{-2} after two weeks (Schlundt et al., 2020). In this study, sonication was used to recover the cells from the plastic biofilm, allowing us to perform culture and molecular analysis in parallel from the same sample. However, we cannot be sure that sonication does not affect the viability of the biofilm population. Thus, the abundance of colony forming units could be underestimated.

We also evaluated the presence of *Pseudomonas* and *Vibrio* bacteria. Both genera include human pathogens, such as *P. aeruginosa, V. cholerae, V. parahaemolyticus* and *V. vulnificus* (Baker-Austin et al., 2018), and marine animal pathogens such as *P. anguilliseptica*, *V. anguillarum*, *V. harveyi*, *V. alginolyticus* or *V. coralliilyticus* (Ji et al., 2020). Some of these bacteria have already been identified on microplastics but not quantified (Kirstein et al., 2016; McCormick et al., 2014; Zettler et al., 2013). Nonetheless, many species of these genera are not pathogenic and can even act as plastic degraders (Wright et al., 2021). This study detected a variable concentration of culturable *Vibrio* spp. in floating plastics representing 9.1 $\times$ 10^1 and 1.1 $\times$ 10^4 cfu in an average sized plastic item (considering the median plastic area in surface water of 2.84 mm^2). However, culturable *Vibrio* spp. were either not detected or detected in low concentrations in plastics from sediments. In this study, we quantified potential *Vibrio* bacteria growing in TCBS, but we did not identify them at the species level, yet if confirmed as potential pathogens, floating plastics may pose a risk, in particular to marine organisms. *Vibrio* spp. have been previously detected in plastics at low densities, although a single marine plastic has been reported in which *Vibrio* spp. represented 24% of the bacterial community (Bryant et al., 2016; Zettler et al., 2013). In our study, the ratio of culturable *Vibrio* spp. relative to the total bacteria measured by qPCR on the 16S rRNA gene ranged from 1:550 to 1:120,000, and from 1:6 to 1:400 in relation to the culturable marine heterotrophic bacteria. The greater relative abundance of culturable marine heterotrophic bacteria and *Vibrio* spp. in some samples may indicate that these plastics have spent longer time in the sea. *Pseudomonas* spp. was detected in low concentrations (maximum of 80 cfu per average plastic), in 75% of the floating plastics, from which the presence of faecal indicators was also detected. Thus, the source of *Pseudomonas* spp. in these plastic samples may also be linked to effluent inputs, which may include potentially pathogenic species for humans, although the risk requires assessment.

To detect faecal indicators, we used a combination of culture and molecular methods. With culture methods, we detected viable bacteria, thus considering the low persistence of bacterial faecal indicators in seawater (Jeanneau et al., 2012), their presence indicates recent contamination. On the other hand, both *E. coli* and Enterococci were detected at a higher abundance by qPCR, given that hold between 6 and 7 copies of the 16S rRNA gene per cell (either alive, viable but non-culturable, or dead) (Johnson et al., 2019). Therefore, they are useful to indicate older contamination. According to the higher levels of cultured faecal indicators in the water column, Besòs coastal area was impacted by the sewage effluent discharge. The WWTP outfall impact was less evident in the Llobregat coastal area, where the levels of culturable faecal indicators were low although, they were widely detected by qPCR.

The same pattern was observed in plastics from these areas. Culturable total coliforms, *E. coli* and Enterococci were detected in floating plastic in both coastal areas. The concentrations of faecal indicators were generally low, but higher levels were detected by qPCR. However, the 2 samples from transect B1 (close to the shore of the Besòs area) stand out, with 15–20 cfu mm^{-2} and 2.1–2.4 $\times$ 10^2 gc mm^{-2} of *E. coli*. The average concentration of *E. coli* in floating plastics of Besòs area was 12 cfu mm^{-2}. Considering the median area of floating plastics was 2.84 mm^2, and assuming a flat surface, our data suggests an average concentration of 68.2 cfu of *E. coli* per plastic collected in this area, and highest values in B1 (between 85.2 and 113.6 cfu). Thus, the concentration of viable *E. coli* in a single microplastic of Besòs area is higher than the concentration of *E. coli* detected in the surface water (mean of 28.6 cfu 100 ml^{-1}). Although the differences were not significant, the plastics retrieved from the Llobregat coastal area harboured a lower abundance of faecal indicator bacteria compared to the Besòs area.

Six of the nine samples positive for the faecal indicators also contained the human-associated crAssphage, indicating these plastics may have been in contact with sewage. The colonisation of plastic nurdles by *E. coli* has already been observed on Scottish beaches and in Guanabara Bay in Brazil (Rodrigues et al., 2019; Silva et al., 2019), however these methods did not produce concentration values.

In addition, bacterial cells interacting in biofilms have been reported as a reservoir of ARGs (Balcázar et al., 2015). Moreover, the selective pressure of several antibiotics has been shown to increase ARGs in plastic biofilms (Wang et al., 2020). Therefore, plastic particles covered with biofilm may pose a risk as a source and disseminator of ARGs (Liu et al., 2021). Using metagenomics approaches, several studies have already detected ARGs in plastic biofilms (Guo et al., 2020; Yang et al., 2019). A total of 64 subtypes of ARGs were identified in plastic debris from the North Pacific Gyre in a proportion ranging from 7.7 $\times$ 10^{-4} to 1.2 $\times$ 10^{-2} copies of ARGs per 16S rRNA gene, with a high prevalence of sulphonamide-related genes (Bryant et al., 2016; Yang et al., 2019). Two studies observed the attachment to plastic and subsequent increase of bacteria carrying the ARGs *sul*I, tetM, and *int*1 in colonisation experiments with sewage (Eckert et al., 2018; Martínez-Campos et al., 2021).

In the present study, the three ARGs: *sul*I, *tet*W and bla_{TEM} were detected in the seawater column of both areas, showing the contribution of human pollution in the dispersion of ARGs (Karkman et al., 2019). Besides, 63% of the floating plastics and plastics from seafloor sediments carried the target ARGs, especially *sul*I and *tet*W, with concentrations ranging from 0.8 to 6.7 $\times$ 10^2 gc mm^{-2}. These concentrations represent a relative abundance of the 16S rRNA gene ranging from 1.3 $\times$ 10^{-3} to 7.9 $\times$ 10^{-7}, which is higher than the observed in water and sediments. This data suggests an enrichment of these genes in the plastic biofilm.

In sediments, the abundance of culturable SRC spores and of faecal indicators detected by qPCR, reflected the presence of bacteria that are dead or in a viable but non-culturable state, indicating the accumulation of old pollution on the seafloor, especially close to the Besòs WWTP outfall (B2 and B3). This supports the theory that it is old pollution that does not pose a concerning risk from the bacterial point of view. The same pattern was observed in plastic from sediments where non-culturable faecal indicators were detected but, they were detected using molecular methods in B2 and B3. All 3 ARGs were also detected in these two plastic sediment samples with higher relative proportions, and barely detected in the other plastic samples. On the other hand, almost all the sediment samples were positive for the three ARGs surveyed in this study, with only three negative samples for bla_{TEM}. Therefore, although there is an enrichment of ARGs in the biofilm from the plastic in sediments, plastics hardly bear a sanitary risk because only non-culturable faecal indicators and *Vibrio* spp. at low concentrations were

detected.

The average abundance of floating plastics found in this study was one order of magnitude lower than abundances recently reported in the nearshore of Barcelona city (0.64 items m^{-2}), close to the areas studied here (de Haan et al., 2022). It also falls within the lower range of average plastic concentrations reported throughout the Mediterranean Sea (Compa et al., 2020; Cózar et al., 2015; de Haan et al., 2019; Pedrotti et al., 2016; among others see review in Simon-Sánchez et al., 2022). Abundance levels are affected by the proximity to densely populated areas, retention by coastal infrastructures (e.g., harbours or breakwaters), distance from the shore, or specific conditions during or before sampling (e.g., windage, alongshore currents) (de Haan et al., 2022, 2019). In addition, to be able to quantify viable bacteria at the sampling moment, we performed rapid sampling and *in-situ* processing of samples, which may have resulted in fewer harvested plastics compared to the number obtained by post-processing in a laboratory (e.g., involving the use of stereoscopes and other equipment).

Our results show that faecal indicators and ARGs can get attached to plastic biofilms, and their relative concentration is higher than that found in water or sediments. Notably, plastics can act like rafts for biofilms which are carried by currents to areas with lower levels of faecal pollution. Recent global simulations of marine plastic transport have described plastic trapping in coastal zones (Lebreton and Andrady, 2019; Onink et al., 2021), where anthropogenic impact is higher, whether from river discharges, inland runoff, or sewage overflows. Although the relative abundance of potentially pathogenic species on microplastics is comparable with that found on floating particles of natural origin (Bowley et al., 2021), the former is more persistent in time and space due to their more durable nature. Plastics with developed biofilms are likely to drift and persist for a long time in coastal waters, and eventually may be beached and/or trapped by coastal infrastructures. In Barcelona city, microplastic hotspots with >1 plastic item m^{-2} have been found near beaches semi-enclosed by breakwaters (de Haan et al., 2022). Tourists and locals use these beaches intensively, so it is essential to evaluate the role of bacterial biofilms as a potential public health risk.

To date, plastisphere-associated pathogenicity for human and marine animals has not been established (Silva et al., 2019), but the risk is plausible and should be studied further. To the best of our knowledge, this is the first study to report abundances of viable faecal indicators in plastics collected from surface seawater. This data can be used together with the concentration of plastics in areas of risk, the exposure pathways for humans and marine animals and infectious doses to perform microbial risk assessment.

5. Conclusions

In this study, we investigated plastic debris (mainly microplastics), faecal indicators and ARGs in water and sediments of two coastal areas receiving sewage effluent and river discharges. The plastic debris was covered by a bacterial biofilm with a concentration of the 16S rRNA gene ranging from 4.9×10^4 to 8.7×10^6 gc mm^{-2}. Species of the *Vibrio* genus, representing a variable percentage of the marine bacteria in biofilms of floating marine plastics, may indicate a different risk for marine animal populations. In contrast, *Vibrio* genus was less prevalent in plastics collected from seafloor sediments, representing a lower risk for marine species.

Faecal bacteria *E. coli* and Enterococci and the human-associated crAssphage were detected in floating plastic biofilms, suggesting that the plastics have been in contact with sewage. The higher abundance detected by qPCR suggests that part of the bacteria may be dead or in a viable but non-culturable state. Plastics collected in Besòs coastal area with a higher concentration of recent faecal pollution coming from the WWTP outfall presented higher concentration of faecal indicators. Moreover, these plastics can drift to areas with lower levels of pollution, increasing the total faecal bacteria load in the water. In fact, we detected more faecal bacteria in one plastic than in 100 ml of the surrounding water. The presence of culturable faecal bacteria in plastics from sediments was low, although possible to be detected using molecular methods. Three ARGs, *sulI, tet*W and bla_{TEM}, were detected in plastic biofilms at higher concentrations than those observed in water and sediments, suggesting an enrichment of these genes in the biofilm.

Although the concentration was low, faecal bacteria and species of the genera *Pseudomonas* and *Vibrio* were detected in marine plastics by culture methods, suggesting that plastic particles could act as a reservoir of human and fish pathogens. Thus, the potential risk of the plastisphere to humans and marine animals should be considered.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We would like to acknowledge the crew of the R/V Garcia de Cid and SOCIB for their invaluable support, the technicians Joel Sans and Ramon Ametller, the managers of UTM-CSIC and the additional hands of Paula de Castro and Miriam Pascual-Benito. We thank the CCiTUB of the UB for providing expert technical help in SEM, in particular, Eva Prats and Rosa Rivera, and Pilar Hermo for FT-IR analysis. We also thank Louise Deering for her manuscript revision. This work has been supported by the Spanish Ministerio de Ciencia e Innovación BACTPLAST project (PID 2019-108957 GA-I00), the TRACE project (TED 2021-130515 B–I00) funded by MCIN/AEI/10.13039/501100011033 and the European Union NextGenerationEU/PRTR, and the Catalan government (2017 SGR 170 and 2017 SGR 315). H. L. is funded by a Joint PhD Training Program of the University of Chinese Academy of Sciences.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120983.

References

Andrady, A.L., 2011. Microplastics in the marine environment. Mar. Pollut. Bull. 62, 1596–1605. https://doi.org/10.1016/j.marpolbul.2011.05.030.

Baker-Austin, C., Oliver, J.D., Alam, M., Ali, A., Waldor, M.K., Qadri, F., Martinez-Urtaza, J., 2018. *Vibrio* spp. infections. Nat. Rev. Dis. Prim. 4, 1–19. https://doi.org/10.1038/s41572-018-0005-8.

Balcázar, J.L., Subirats, J., Borrego, C.M., 2015. The role of biofilms as environmental reservoirs of antibiotic resistance. Front. Microbiol. 6, 1216. https://doi.org/10.3389/fmicb.2015.01216.

Ballesté, E., Pascual-Benito, M., Martín-Díaz, J., Blanch, A.R., Lucena, F., Muniesa, M., Jofre, J., García-Aljaro, C., 2019. Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments. Water Res. 155, 233–244. https://doi.org/10.1016/j.watres.2019.02.042.

Borrelle, S.B., Ringma, J., Law, K.L., Monnahan, C.C., Lebreton, L., McGivern, A., Murphy, E., Jambeck, J., Leonard, G.H., Hilleary, M.A., Eriksen, M., Possingham, H. P., Frond, H. De, Gerber, L.R., Polidoro, B., Tahir, A., Bernard, M., Mallos, N., Barnes, M., Rochman, C.M., 2020. Predicted growth in plastic waste exceeds efforts to mitigate plastic pollution. Science 369, 1515–1518. https://doi.org/10.1126/science.aba3656.

Bowley, J., Baker-Austin, C., Porter, A., Hartnell, R., Lewis, C., 2021. Oceanic hitchhikers – assessing pathogen risks from marine microplastic. Trends Microbiol. 29, 107–116. https://doi.org/10.1016/j.tim.2020.06.011.

Brahney, J., Hallerud, M., Heim, E., Hahnenberger, M., Sukumaran, S., 2020. Plastic rain in protected areas of the United States. Science 368, 1257–1260. https://doi.org/10.1126/science.aaz5819.

Bryant, J.A., Clemente, T.M., Viviani, D.A., Fong, A.A., Thomas, K.A., Kemp, P., Karl, D. M., White, A.E., DeLong, E.F., 2016. Diversity and activity of communities inhabiting

plastic debris in the North Pacific Gyre. mSystems 1, 1–19. https://doi.org/10.1128/msystems.00024-16.
Cahn, M.L., Conner, M.M., Schmitz, O.J., Stephenson, T.R., Wehausen, J.D., Johnson, H. E., 2011. Disease, population viability, and recovery of endangered Sierra Nevada bighorn sheep. J. Wildl. Manag. 75, 1753–1766. https://doi.org/10.1002/jwmg.232.
Calero-Cáceres, W., Melgarejo, A., Colomer-Lluch, M., Stoll, C., Lucena, F., Jofre, J., Muniesa, M., 2014. Sludge as a potential important source of antibiotic resistance genes in both the bacterial and bacteriophage fractions. Environ. Sci. Technol. 48, 7602–7611. https://doi.org/10.1021/es501851s.
Carson, H.S., Nerheim, M.S., Carroll, K.A., Eriksen, M., 2013. The plastic-associated microorganisms of the North Pacific gyre. Mar. Pollut. Bull. 75, 126–132. https://doi.org/10.1016/j.marpolbul.2013.07.054.
Compa, M., Alomar, C., Mourre, B., March, D., Tintoré, J., Deudero, S., 2020. Nearshore spatio-temporal sea surface trawls of plastic debris in the Balearic Islands. Mar. Environ. Res. 158, 104945 https://doi.org/10.1016/j.marenvres.2020.104945.
Cózar, A., Echevarría, F., González-Gordillo, J.I., Irigoien, X., Úbeda, B., Hernández-León, S., Palma, Á.T., Navarro, S., García-de-Lomas, J., Ruiz, A., Fernández-de-Puelles, M.L., Duarte, C.M., 2014. Plastic debris in the open ocean. Proc. Natl. Acad. Sci. U. S. A. 111, 10239–10244. https://doi.org/10.1073/pnas.1314705111.
Cózar, A., Sanz-Martín, M., Martí, E., González-Gordillo, J.I., Ubeda, B., Gálvez, J.Á., Irigoien, X., Duarte, C.M., 2015. Plastic accumulation in the Mediterranean Sea. PLoS One 10, e0121762. https://doi.org/10.1371/journal.pone.0121762.
de Haan, W.P., Sanchez-Vidal, A., Canals, M., 2019. Floating microplastics and aggregate formation in the western Mediterranean Sea. Mar. Pollut. Bull. 140, 523–535. https://doi.org/10.1016/j.marpolbul.2019.01.053.
de Haan, W.P., Uviedo, O., Ballesteros, M., Canales, Í., Curto, X., Guart, M., Higueras, S., Molina, A., Sanchez-Vidal, A., 2022. Floating microplastic loads in the nearshore revealed through citizen science. Environ. Res. Lett. 17, 045018 https://doi.org/10.1088/1748-9326/ac5df1.
Dussud, C., Meistertzheim, A.L., Conan, P., Pujo-Pay, M., George, M., Fabre, P., Coudane, J., Higgs, P., Elineau, A., Pedrotti, M.L., Gorsky, G., Ghiglione, J.F., 2018. Evidence of niche partitioning among bacteria living on plastics, organic particles and surrounding seawaters. Environ. Pollut. 236, 807–816. https://doi.org/10.1016/j.envpol.2017.12.027.
Eckert, E.M., Di Cesare, A., Kettner, M.T., Arias-Andres, M., Fontaneto, D., Grossart, H.P., Corno, G., 2018. Microplastics increase impact of treated wastewater on freshwater microbial community. Environ. Pollut. https://doi.org/10.1016/j.envpol.2017.11.070.
Enyoh, C.E., Verla, A.W., Verla, E.N., Ibe, F.C., Amaobi, C.E., 2019. Airborne microplastics: a review study on method for analysis, occurrence, movement and risks. Environ. Monit. Assess. 191, 668. https://doi.org/10.1007/s10661-019-7842-0.
Fernández-Orth, D., Miró, E., Brown-Jaque, M., Rodríguez-Rubio, L., Espinal, P., Rodriguez-Navarro, J., González-López, J.J., Muniesa, M., Navarro, F., 2019. Faecal phageome of healthy individuals: presence of antibiotic resistance genes and variations caused by ciprofloxacin treatment. J. Antimicrob. Chemother. 74, 854–864. https://doi.org/10.1093/jac/dky540.
GarcíaAljaro, C., Ballesté, E., Muniesa, M., Jofre, J., 2017. Determination of crAssphage in water samples and applicability for tracking human faecal pollution. Microb. Biotechnol. 10, 1775–1780. https://doi.org/10.1111/1751-7915.12841.
Garcia-Aljaro, C., Martin-Diaz, J., Viñas-Balada, E., Calero-Caceres, W., Lucena, F., Blanch, A.R., 2017. Mobilisation of microbial indicators, microbial source tracking markers and pathogens after rainfall events. Water Res. 112, 248–253. https://doi.org/10.1016/j.watres.2017.02.003.
Guo, X. pan, Sun, X. li, Chen, Y. ru, Hou, L., Liu, M., Yang, Y., 2020. Antibiotic resistance genes in biofilms on plastic wastes in an estuarine environment. Sci. Total Environ. 745, 140916 https://doi.org/10.1016/j.scitotenv.2020.140916.
Hermsen, E., Mintenig, S.M., Besseling, E., Koelmans, A.A., 2018. Quality criteria for the analysis of microplastic in biota samples: a critical review. Environ. Sci. Technol. 52, 10230–10240. https://doi.org/10.1021/acs.est.8b01611.
Huijsdens, X.W., Linskens, R.K., Mak, M., Meuwissen, S.G.M., Vandenbroucke-Grauls, C. M.J.E., Savelkoul, P.H.M., 2002. Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR. J. Clin. Microbiol. 40, 4423–4427. https://doi.org/10.1128/JCM.40.12.4423-4427.2002.
ISO, 2000a. Water Quality – Detection and Enumeration of *Escherichia coli* and Coliform Bacteria – Part 1: Membrane Filtration Method. ISO 9308-1: 2000).
ISO, 2000b. Water Quality – Detection and Enumeration of Intestinal Enterococci – Part 2: Membrane Filtration Method. ISO 7899-2: 2000).
Jeanneau, L., Solecki, O., Wery, N., Jarde, E., Gourmelon, M., Communal, P.Y., Jadas-Hecart, A., Caprais, M.P., Gruau, G., Pourcher, A.M., 2012. Relative decay of fecal indicator bacteria and human-associated markers: a microcosm study simulating wastewater input into seawater and freshwater. Environ. Sci. Technol. 46, 2375–2382. https://doi.org/10.1021/es203019y.
Ji, Q., Wang, S., Ma, J., Liu, Q., 2020. A review: progress in the development of fish *Vibrio* spp. vaccines. Immunol. Lett. 226, 46–54. https://doi.org/10.1016/j.imlet.2020.07.002.
Jiang, P., Zhao, S., Zhu, L., Li, D., 2018. Microplastic-associated bacterial assemblages in the intertidal zone of the Yangtze Estuary. Sci. Total Environ. 624, 48–54. https://doi.org/10.1016/j.scitotenv.2017.12.105.
Johnson, J.S., Spakowicz, D.J., Hong, B.-Y., Petersen, L.M., Demkowicz, P., Chen, L., Leopold, S.R., Hanson, B.M., Agresta, H.O., Gerstein, M., Sodergren, E., Weinstock, G.M., 2019. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. Nat. Commun. 10, 5029. https://doi.org/10.1038/s41467-019-13036-1.
Karkman, A., Pärnänen, K., Larsson, D.G.J., 2019. Fecal pollution can explain antibiotic resistance gene abundances in anthropogenically impacted environments. Nat. Commun. 10, 1–8. https://doi.org/10.1038/s41467-018-07992-3.
Kay, P., Hiscoe, R., Moberley, I., Bajic, L., McKenna, N., 2018. Wastewater treatment plants as a source of microplastics in river catchments. Environ. Sci. Pollut. Control Ser. 25, 20264–20267. https://doi.org/10.1007/s11356-018-2070-7.
Kirstein, I.V., Kirmizi, S., Wichels, A., Garin-Fernandez, A., Erler, R., Löder, M., Gerdts, G., 2016. Dangerous hitchhikers? Evidence for potentially pathogenic Vibrio spp. on microplastic particles. Mar. Environ. Res. 120, 1–8. https://doi.org/10.1016/j.marenvres.2016.07.004.
Koelmans, A.A., Mohamed Nor, N.H., Hermsen, E., Kooi, M., Mintenig, S.M., De France, J., 2019. Microplastics in freshwaters and drinking water: critical review and assessment of data quality. Water Res. 155, 410–422. https://doi.org/10.1016/j.watres.2019.02.054.
Koelmans, A.A., Redondo-Hasselerharm, P.E., Nor, N.H.M., de Ruijter, V.N., Mintenig, S. M., Kooi, M., 2022. Risk assessment of microplastic particles. Nat. Rev. Mater. 7, 138–152. https://doi.org/10.1038/s41578-021-00411-y.
Lachmayr, K.L., Kerkhof, L.J., DiRienzo, A.G., Cavanaugh, C.M., Ford, T.E., 2009. Quantifying nonspecific TEM β-lactamase (bla_{TEM}) genes in a wastewater stream. Appl. Environ. Microbiol. 75, 203–211. https://doi.org/10.1128/AEM.01254-08.
Law, K.L., Thompson, R.C., 2014. Microplastics in the seas. Science 345, 144–145. https://doi.org/10.1126/science.1254065.
Lebreton, L., Andrady, A., 2019. Future scenarios of global plastic waste generation and disposal. Palgrave Commun 5, 1–11. https://doi.org/10.1057/s41599-018-0212-7.
Lebreton, L.C.M., Van Der Zwet, J., Damsteeg, J.W., Slat, B., Andrady, A., Reisser, J., 2017. River plastic emissions to the world's oceans. Nat. Commun. 8, 1–10. https://doi.org/10.1038/ncomms15611.
Li, C., Busquets, R., Campos, L.C., 2020. Assessment of microplastics in freshwater systems: a review. Sci. Total Environ. 707, 135578 https://doi.org/10.1016/j.scitotenv.2019.135578.
Liquete, C., Canals, M., Ludwig, W., Arnau, P., 2009. Sediment discharge of the rivers of Catalonia, NE Spain, and the influence of human impacts. J. Hydrol. (Amst.) 366, 76–88. https://doi.org/10.1016/j.jhydrol.2008.12.013.
Liu, Y., Liu, W., Yang, X., Wang, J., Lin, H., Yang, Y., 2021. Microplastics are a hotspot for antibiotic resistance genes: progress and perspective. Sci. Total Environ. 773, 145643 https://doi.org/10.1016/j.scitotenv.2021.145643.
Lu, Y., Yuan, J., Lu, X., Su, C., Zhang, Y., Wang, C., Cao, X., Li, Q., Su, J., Ittekkot, V., Garbutt, R.A., Bush, S., Fletcher, S., Wagey, T., Kachur, A., Sweijd, N., 2018. Major threats of pollution and climate change to global coastal ecosystems and enhanced management for sustainability. Environ. Pollut. 239, 670–680. https://doi.org/10.1016/j.envpol.2018.04.016.
Martínez-Campos, S., González-Pleiter, M., Fernández-Piñas, F., Rosal, R., Leganés, F., 2021. Early and differential bacterial colonization on microplastics deployed into the effluents of wastewater treatment plants. Sci. Total Environ. 757, 143832 https://doi.org/10.1016/j.scitotenv.2020.143832.
McCormick, A., Hoellein, T.J., Mason, S.A., Schluep, J., Kelly, J.J., 2014. Microplastic is an abundant and distinct microbial habitat in an urban river. Environ. Sci. Technol. 48, 11863–11871. https://doi.org/10.1021/es503610r.
Muyzer, G., Hottentrager, S., Teske, A., Wawer, C., 1996. In: Akkermans, A., van Elsas, J. D., de Bruijn, F. (Eds.), Denaturing Gradient Gel Electrophoresis of PCR Amplified 16s rDNA-A New Molecular Approach to Analyze the Genetic Diversity of Mixed Microbial Communities, vol. 4. Molecular Microbial Ecology Manual. KluwerAcademic Publishing, Dordrecht, p. 3, 4.1-3.4.4.22.
Muyzer, G., Teske, A., Wirsen, C.O., Jannasch, H.W., 1995. Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. Arch. Microbiol. 164, 165–172. https://doi.org/10.1007/BF02529967.
Obbard, R.W., 2018. Microplastics in Polar Regions: the role of long range transport. Curr Opin Environ Sci Health 1, 24–29. https://doi.org/10.1016/j.coesh.2017.10.004.
Onink, V., Jongedijk, C.E., Hoffman, M.J., van Sebille, E., Laufkötter, C., 2021. Global simulations of marine plastic transport show plastic trapping in coastal zones. Environ. Res. Lett. 16 https://doi.org/10.1088/1748-9326/abecbd.
Pedrotti, M.L., Petit, S., Elineau, A., Bruzaud, S., Crebassa, J.C., Dumontet, B., Martí, E., Gorsky, G., Cózar, A., 2016. Changes in the floating plastic pollution of the mediterranean sea in relation to the distance to land. PLoS One 11. https://doi.org/10.1371/journal.pone.0161581.
R Core Team, 2016. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. https://doi.org/10.1007/978-3-540-74686-7.
Rodrigues, A., Oliver, D.M., McCarron, A., Quilliam, R.S., 2019. Colonisation of plastic pellets (nurdles) by *E. coli* at public bathing beaches. Mar. Pollut. Bull. 139, 376–380. https://doi.org/10.1016/j.marpolbul.2019.01.011.
Schlundt, C., Mark Welch, J.L., Knochel, A.M., Zettler, E.R., Amaral-Zettler, L.A., 2020. Spatial structure in the "Plastisphere": molecular resources for imaging microscopic communities on plastic marine debris. Mol Ecol Resour 20, 620–634. https://doi.org/10.1111/1755-0998.13119.
Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.
Silva, M.M., Maldonado, G.C., Castro, R.O., de Sá Felizardo, J., Cardoso, R.P., Anjos, R.M. dos, Araújo, F.V. de, 2019. Dispersal of potentially pathogenic bacteria by plastic debris in Guanabara Bay, RJ, Brazil. Mar. Pollut. Bull. 141, 561–568. https://doi.org/10.1016/j.marpolbul.2019.02.064.
Simon-Sánchez, L., Grelaud, M., Franci, M., Ziveri, P., 2022. Are research methods shaping our understanding of microplastic pollution? A literature review on the seawater and sediment bodies of the Mediterranean Sea. Environ. Pollut. 292 https://doi.org/10.1016/j.envpol.2021.118275.

Song, J., Jongmans-Hochschulz, E., Mauder, N., Imirzalioglu, C., Wichels, A., Gerdts, G., 2020. The Travelling Particles: Investigating microplastics as possible transport vectors for multidrug resistant *E. coli* in the Weser estuary (Germany). Sci. Total Environ. 720, 137603 https://doi.org/10.1016/j.scitotenv.2020.137603.

Sun, J., Dai, X., Wang, Q., van Loosdrecht, M.C.M., Ni, B.J., 2019. Microplastics in wastewater treatment plants: detection, occurrence and removal. Water Res. 152, 21–37. https://doi.org/10.1016/j.watres.2018.12.050.

U.S. EPA, 2012. Method 1611: Enterococci in Water by TaqMan® Quantitative Polymerase Chain Reaction (qPCR) Assay. Office of Water EPA-821-R-12-008.

Wang, S., Xue, N., Li, W., Zhang, D., Pan, X., Luo, Y., 2020. Selectively enrichment of antibiotics and ARGs by microplastics in river, estuary and marine waters. Sci. Total Environ. 708, 134594 https://doi.org/10.1016/j.scitotenv.2019.134594.

WHO, 2019. Microplastics in Drinking-Water. Licence: CC BY-NC-SA 3.0 IGO. World Health Organization, Geneva.

Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

Wickham, H., 2007. Reshaping data with the {reshape} package. J. Stat. Software 21, 1–20.

Wright, R.J., Langille, M.G.I., Walker, T.R., 2021. Food or just a free ride? A meta-analysis reveals the global diversity of the Plastisphere. ISME J. 15, 789–806. https://doi.org/10.1038/s41396-020-00814-9.

Wu, X., Pan, J., Li, M., Li, Y., Bartlam, M., Wang, Y., 2019. Selective enrichment of bacterial pathogens by microplastic biofilm. Water Res. 165, 114979 https://doi.org/10.1016/j.watres.2019.114979.

Yang, Y., Liu, G., Song, W., Ye, C., Lin, H., Li, Z., Liu, W., 2019. Plastics in the marine environment are reservoirs for antibiotic and metal resistance genes. Environ. Int. 123, 79–86. https://doi.org/10.1016/j.envint.2018.11.061.

Zettler, E.R., Mincer, T.J., Amaral-Zettler, L.A., 2013. Life in the “Plastisphere”: microbial communities on plastic marine debris. Environ. Sci. Technol. 47, 7137–7146. https://doi.org/10.1021/es401288x.