Science of the Total Environment 869 (2023) 161703

Contents lists available at ScienceDirect

Science of the Total Environment

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

Plastisphere assemblages differ from the surrounding bacterial communities in transitional coastal environments

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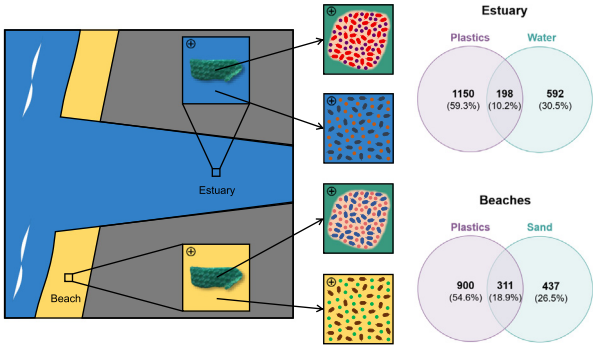
HIGHLIGHTS

- Plastisphere was studied in transitional ecosystems using next-generation sequencing.
- Plastics-associated bacteria differed from their surrounding environments.
- Key pathogens and sludge related bacteria were found exclusively on (micro)plastics.
- Microplastics can disperse pathogenic bacteria in transitional ecosystems.
- Possible existence of substantial sea-river loads of (micro)plastics in estuaries.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Damia Barcelo

Keywords:
Plastic pollution
Microplastics
Estuary
Sandy beaches
Bacterial communities

ABSTRACT

Marine plastic contamination is currently considered ubiquitous in aquatic environments. These particles present a resistant and hydrophobic substrate known to promote microbial colonisation and biofilm formation in aquatic ecosystems, the so-called "Plastisphere", raising concerns about its potential ecological risks. The novelty of this topic translates into a relatively low number of studies, including for transitional coastal ecosystems, such as sandy beaches or estuarine habitats. Therefore, a sampling campaign was conducted in two transitional coastal ecosystems - the Mondego estuary (Portugal) - and adjacent sandy beaches (winter 2020). After visual sorting and filtering of suspected particles under sterile conditions DNA extraction and 16S rRNA amplicon high throughput sequencing was used to profile the bacterial communities on the surface of plastic particles and from those found on the water and sediments from the sampled transitional coastal ecosystems. All particles were characterised according to type, colour and size, and the chemical nature of the particles was determined by FTIR-ATR or μ-FTIR spectroscopy after DNA extraction. All samples contained plastics in several sizes (micro and mesoplastics), shapes (higher abundances of fragments on beaches and fibres in the estuarine waters), colours and polymers. Although no significant differences were detected in the α-diversity indexes of the bacterial communities between plastics and their surrounding environments, data showed the occurrence of unique key bacterial groups on plastics from both environments, such as pathogens (e.g., *Lactococcus*, *Staphylococcus* and *Streptococcus*) and groups commonly associated with wastewater treatment plants (e.g., members of the phylum Firmicutes). This highlights the concerns for plastics to act as vectors of transmission and spread of these bacterial groups in transitional coastal ecosystems. Furthermore, it raises the possibility that

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http://dx.doi.org/10.1016/j.scitotenv.2023.161703
Received 7 October 2022; Received in revised form 2 January 2023; Accepted 15 January 2023
Available online 25 January 2023

(micro)plastics entering the estuary from the sea play a substantial contribution to overall dynamics of (micro)plastics and their microbial assemblages in the estuarine system.

1. Introduction

Plastic debris, and especially microplastics (particle size <5 mm) are widely documented as contaminants in worldwide ecosystems, including terrestrial, marine, coastal, freshwater, polar and even in the atmosphere (Cole et al., 2011; de Souza Machado et al., 2018; Evangeliou et al., 2020; Li et al., 2018; Ling et al., 2017; Mishra et al., 2021). These particles can be classified as primary or secondary microplastics. Primary microplastics originate from direct environmental emissions, including particles manufactured intentionally within this reduced size range (for example granular pellets or microbeads in cosmetics) or generated from spills during production, use or maintenance, such as from wear and tear of car tires or through the releases of synthetic textiles from washing or wearing (Boucher and Friot, 2017; Sundt et al., 2014). Boucher and Friot (2017) estimated losses of 3.2 million tons/year of these particles into the environment (48 % released into the ocean and 52 % remaining on land). On the other hand, secondary microplastics originate from the fragmentation of larger plastic litter once exposed to the marine environment (Boucher and Friot, 2017; Sundt et al., 2014). Lebreton et al. (2019) estimated that one-third of the total predicted emissions of buoyant macroplastics since 1950 may already have degraded into microplastics, 22.3–60.4 million tons from the shoreline and 0.29–0.80 million tons from the ocean. Furthermore, even in an extremely ambitious scenario (no further emissions of macroplastics), the microplastic contamination levels in the environment will continue to increase (Lebreton et al., 2019).

The small size of microplastics, their durability and their resistance to degradation, which allows them to exist in the environment for decades, have led to increased concerns about their ecological impacts (Curren and Leong, 2019; Rochman, 2016), particularly regarding microplastic ingestion by animals, adsorption/release of toxins and microbial colonisation (Koelmans et al., 2014; Wright et al., 2013; Zettler et al., 2013). These are interconnected concerns that still present unknown impacts on biodiversity and aquatic food webs, for human health and food security.

For nearly 50 years, it has been known that plastic debris, including microplastics, are carriers of microbial communities (Carpenter et al., 1972), later termed “Plastisphere” (Zettler et al., 2013). However, only recently the role of microbial interactions with microplastics in the environment has been investigated in more detail (Oberbeckmann and Labrenz, 2020; Amaral-Zettler et al., 2021). In this regard, research has been focused on three main areas: (a) the establishment of plastic-specific biofilms; (b) colonisation and enrichment of pathogenic bacteria coupled to a vector function of microplastics; and (c) the microbial degradation of microplastics in the environment (Bowley et al., 2021; Oberbeckmann and Labrenz, 2020). Several studies have reported that plastics are selected by specific bacterial communities, which differ from organic particle-attached (PA) and free-living (FL) communities (Bryant et al., 2016; Dussud et al., 2018; Oberbeckmann et al., 2015, 2016; Pinto et al., 2019; Zettler et al., 2013). However, the relevance of the different factors involved in the selection of the microbial assemblages on (micro)plastics such as geography, time, substrate and environment remain controversial. It was recently reported that the influence of the geographical region is greater than that of the surface characteristics when comparing plastic polymers with natural particle surfaces (Amaral-Zettler et al., 2021; Oberbeckmann and Labrenz, 2020; Zhang et al., 2022). Furthermore, various studies have reported the presence of potentially pathogenic bacterial strains on environmental microplastic samples, such as *Vibrio* spp., *Aeromonas* spp., *Arcobacter* spp., *Pseudoalteromonas* spp., *Shewanella* spp., *Alteromonas* spp., *Tenacibaculum* spp., *Phormidium* spp. or *Leptolyngbya* spp., that have been collected from various locations worldwide in both seawater and freshwater environments (Amaral-Zettler et al., 2020, 2021; Bowley et al., 2021). Heavy metals (e.g., aluminium, copper, zinc), as well as other pollutants (e.g., persistent organic pollutants), have been shown to sorb onto plastic surfaces, which may influence selection processes and horizontal gene transfer within attached bacterial communities (Bowley et al., 2021). Arias-Andres et al. (2018) reported an increased frequency of plasmid transfer in microplastic-associated bacteria compared to free-living bacteria or those in natural aggregates, which may aid the spread of antimicrobial resistance although the differences to bacteria associated with natural particles (e.g., wood, cellulose, or glass) are still unknown.

Despite the current interest in this topic, data is still lacking for a representative set of samples from different environments, including coastal and riverine areas, to assess the risks for ecosystems, food safety and public health (Frère et al., 2018). In particular, transitional coastal areas, namely beaches and estuaries, provide key ecosystem services and are highly dynamic systems, generally densely populated, vulnerable to a multitude of anthropogenic stressors such as recreational activities, waste disposal, land reclamation, aquaculture, fishing activities and pollution (Frias et al., 2021). Furthermore, it is known that (micro)plastic pollution accumulates heavily in coastal areas (Harris, 2020). Estuaries and beaches are both hotspots and pathways for plastic pollution, capturing and transferring (micro)plastics from land to marine ecosystems for estuaries, and in both directions for beaches (Bessa et al., 2018; Naidoo et al., 2015). This poses concerns about the presence, retention and concentration of potentially pathogenic strains associated with plastics from both ocean and land-based (including from waste-water treatment plants - WWTPs), in these transitional ecosystems. The ecological importance as well as of human activities in these areas highlights the need for further research. In the last few years, substantial contributions and advances have been made regarding this topic. However, there are still numerous questions and knowledge gaps that require further research to add to the worldwide studies performed to date.

The present study aimed to profile the (micro)plastic-associated bacterial communities collected from two transitional coastal ecosystems in the centre of Portugal, (a) the Mondego River estuary and (b) three beaches adjacent to the Mondego River mouth, and to identify the key bacterial groups present in recovered plastic particles. We also aim to compare the plastic-associated bacterial communities with those found on their environmental matrix, estuarine water and beach sand, respectively, with spatiotemporal proximity.

2. Material and methods

2.1. Sample collection

Sample collection (water and sand) was conducted in the coastal area of the Figueira da Foz municipality (Centre of Portugal) during two sampling campaigns on November 25th, 2020, and December 4th, 2020. Two transitional coastal ecosystems were selected: the Mondego estuary and three adjacent sandy beaches. In the estuarine sampling campaign three representative sites were sampled: E1, in the south arm of the estuary (40°7′47.116″N, 8°51′4.565″W); E2, in the north arm of the estuary (40°8′23.732″N, 8°48′52.858″W); and E3, upstream of the bifurcation of the estuary into two arms (40°7′16.519″N, 8°46′17.899″W) (Fig. 1). Samples from surface water were collected using a plankton net (335 μm mesh, circular net opening of 0.50 m of diameter) dragged horizontally during 10 min and stored in sterilised PET bottles on board. Additionally, four litres of surface water were also collected at each sampling site and filtered through 0.2 μm Pall filters under sterile conditions. Filters were stored at −20 °C and used for subsequent DNA extraction to assess both FL and PA bacterial communities present in the estuarine waters. On the sandy beaches, three representative sites were selected: B1, on

Fig. 1. Overview of the Mondego estuary and adjacent coastline along the central coast of Portugal and sampling sites in both environments: estuarine (E1–E3) and sandy beaches (B1–B3).

the Forte de Santa Catarina beach (40°8′49.436″N, 8°52′2.559″W); B2, on the Cabedelo beach (40°8′7.601″N, 8°51′44.846″W); and B3, on the Quiaios beach (40°13′14.893″N, 8°53′30.913″W). Sand samples were collected from an area of 0.25 m^2 (using a 0.5 m wood square) in the strandline using a sterile metal spoon (washed with ethanol) and stored into sterile glass jars. For DNA extraction of the bacterial communities present in the sediment, one sterile microtube was filled from each sand sample and stored at −20 °C for further processing in the laboratory.

2.2. (Micro)plastics extraction

All collected particles were processed within 24 h upon sample collection, in rigorous sterile conditions throughout their manipulation and with minimal freezing steps to avoid losses, contamination or DNA alteration. Manual microparticles extraction was performed using sterilised material (Petri dishes, filters, microtubes, trays and forceps), with forceps being systematically rinsed in ethanol and flamed between manipulations of each particle. Particles were characterised visually according to their colour and type (foam, pellet, film, fragment or fibre). Estuarine water samples were filtered through 1.2 μm Whatman GF/C microfiber filter papers, which were transferred into Petri dishes followed by manual microparticles extraction, under a dissecting microscope. The microparticles were sorted into sterile microtubes and stored at −80 °C until DNA extraction. The sand samples were poured into white sterile trays and 6 to 7 particles, enough for a sufficient quantity of amplifiable DNA based on the work of Frère et al. (2018), detected by naked eye were picked from each sample with forceps into microtubes and stored at −80 °C until DNA extraction. To avoid sample contamination, specifically airborne fibre contamination in the laboratory, standard practices were followed, which included cleaning all equipment with RO-water (Reverse Osmosis), limiting the use of plastic laboratory equipment/consumables and synthetic clothing, performing all steps in a laminar flow cabinet (according to the protocol described by Bessa et al. (2019) in a room with restricted access and processing the samples in the shortest time possible (one day) avoiding additional manipulations.

2.3. DNA extraction

DNA extraction was performed on the plastic-like particles collected and isolated from estuarine samples and sand samples and also from the

estuarine waters and sediment from sandy beaches. The DNA of microbial communities was extracted using Qiagen Powersoil DNA extraction kits (Qiagen GROUP), following the manufacturer's instructions. Before extraction, an intermediate step was carried out to release the FL and PA bacteria from the 0.22 μm Pall filters by macerating them in sterile zipper plastic bags with Milli-Q water. The extracted DNA was then eluted in sterile DNA-Free PCR-Grade water and stored at −20 °C for further downstream applications.

2.4. Sample preparation and Illumina sequencing

Samples were prepared for Illumina Sequencing by 16S rRNA gene amplification of the bacterial community (Caporaso et al., 2011; McCormick et al., 2014). The DNA was amplified for the hypervariable V4 region with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. Firstly, PCR reactions were performed for each sample using KAPA HiFi HotStart PCR Kit according to the manufacturer's suggestions, 0.3 μM of each PCR primer: forward primer 515F-Y (5′-GTGYCAGCMGCCGCGGTAA-3′) and reverse primer 806rB (5′-GGAC TACNVGGGTWTCTAAT-3′) (Caporaso et al., 2011; McCormick et al., 2014) and 12.5 ng of template DNA, in a total volume of 25 μL. The PCR conditions involved a 3 min denaturation step at 95 °C, followed by 30 cycles of 98 °C for 20 s, 64 °C for 30 s and 72 °C for 30 s and a final extension at 72 °C for 5 min. Second PCR reactions added indexes and sequencing adapters to both ends of the amplified target region according to the manufacturer's recommendations (Illumina 2013, n.d.). Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalised at Genoinseq (Cantanhede, Portugal), using a SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau et al., 2017), pooled and pair-end sequenced in the Illumina MiSeq sequencer with the V3 chemistry, according to the manufacturer's instructions (Illumina, San Diego, CA, USA).

2.5. Processing sequences

The set of Illumina-sequenced paired-end fastq files, received without barcodes, were imported in R (version 4.04) and analysed, demultiplexed, primer sequences removed, chimaera-filtered and Amplicon Sequence Variants (ASVs) were obtained using DADA2 package (version 1.18) (Callahan et al., 2016). Following the package instructions, sequences quality was inspected by checking the quality plots, subsequently trimming the last 20 bp for forward reads and allowing a max estimated error ("maxEE" option) higher than 2 per 100 bp for forward and reverse reads, which were truncated at position 240. The ASVs were assigned with RDP Taxonomy 18 database, which provides quality-controlled, aligned and annotated Bacterial and Archaeal 16S rRNA sequences (Wang et al., 2007). The RDP Classifier tool was used with an 80 % confidence cut-off. For species identification, the RDP Sequence Match tool was used and the sequences with 100 % similarity were selected. Non-assigned sequences, archaeal and eukaryotic sequences were removed. To ensure an equal sampling depth for all samples, the ASVs were rarefied to the same number (n = 23,695) using the Phyloseq R package (version 3.3.3) (McMurdie and Holmes, 2013) and rarefaction curves were visualised using the ggplot2 R package (version 1.34) (Wickham, 2016). Raw sequence data were deposited in the Sequence Read Archive (SRA) database at the NCBI under BioProject accession number PRJNA706887.

2.6. (Micro)plastics characterisation

Post DNA extraction particles were characterised according to shape (i.e., fibre, film fragment, pellet or fibre bundle), colour and measured at their largest cross-section (mm) under a dissecting microscope coupled with an image analysis system IC80 HD Camera with Leica Application Suite (LAS) software.

The chemical composition of the microplastics was evaluated by Fourier transform infrared spectroscopy (FTIR), in the mid-IR interval (400–4000 cm^{-1}), at the vibrational spectroscopy laboratory of the "Molecular Physical-Chemistry" R&D Unit (QFM-UC, Coimbra, Portugal), using a Bruker Optics Vertex 70 FTIR spectrometer purged by CO_2-free dry air. Depending on particle dimension, either attenuated reflectance (FTIR-ATR) or microFTIR measurements were performed. FTIR-ATR spectra were acquired using a Bruker Platinum ATR single reflection diamond accessory and a Ge on KBr substrate beamsplitter with a liquid nitrogen-cooled wide band mercury cadmium telluride (MCT) detector. Each spectrum was the sum of 64 scans, at 2 cm^{-1} resolution, and the 3-term Blackman–Harris apodization function was applied. Under these conditions, the wavenumber accuracy was better than 1 cm^{-1}. The spectra were corrected for the frequency dependence of the penetration depth of the electric field in ATR (considering a mean refraction index of 1.25). The microFTIR experiments were performed in a Bruker Hyperion 2000 microscope, in reflectance mode, with a nitrogen-cooled wideband MCT detector, coupled to the same spectrometer. Each acquisition was performed with 4 cm^{-1} resolution and 256 scans using a 15× Cassegrain objective.

The Bruker OPUS Spectroscopy Software (8.1 version) was used to preprocess the spectra (baseline correction, ATR correction and normalisation relative to the most intense band, for each sample). Spectra were then processed using the OMNIC software and compared with a commercial spectral library (Hummel Polymer Spectral Library, Thermo Fisher Scientific Inc.) and the BASEMAN library developed by Primpke et al. (2018). Only particles with a match higher than or equal to 70 % were accepted and classified as "Synthetic polymer", considering the component with the highest agreement value (Cowger et al., 2020; Kanhai et al., 2018; Thiele et al., 2021; Woodall et al., 2014). Particles with matches lower than 70 % were rejected and classified as "Unidentified" (polymer not assigned - N.A. in Table 1).

2.7. Data analysis

Venn diagrams of the percentage and number of shared and unique ASVs between grouped samples in both sampling environments were generated using the R package Venn (R Core Team, 2016). A principal coordinate analysis (PCoA) was performed using PAST program (version 4.02) (Hammer et al., 2001) to evaluate the differences in the microbial community compositions using the Bray-Curtis (BC) similarity index as an estimator of the taxonomic distance between samples. Hierarchical clustering based on BC distances was performed for all samples. All analyses were carried out using the ASVs frequency matrices at the genera level. Alpha diversity indices Shannon, Simpson and Pielou were calculated using the R package Vegan (version 2.5.7) (R Core Team, 2016). Also, a student's *t*-test ($p < 0.05$) was used to check differences in alpha diversity indexes among samples using *t*-test in R. Statistical comparison of bacterial communities between samples was performed by permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001). Relative abundance graphs of the bacterial taxa mean abundances were performed for all samples at all taxonomic levels (phylum, class, order, family and genus) using GraphPad Prism (version 8.0.1). Furthermore, data regarding the extracted particles used for DNA analyses were compiled for all matrices based on the number of particles, colour, size and polymer type, as well as on the calculation of the concentrations.

3. Results and discussion

3.1. Plastics and environmental matrices shared a low proportion of taxa

After quality filtering and chimaera removal of the initial 1,056,552 reads, 633,757 good quality reads with an average of 256 bp were retained (mean reads per sample = 52,813), ranging from 30,322 to 84,039 reads in samples B2S and E3W, respectively. All samples presented rarefaction curves with a stationary phase indicating sufficient depth of sequencing to account for most of the taxa amplified in both the plastics and the environmental matrices (estuarine water and beach sand) (Supplementary Fig. 1). Good quality reads were taxonomically classified using The Ribosomal Database Project (RDP), recovering Archaea (477 ASVs), Eukaryotes (37 ASVs) and Bacteria (9486 ASVs) taxa. Reads assigned to

Table 1
Categorization of the extracted particles (n = 48) according to type, colour size (mm) and polymer type found in both environments (Mondego estuary and adjacent sandy beaches). N.A. - "Not assigned" with matches lower than 70 %.

Environment	Station	Shape	Colour	Size (mm)	Polymer
Beach	B1	Fragment	White	4.99	Polystyrene
		Fragment	White	7.14	Polystyrene
		Fragment	White	4.77	Polystyrene
		Fibre	Blue	5.26	Polypropylene
		Pellet	White	4.11	Polyethylene
		Fragment	Blue	3.45	Polypropylene
		Fragment	Green	12.25	Polypropylene
	B2	Fragment	White	4.42	Polystyrene
		Fragment	White	5.36	Polystyrene
		Fragment	White	3.86	Polypropylene
		Fragment	Transparent	4.49	Polystyrene
		Fragment	Purple	4.86	Polyethylene
		Fragment	White	10.30	Polypropylene
		Fragment	Purple	4.78	Polyethylene
		Fragment	Green	13.95	Polypropylene
	B3	Fragment	Yellow	13.98	Polyethylene
		Fragment	Yellow	4.59	Polyethylene
		Fragment	Blue	5.01	Polypropylene
		Fragment	Blue	5.88	Polyethylene
		Fibre	Blue	4.21	Polypropylene
Estuary	E1	Fragment	Red	4.193	Polypropylene
		Fibre bundle	Blue	>5 mm	Polyester
		Film	White	6.22	Polyethylene
		Fragment	White	2.61	Polystyrene
		Fragment	White	3.56	Polystyrene
		Fragment	White	3.78	Polystyrene
	E2	Fragment	Black	0.85	N.A.
		Fibre	Blue	0.94	Polyester
		Fibre	Blue	0.94	Polyester
		Fibre	Blue	0.35	Polyester
		Fibre	Blue	0.61	Polyester
		Fibre	Blue	1.22	Polyester
		Fibre	Black	0.68	Polyester
		Fibre	Blue	0.87	Polyester
		Fibre	Black	2.67	N.A.
		Fibre	Black	1.31	N.A.
		Fibre	Black	4.15	N.A.
		Fibre	Black	0.49	N.A.
		Fibre	Blue	1.01	N.A.
		Fibre	Blue	1.07	N.A.
	E3	Fibre bundle	Blue	>5 mm	Polyester
		Fibre	Black	2.71	N.A.
		Fibre	Red	1.95	N.A.
		Fibre	Blue	3.02	N.A.
		Fibre	Blue	2.91	N.A.
		Fibre	Blue	2.29	N.A.
		Fibre	Blue	3.22	Polyester
		Fragment	Blue	0.22	Polyethylene

Cyanobacteria/Chloroplasts were detected, mainly in estuarine waters, accounting for 12 % on average of the total relative abundance. No mitochondrial reads were detected. Archaea and Eukaryotes reads were removed from the analyses since the goal was the study of the bacterial communities, and the bacterial reads were rarefied into the minimum sequencing depth. From each sample, 23,695 sequences were retrieved and taxonomically annotated, revealing 8999 different ASVs; of which 3279 belonged to 36 phyla, 84 classes, 151 orders, 313 families, and 818 genera. The remaining 5720 ASVs were considered unassigned at different levels (sequences with <80 % similarity): 2402 at the phylum level, 974 at the class level, 869 at the order level, and 1475 at the family level.

Plastics and their respective environmental matrices (estuarine waters and beach sand) shared a relatively low percentage of ASVs: 10.2 % in the estuary and 18.9 % on the beaches (Fig. 2). On the contrary, plastic samples in both environments presented high percentages of unique ASVs (59.3 % and 54.6 %, respectively) and the environmental matrices also presented noteworthy percentages of unique ASVs (30.5 % and 26.5 %, respectively) (Fig. 2).

Concerning the estuarine environment, the low values of shared ASVs are expected, according to the estuarine dynamics determined by the tidally averaged forcing and the river discharges, that determines the mixing water behaviour of these ecosystems. Accordingly, the Mondego estuary has significant oceanic and river contributions and therefore is characterised as a transitional well-mixed water body (Mendes et al., 2021) that could determine the emission of (micro)plastics from upstream areas with distinct microbiota when compared with the water in downstream areas.

These results are in line with a study conducted by Wu et al. (2020) in the Haihe Estuary (China), that reported a similar lower percentage of shared operational taxonomic units (OTUs) between the microplastics and the estuarine waters (14.8 %).

In parallel, the results of the present study also showed a lower value of common ASVs between microplastics and mesoplastics and beach sediments (Fig. 2). Since these beaches are located close to the Mondego estuary, they can also receive the influence of the riverine and estuarine areas regarding pollutant transfer to coastal areas.

Similar results were obtained by Basili et al. (2020) in a study conducted on the beaches of Italy for unique ASVs on sand samples (24.5 %) but reported a substantially lower percentage of shared ASVs between sand and the microplastics (6.5 %). When comparing our results with the aforementioned works, as well as with the works of Frère et al. (2018), conducted in a relatively enclosed bay (Bay of Brest, France), and Xu et al. (2019), a one-year incubation experiment in China's coastal waters, is plausible to suggest that a higher water residence time or slower circulation in a relatively closed geographical region is reflected by a higher percentage of shared taxa between the microplastics and its respective environmental matrix, as well as a lower percentage of unique taxa in the environmental matrix

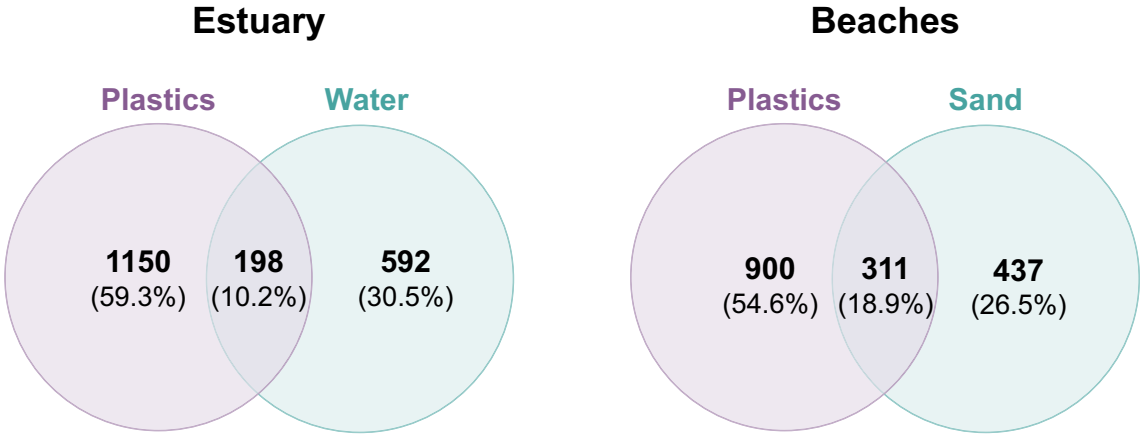

Fig. 2. Venn diagrams representing the number and percentage (%) of unique and shared ASVs between grouped plastics and their respective environmental matrix (water and sand) for both sampled environments: the Mondego estuary and adjacent beaches.

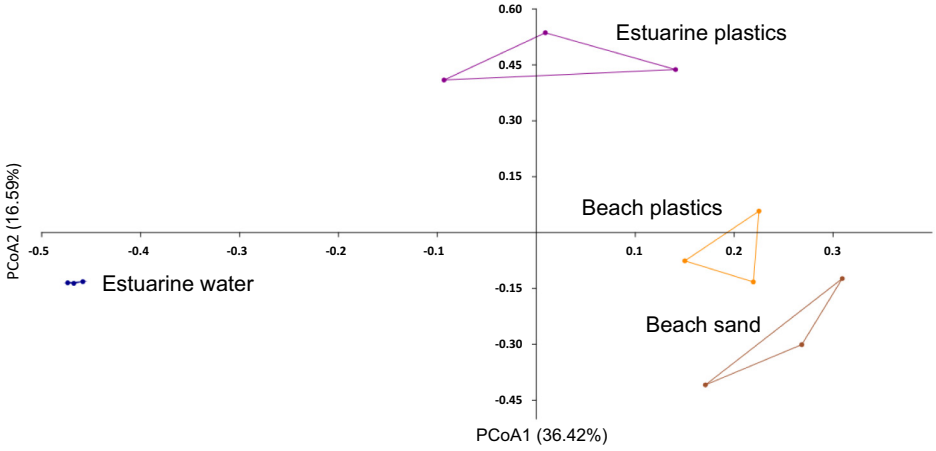

Fig. 3. Principal coordinate analysis (PCoA) of the bacterial communities from plastic particles from beaches and estuarine area, beach sediments and estuarine water fractions based on Bray-Curtis (BC) similarity index as an estimator of taxonomic distance within and between sample types. Each symbol refers to a bacterial community. The individual communities are coloured based on sample type and more similar communities are closer together in the ordination plot. Percentage of the diversity distribution explained by each axis is indicated on the plot: PCoA1 (36.42 %); PCoA2 (16.59 %).

samples. The local environmental conditions were already suggested to serve as a bacterial source for plastic biofilms formation (De Tender et al., 2017). However, it is important to note that the dynamics of both environments are very different, contributing to biofilm formation in distinct ways.

3.2. Plastisphere from the estuary was more similar to those found in beach samples

To visualise the differences between the bacterial communities of plastics collected from both environments, a PCoA was performed (Fig. 3). This analysis revealed that both types of samples were distinct for both environments, with higher proximity between beach plastics and beach sand (Fig. 3). The bacterial communities found in the estuarine waters (FL + PA) were very distinct from the ones associated with the estuarine plastics by both components 1 and 2, which explained 36.42 % and 16.59 % of the variance, respectively (Fig. 3). Curiously, the bacterial communities found in the estuarine waters clustered very close between sampling locations but clustered away from all the remaining samples, showing that the bacterial communities associated with estuarine plastics presented a higher similarity with the ones found in beach samples (plastics and sand) than with the ones found in the estuarine waters itself. Once again, the explanation might reside in the proximity of the beaches to the mouth of the Mondego estuary, from which they can also be influenced regarding pollutant transfer to coastal areas.

3.3. Bacterial communities diversity

Overall, the α-diversity indexes values were higher for plastics samples, but no significant differences were found between plastic's bacterial communities and those found in their environmental matrices with exception of the Simpson index in the estuarine samples (Student's *t*-test: $p > 0.05$) (Supplementary Table 1). It is not clear yet whether plastic-associated bacterial communities present an increased or decreased diversity compared with their counterparts on natural particles or their environmental matrices. While some studies from aquatic ecosystems have reported similar or even higher α-diversities on microplastics, other studies have postulated the opposite (Oberbeckmann and Labrenz, 2020). On the other hand, it has been reported that microplastic biofilms are shaped primarily by biogeographical and environmental factors, such as salinity and nutrient concentration (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2018),

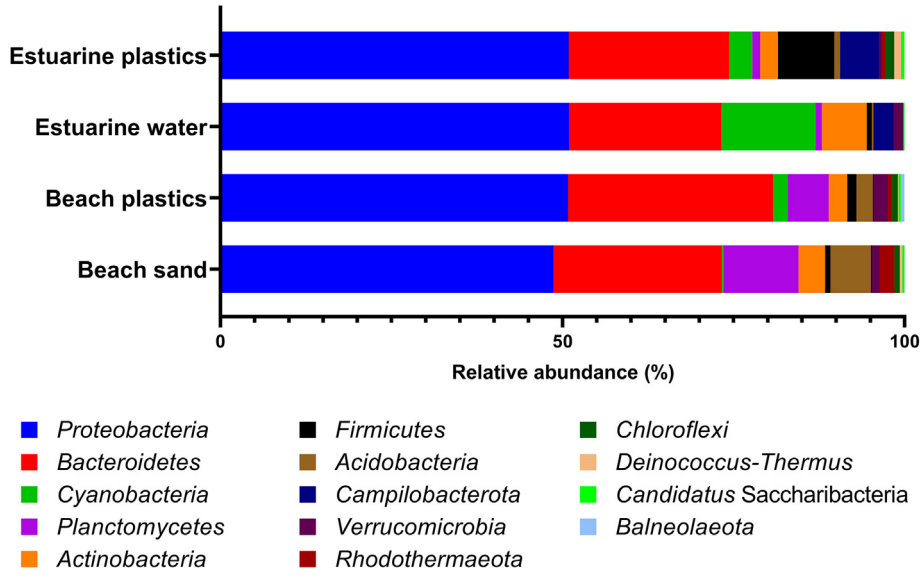

Fig. 4. Relative abundance of bacterial phyla of Plastisphere and environmental samples (estuarine water and beach sand). Phyla representing <0.1 % are not represented.

Amaral-Zettler et al. (2021) demonstrated that regional gradients can affect the bacterial communities in the Mediterranean Sea with clear differences between Plastisphere communities in rivers, ports, and seas in these areas. Although significant differences were not observed in bacterial communities nor in bacterial community diversity indexes between samples within the same environment (estuarine and sandy beach), this might be an expected result since both bacterial communities (plastics and environmental matrix) are under the influence of the same environmental factors at the time of the sampling events and there is no information regarding their residence time on these locations. Nevertheless, in this study, the taxa identity (richness) was considered more relevant than the taxa abundance (diversity) since the identity may give indications about the origin of the plastics and its associated bacteria.

3.4. Presence of potentially pathogenic genera unique to (micro)plastics

In this study, plastics harboured different bacterial communities as compared to their respective environmental matrices (estuarine water and beach sand) in terms of the presence/absence of key bacterial members, as well as in terms of their relative abundances (Figs. 4 and 5).

At the phylum level, the bacterial communities were dominated by Proteobacteria (49 % to 51 %) and Bacteroidetes (22 % to 30 %) in both (micro)plastics and their respective environmental matrices (Fig. 4). Furthermore, in the estuary, plastics presented a substantial abundance of Firmicutes (8 %) when compared to the waters (<1 %). The latter phyla are frequently the main phyla detected in microbial communities of microplastic biofilms from aquatic environments (Delacuvellerie et al., 2019; Dussud et al., 2018; Frère et al., 2018; Gong et al., 2019; Jiang et al., 2018; Kirstein et al., 2018; Zettler et al., 2013). Here, however, Proteobacteria and Bacteroidetes were also the most abundant phyla in the bacterial communities of both environments to a very similar extent, which indicates their dominance in these transitional ecosystems.

On the other hand, the phylum Firmicutes only had a representative abundance associated with the estuarine plastics (Fig. 4). Typical sewage-associated microorganisms belong predominantly to this phylum (e.g., Streptococcus, Lactobacillus, Blautia, Lachnospiraceae, Enterococcus, Ruminococcus) (Oberbeckmann et al., 2015). This association might indicate a potential contribution of wastewater treatment plants (WWTPs), as a source of these bacterial communities and using microplastics as transport vectors for the overall estuarine microplastics contamination. In the case of the Mondego estuary, this might be relevant since two WWTPs operate within the estuarine area used as sampling stations, which provides only secondary water treatment and without the capacity to treat industrial wastewater (Teixeira, 2016).

In the beach environment, samples presented an evenly distributed relative abundance of genera. In contrast, in the estuarine environment this was not observed, with the estuarine waters presenting three genera (*Candidatus* Pelagibacter, *Litoreibacter* and *Foliisarcina*) that accounted for nearly 70 % of the relative abundance. In the estuarine plastics these genera only accounted for 5 % of the relative abundance (Fig. 5).

The genus *Candidatus* Pelagibacter, was highly abundant in the estuarine waters but scarce in the estuarine plastics and practically absent from the beach environment (Fig. 5). In fact, this genus is known to dominate marine open waters but tends to be scarce on plastics debris (Amaral-Zettler et al., 2020, 2021).

In addition, the genera *Erythrobacter*, *Maribacter* and *Pseudoalteromonas* were more abundant on plastics than in their environmental matrices. The genus *Erythrobacter*, which is one of the most common and abundant members of the "Plastisphere" (Curren and Leong, 2019), was more abundant on plastics, especially in the estuarine environment (19 %), but was practically absent from the estuarine waters, reinforcing the observation that this genus is a core member of the "Plastisphere". Interestingly, the genus *Maribacter* had a higher abundance on microplastics particles, especially in the Mondego estuary, where it had a substantial abundance (12.7 %) but was completely absent from the waters. *Maribacter* species have been isolated from diverse environments, most of which are marine-associated habitats such as seawater and sediment adjacent to the sea (Nedashkovskaya et al., 2004; Thongphrom et al., 2016). This raises the question of how estuarine microplastics have acquired these bacterial genera. Commonly, it is only mentioned a river-sea trajectory of transport of (micro)plastics, but it is plausible that the saltwater intrusion into the estuary during high tides may also transport these particles from the ocean into the estuaries and contribute to the overall (micro)plastic estuarine dynamics. The sampling campaign in the Mondego estuary occurred close to the maximum point of high tide and during the autumn, in which the influence of the saltwater intrusion extends further than the furthest estuarine sampling location from the river mouth. However, the methodologies and the results obtained here are not sufficient to assess this possibility, for which a wide spatial-temporal scale would be required.

The genus *Pseudoalteromonas* presented a higher abundance on microplastic samples (3.6–5.8 %) than in their environmental matrices (<1 %). This genus has been reported to harbour potential pathogens and for being a commonly detected member on polypropylene (PP) as well as on polyethylene (PE) plastics but not in the background waters (Bowley

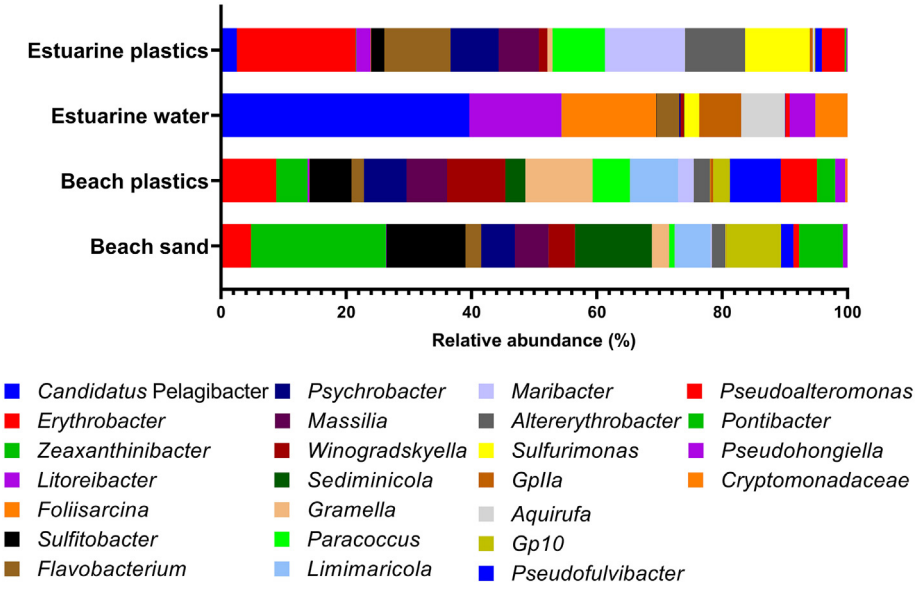

Fig. 5. Relative abundance of bacterial genera of Plastisphere and environmental samples (estuarine water and beach sand). Genera representing <1 % are not represented.

et al., 2021; Oberbeckmann et al., 2015), which is in concordance with the reported in the present study. The genus *Flavobacterium* was found in both microplastic samples but in the estuarine environment had a three-fold higher relative abundance on microplastics than in the waters. This genus can harbour fish pathogens and has been reported to be abundant in PE microplastic biofilms (Gong et al., 2019). Additionally, other potential pathogenic genera unique to the microplastic samples on both transitional ecosystems were found: *Lactococcus* and *Staphylococcus* on the estuary; *Acinetobacter*, *Mycobacterium*, *Shewanella*, *Staphylococcus* and *Streptococcus* on the beaches. The presence of potentially pathogenic genera unique to microplastics highlights the potential for these particles to act as dissemination vectors of key bacterial groups in transitional ecosystems. This poses potential ecological risks in these environments, as well as to human health, that require further attention and research. For instance, on beaches with high potential for recreational use, especially during the bathing season where the human presence is higher, this can increase the risk of human exposure to these potential pathogens through plastics but also for seabirds and/or other species that are commonly found feeding on the seashore. Finally, were also found WWTP/sewage-associated genera unique to the microplastic samples on both transitional ecosystems: *Aquabacterium*, *Blautia*, *Lactobacillus*, *Lactococcus*, *Prosthecobacter*, *Reyranella*, *Iamia* and *Staphylococcus* in the estuary; *Acinetobacter*, *Fluviivola*, *Mycobacterium*, *Paludibacter*, *Reyranella*, *Staphylococcus* and *Streptococcus* in mesoplastics from beaches. WWTPs are regarded as an important pathway of microplastics and associated key bacterial communities entering natural aquatic systems that can be used as a baseline for mitigation programmes such as microplastics-targeted treatment processes. Those programmes deserve future attention as mitigation actions for reducing the amount of (micro)plastic discharged from WWTPs and released from sewage sludge.

3.5. Plastic particles characterisation

All samples from both environments contained microplastics (<5 mm) but also mesoplastics (>5 mm) with great spatial variability, with fragments being dominant in the beaches (frequency of occurrence of 85 %) and fibres the main type found in the Mondego estuary (frequency of occurrence of 68 %) in many different colours and sizes (Table 1, Supplementary Fig. 5).

The plastic chemical composition from all examined particles showed the dominating presence of polyester (21%), expanded polystyrene (19%), polypropylene (19%) and polyethylene (16 %), of the total particles analysed (Supplementary Fig. 5). The remaining particles presented a match lower than 70 %, being defined as N.A. ("Not assigned") (Table 1). This situation is justified by the logistical and technical constraints previously mentioned (see Section 2.6). Ideally, polymer characterisation should be performed before DNA extraction due to the destructive nature of the solvents used for plastics. However, due to logistical and technical constraints in maintaining sterile conditions throughout the processes, it was necessary to perform DNA extraction from the plastic pool for each site and only subsequently discriminate against the nature of the polymer. In this study, the goal was not to discriminate bacterial communities according to polymer type but to ensure that the extracted particles were plastic polymers. Despite these constraints, the careful criteria used during the extraction procedure (The Hot Needle test) in the initial phase of this work regarding the appearance and physical characteristics of microfibres allows one to be confident in its synthetic nature, or at least in its anthropogenic nature (Finnegan et al., 2022). More specifically, particles with homogenous thickness across its length, a homogenous colour, gloss or absence of cellular or organic structures where not selected, as well as the use of the fibres Hot Needle Test, in which plastic particles change the structure or move when in contact with the needle (Bessa et al., 2019). Previous experimental studies have determined that polymers can indeed shape different bacterial communities, but at the same time it is virtually impossible to have all these similarities in terms of polymers when planning an experimental design in natural systems, if those particles are not occurring in these habitats during the sampling periods. Therefore, we assumed that we are collecting what is currently available and more frequent in these natural systems, keeping in mind that those differences (according to polymer, shape, colour and size) can play an important role in shaping those communities.

4. Conclusions

In general, the present work provides new insights into the comprehension of (micro)plastic contamination in transitional coastal ecosystems. The Plastisphere analysed in the present study revealed the occurrence of key bacterial groups, such as potential pathogens and WWTPs/sewage-associated, that were unique to microplastics and mesoplastics in the analysed transitional coastal ecosystems. This evidence reinforces the concern of (micro)plastics as vectors of transmission and spread of these bacterial groups and their potential ecological consequences in these ecosystems as well as for human health. Furthermore, the results suggest the existence of a substantial contribution of a sea-river trajectory to the overall estuarine microplastic dynamics. However, further research is required to confirm this possibility.

Overall, this study highlights the importance of the study of (micro)plastic-associated bacterial communities as a "storyteller" factor in plastic environmental contamination. With the levels of (micro)plastic contamination in the environment expected to increase, further research on the Plastisphere and its potential ecological and human health risks is required, especially in settings that represent important human activities and ecological services, such as the transitional coastal ecosystems. This work provided new insights and possibilities on these topics, but further research is required to answer the open questions: (i) How do tidal range and seasonality affect the (micro)plastic-bacterial communities in transitional ecosystems and the sea-river trajectory of plastics into estuarine systems? (ii) How similar/different are the estuarine microplastics-bacterial communities from the bacterial communities of known microplastic sources, such as WWTPs/sewage effluents? (iii) Do microplastics ingested by organisms such as fish or seabirds also harbour these key bacterial groups? If so, does this translate into a higher risk of infections or disease for the individual or the community? Does this occur in estuarine commercial species, and does it present any risk for human health? (iv) Does the current concentration of (micro)plastics and different polymer types on beaches present measurable risks for human health?

CRediT authorship contribution statement

José Marques: Conceptualization, Investigation, Writing - Original draft preparation; **Aitana Ares:** Formal analysis; Writing - Review & Editing; **Joana Costa:** Conceptualization, Resources, Writing - Review & Editing, Supervision; **M. P. M. Marques:** Resources, Formal analysis; Writing - Review & Editing; **L. A. E. Batista de Carvalho:** Formal analysis; Writing - Review & Editing; **Filipa Bessa:** Conceptualization, Resources, Writing- Reviewing and Editing, Supervision.

Data availability

Data will be made available on request.

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.

Acknowledgements

The work of FB is supported by the University of Coimbra through the contract IT057-18-7252 and Portuguese Foundation for Science and Technology (FCT), I.P. through the strategic project UIDB/04292/2020 and UIDP/04292/2020 granted to MARE and LA/P/0069/2020, granted to the Associate Laboratory ARNET. The NGS data sequencing and the work

of AA and JC were supported by the R&D Unit Center for Functional Ecology-Science for People and the Planet (CFE), with reference UIDB/04004/2020, financed by FCT/MCTES through national funds (PIDDAC). The work at the Molecular Physical-Chemistry R&D Unit was supported by FCT through the strategic project UIDB/00070/2020.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.161703.

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