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Marine Bacteria Associated with Colonization and Alteration of Plastic Polymers

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Abstract: The aim of this work was molecular identification of bacteria associated with marine sand at the drift line, where most plastic debris is deposited, and evaluation of the alteration of plastic polymers by them. Bacterial communities growing on plastic polymer surfaces may differentially cause surface alteration through exopolysaccharide production. This alteration can be analyzed by changes in spectra regions of colonized polymers compared to uncolonized polymers using Fourier Transform Infrared Spectroscopy (FTIR). In this study, bacteria located in sand at the drift line above sea water, where microplastics are most abundant, were isolated and identified through 16S rRNA. Six of the identified species produced exopolysaccharides, namely *Bacillus thuringiensis*, *B. cereus*, *Bacillus* sp. *Proteus penneri*, *Alcaligenes faecalis* and *Myroides gitamensis*. These bacteria species were inoculated into plates, each containing two frequently reported types of polymers at the drift line. Specifically, the two types of plastic polymers used were polypropylene and polystyrene spheres in whole and mechanically crushed states. Differences in bacterial growth were reported as inferred from weight increase of polypropylene and polystyrene spheres after 1-year long culture. Results also showed that *Alcaligenes faecalis*, *Bacillus cereus* and *Proteus penneri* colonized polypropylene spheres and modified spectra regions of FTIR. It is concluded that bacteria located in sand can be considered plastic-altering bacteria as changes in FTIR-spectra of polymers can be related to bioalteration.

Keywords: bacteria; EPS; FTIR; in vitro colonization; plastic polymer

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1. Introduction

Bacteria are pioneering organisms in plastic surface colonization. Some approaches have been carried out for study of colonization of plastic debris by bacteria [1,2] and the existence of bacteria growth and plastic-associated microbial assemblages on diverse polymer surfaces such as polypropylene, polystyrene and polyethylene has been demonstrated [3–5].

Bacteria colonization is associated with production of extracellular polysaccharidic substances (EPS) which reduce ionic repulsive forces between bacteria and polymer surfaces [6]. In some cases, EPS production has been detected while no polymer alteration was observed [7]. Otherwise, bacteria, such as *Pseudomonas* sp., *Kocuria* sp. and *Bacillus* sp., can form biofilms and facilitate the breakdown of polymer-associated chemical compounds [8]. Hence, bacteria could play a role in the colonization and possible alterations of polymer because of their occurrence on marine plastic debris. Studies of plastic-colonizing bacteria would thus provide useful information for knowing how bacteria act and their potential influence on biofilm abundance and plastic alteration.

Marine bacteria associated with plastics are predominantly identified through molecular characterization using the 16S rRNA gene. Bacteria from sand from coastal areas, where plastics accumulate most [9], can be thus assumed as the main colonizers of ≤ 5 mm plastic surfaces as identification of bacteria detached from microplastics during the separation procedure in a laboratory is not feasible.

In addition, microplastics take a longer time to alter due to the presence of salt and due to slow buildup of heat in the samples in sea water [10,11]; thus, its persistence provides

a habitable environment for microorganisms. Plastic debris can suffer cleavage of bonds through processes of hydrolysis and oxidation and address the formation of new chemical groups [12]. Polymer oxidation from biotransformation results in toxic compounds that are harmful for human health and impact natural environments [13]. Moreover, polymer alteration includes changes in polymer chemical structure that can lead to chemical bond weaking and loss of properties [14]. Polystyrene (PS) and polypropylene (PP) are polymers used in several industries and with commercial applications. They are the kind of plastic most reported at the drift line above sea water level [2].

Analytical procedures to monitor plastic polymer alteration include a microbiological approach such as EPS determination and chemical analyses through Fourier transform infrared spectroscopy (FTIR) [15]. Fourier transform infrared spectroscopy analysis can show chemical modifications of the polymer structure and the formation of new chemical groups because of microbial attack [16,17].

This study aimed at isolation and molecular identification of bacteria that are able to colonize plastic polymer in order to evaluate the alteration of plastic polymers by bacteria. Our goal is to identify predominant bacteria through the 16S rRNA gene that are associated with marine sand at the drift line where most plastic debris is deposited. Moreover, polymer colonization by bacteria is evaluated in in vitro assays through EPS production and by increment in weight of polymers. Commercial polystyrene and polypropylene spheres are chosen as representative polymers and structural alteration due to in vitro colonization by bacteria is analyzed through FTIR.

2. Materials and Methods

2.1. Sampling Site and Characterization of Microplastic Type

Marine sand was collected at the drift line above sea water level, during low tide, along the northeast coast of Gran Canaria in the Canary Islands (28°04′33.5″ N, 15°24′51.7″ W). Sampling was carried out between 2 cm and 5 cm depth for a year and sand was then kept refrigerated at 4 °C and transported to a laboratory. Marine sand was then dried in a standard hot-air drying oven at 55 °C for 5–6 h. Sand was sieved with a 4 mm mesh pore size and the potential aggregates were smoothed with a spoon during sieving.

To characterize most abundant kinds of microplastics in the drift line, microplastics were extracted through a saturated salt solution according to [18]. Thus dry sand < 4 mm fraction and saturated NaCl solution (1:4 sand weight/volume salt solution) was stirred for 5 min at 92× *g* at room temperature. Supernatant was carefully filtered using a vacuum pump through a 47 mm Millipore 0.45 µm filter. The filter was then examined under a stereomicroscope and microplastics were taken to be analyzed under Fourier Transform Infrared Spectroscopy (FTIR) to determine the kind of plastic. Sodium chloride solution was prepared 48 h prior to use and then filtered to remove impurities. The control was carried out filtering the same volume of salt solution as that used in the sand:salt solution, but without sand.

2.2. Bacteria Molecular Identification

Bacteria adhering to marine sand were detached through ultrasound bath and orbital shaker using a 1:2 ratio (*w*/*v*) of sand:double distilled and autoclaved water. Ultrasound bath was carried out for 1 min × 5 times each, at room temperature and orbital shaking was performed overnight at 23 °C. Detached bacteria in supernatant (200 µL) were plated in heterotrophic marine agar culture media. Bacteria plates were then cultivated at 23 °C and individual colonies were successively plated from each plate to obtain the pure colonies. The bacteria pure colonies were then molecularly characterized. When bacteria biomass needed to be increased, pure colonies were cultivated in sterile phosphate-buffered saline (PBS) solution containing tryptone (10 g L^{-1}) and yeast extract (5 g L^{-1}) at 23 °C for 24 h. The culture was then centrifuged at 15,000× *g* for 15 min and the resulting pellet was taken as a source of template DNA. All media and reagents were purchased from Scharlab (Barcelona, Spain).

Pure colonies and pellets were homogenized in liquid nitrogen and incubated in 800 µL of DNA isolation solution containing 100 mM Tris-HCl (pH 8.2), 4 M NaCl, 20 mM EDTA, CTAB (2%, *w*/*v*), PVPP (0.1%, *w*/*v*), SDS (0.1%, *w*/*v*) and mercaptoethanol (2%) in a water bath at 65 °C for 1 h according to the Murray and Thompson procedure [19] with modifications. A volume of chloroform:isoamyl alcohol solution (24:1 *v*/*v*) was added and the samples were gently mixed by inversion at intervals of 20 s. The mixture was then centrifuged for 10 min at 827× *g* in a Beckman Coulter Allegra X-22R centrifuge (Beckman Coulter Inc., Brea, CA, USA). Successive washings with chloroform:isoamyl alcohol (24:1 *v*/*v*) solution were performed. The supernatant was then placed in a fresh tube and an equal volume of n-propanol (−20 °C) was added, mixed gently and centrifuged at 15,000× *g* for 30 min. The resulting pellet, containing DNA, was washed with ethanol (80% *v*/*v*, molecular grade), dried and suspended in sterile deionized water. DNA yields were assessed using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All samples were in triplicate. Purity DNA was valued by smear absence, migrating on a 0.8% (*w*/*v*) agarose gel.

The molecular identification was based on the amplification of the 16S rRNA gene (Table 1), amplified by a polymerase chain reaction in a GenAmp 2400 thermal cycler (PerkinElmer Inc., Waltham, MA, USA) with 30 cycles consisting at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1.5 min, followed by a final extension step of 5 min at 72 °C. Each PCR reaction mixture contained 0.5 U Takara Ex Taq DNA polymerase (TaKaRa Shuzo Co., Shiga, Japan), 2.5 mM of each dNTP, 10 µL Takara Ex Taq PCR buffer with $MgCl_2$, 10 µM forward and reverse primers (Table 1) and 90–95 ng DNA template.

Table 1. Sequences of the forward (F) and reverse (R) primers, for 16S rRNA gene. Sequences were retrieved from [20,21].

Gene	Primer Name	Sequence (5′-3′)
16S rRNA	Forward	TTCGGAATAACAGTTG
	Reverse	CGGCTGGATCTAAGGA
	Forward	GAGTTTGATCCTGGCTCAG
	Reverse	ACG GHT ACC TTG TTA CGA CTT
	Forward	AGAGTTTGATCMTGGCTCAG
	Reverse	TACGGYTACCTTGTTACGACTT
	Forward	CCAGCAGCGCTAATACG
	Reverse	TACCAGGGTATCTAATCC

PCR products were visualized with agarose gel electrophoresis. In addition, amplification products ranging from 800 to 1,200 bp were obtained and purified using the QIAEX agarose gel extraction kit (Qiagen Inc., Hilden, Germany). The fragments were then ligated to the pGEM-T-easy cloning vector (Promega, Fitchburg, WI, USA) and cloned in JM109 cells according to the manufacturer's instructions (Promega). Plasmids were isolated using a plasmid purification kit (Qiagen Inc.). The insert in the plasmid was checked by PCR using primers M13F and M13R (Promega). The insert was then sequenced on both strands using an ABI-310 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA) and BigDye Terminator v3.1. Nucleotide sequences were submitted to NCBI GenBank BLAST search and identified through similarity values. Sequences were then aligned to reference sequences using MAFFT v7.222 [22] with default parameters, followed by visualization and manual trimming employing UGENE v33.0 [23] to delimit unequivocal aligned regions. The phylogenetic trees were estimated and constructed using Maximum Likelihood (ML) criteria implemented in IQTREE v2.1.3 [24]. The best substitution model was selected with Modelfinder [25] performed in IQTREE v2.1.3 and 1.000 ultrafast bootstrap replicates were sampled to assess the robustness of the phylogeny [26]. Support values from bootstrapping runs (MLB) were mapped on the best trees. The phylogenetic trees were finally viewed and edited using FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree, accessed on 11 February 2022). The best-fit evolutionary model was selected under the

Bayesian Information Criterion (BIC). The criterion for differentiating bacteria was a value of 16S rDNA gene sequence similarity over 70% [27].

2.3. Bacteria In Vitro Colonization

To value polymer colonization by bacteria, a selected pure colony was individually inoculated in supplemented PBS 25 mL. When log-growth was reached (OD ~ 1.5–2 at 660 nm and ca. (1–4) $\times$ 10^9 UFC mL^{-1}), a bacteria 100 µL aliquot was then inoculated in a Petri plate containing PBS media (15 mL) and plastic polymer spheres. Selected pure colonies of bacteria were *Myroides* belonging to phylum Bacteriodetes, *Proteus and Alcaligenes* belonging to Proteobacteria *and Bacillus* sp. belonging to Firmicutes. All bacteria were molecularly isolated and identified as described above. Polymer spheres (ca. 12 of 4 mm diameter) were independently added in each plate as whole spheres or as mechanically crushed polymer spheres. Plastic polymer spheres were made of polypropylene (PP, average MW 12,000, Sigma Co., St Louis, MO, USA) or polystyrene (PS, average MW 35,000; Sigma Co.). Plates with polymer spheres and inoculated with bacteria (henceforth test spheres) were then sealed and placed in an orbital shaker at 110 rpm at 25 ºC. Plates containing polymer spheres without bacteria (henceforth control spheres) were used as controls. All spheres were cleaned with pure ethanol and autoclaved for 20 min at 121 ºC prior to use. Bacteria were allowed to colonize polymer spheres and colonization was evaluated through bacteria growth. Plastic polymer spheres were weighted (n = 5) and the weight was compared to those without bacteria that were used as control plates at 12 months. It was assumed that an increase in weight spheres was due to bacteria growth over spheres.

2.4. Determination of Exopolysaccharides (EPSs)

Alteration of plastic polymer surfaces was valued through bacterial exopolysaccharide production. Exopolysaccharide determination was performed according to Dubois et al. [28] with modifications. Bacteria pure colonies were individually cultured in 5 mL of PBS for 48 h. Then, 2 mL supernatant was placed in a new tube and 96% cold ethanol was added in a ratio 1:1. To precipitate polysaccharides, tubes were stored overnight at 4 °C and then centrifuged at 1500$\times$ g for 5 min. The EPS pellet was resuspended in 400 µL of distilled water. Then, phenol 10 µL (80%) and sulfuric acid 1000 µL (99%) were added. The mix reaction was incubated in a water bath at 35 °C for 15–25 min. Absorbance at 490 nm was determined to reveal the presence of hexoses. For determination, a calibration curve was made using hexose (Sigma Aldrich) as a standard. All samples were analyzed by triplicate and measured in a Beckman DU 530 UV-Vis Spectrophotometer.

2.5. Structural Changes of Polymers through FTIR

Structural changes of polypropylene and polystyrene spheres were performed using an FTIR. The spectra region was analyzed from 3200 cm^{-1} to 700 cm^{-1}. In order to avoid disruptions due to natural organic matter adhering to spheres, all spheres were washed with an aqueous solution of ethanol (70%) and oxygen peroxide (3%), allowed to dry for 1 day and then grinded to reach fine powder 5 mg. Polymer sphere spectra, from plates within bacteria and those from plates without bacteria were analyzed and compared to test the effect of colonization by bacteria. A comparison between whole and mechanically crushed polymer spheres from plates within bacteria was carried out to test bacteria affinity for whole or crushed spheres.

2.6. Data Analysis

A one-way ANOVA followed by post hoc tests (Tukey HSD and Dunnett T3) were used to detect significant differences ($p < 0.05$) in EPS concentration and weights of whole and mechanically crushed spheres made of polypropylene and polystyrene.

3. Results and Discussion

3.1. Bacteria Molecular Identification

The occurrence of microplastics in marine ecosystems is an undisputed fact; however, identification of bacteria associated with these tiny plastic polymers is lacking. It is also worth mentioning that characterization of the bacteria community directly associated with microplastics is not possible as bacteria can be removed during the separation process with a fully saturated salt solution.

As a starting point, bacteria sampled and identified in different seasons are assumed as key bacteria species able to alter microplastics. This is because (i) bacteria adhering to marine sand at the drift line above sea water level are prevalently associated with microplastic particles which, in turn, are also deposited the most in the same drift coast line [2,29,30]; (ii) bacteria are spread out through covering plastic particles [31]; and (iii) bacteria composition in this drift line can be representative of different plastic surfaces as the bacteria community depends on the kind of polymer and even the sampling period [32]. Different studies have reported that 20% plastic debris in coastal areas is deposited on beaches [33–35].

Seventeen bacteria pure colonies from marine sand were characterized in culture and identified through the 16S rRNA gene. Four universal primer combinations, used in this work, have been previously validated (Table 1), as they match different positions of the genome, identifying different areas of the 16S rRNA region [36–38]. Sequences from PCR fragments were assigned to groups based on BLAST similarities. Colony identification was classified with five bacteria genera, *Alcaligenes*, *Proteus*, *Myroides*, *Kocuria* and *Bacillus*, which contained most species with more than 50% identified clones. Moreover, species from *Kocuria* genera were doubtfully identified, as the existence of possible chimeras revealed. Other genera were also determined to be less prevalent in the sampling.

Bacteria species were molecularly identified as *Myroides gitamensis*, *Proteus penneri*, *Bacillus* sp., *Bacillus thuringiensis*, *Bacillus cereus* and *Alcaligenes faecalis* in BLAST. The phylogenetic analysis positioned *Myroides gitamensis* LP5 sequence in a clade within other 16S rRNA Myroides (Figure S1; Table S1). There was a total of 715 positions in the final dataset, of which 516 were conserved, 113 were parsimony-informative and 86 were parsimony-uninformative. The best-fit evolutionary model was TPM3E + F + R2. This result (Figure S1), with a value of 16S rDNA gene sequence similarity of 78%, demonstrated that *Myroides gitamensis* could be regarded as representing a species within the genus.

The *Proteus penneri* dataset was constructed with 15 reference sequences (Table S2). This dataset had a length of 799 characters, of which 738 were conserved, 26 were parsimony-informative and 35 were parsimony-uninformative. The best-fit evolutionary model was HKY + F + I [39]. The phylogenetic analysis positioned *P. penneri LP3* in a clade within other *P. penneri* and had a similarity value of 73% (Figure S2).

The tree of *Alcaligenes faecalis* was constructed with 19 reference sequences (Table S3). This dataset had a length of 873 characters, of which 795 were conserved, 41 were parsimony-informative and 37 were parsimony-uninformative. The best-fit evolutionary model was HKY + F + I [39]. *A. faecalis LP6* was positioned in a clade for the same species with a value of 70% (Figure S3).

Significantly, the phylogenetic analysis positioned *Bacillus* sequences in three clades within *B. thuringiensis* (*B. thuringiensis LP1*; value 76%), *B. cereus* (*B. cereus LP2*; value 78%) and *Bacillus* sp. (*Bacillus* sp. *LP4*; value 66%). The dataset, constructed with 15 reference sequences (Table S4), had a length of 968 characters, of which 805 were conserved, 67 were parsimony-informative and 96 were parsimony-uninformative. The best-fit evolutionary model was HKY + F [39]. Unlike *B. thuringiensis* and *B. cereus* which were assigned to two distinct clades with high similarity values, *Bacillus* sp. can be disregarded as no distinctions between *B. cereus*, *B. paramycoides* and *B. pseudomycoides* were found (Figure S4).

From phyla identified in this work, Proteobacteria that comprises *P. penneri* and *Alcaligenes faecalis* and Firmicutes with *Bacillus* spp. are one of the most reported on marine plastic debris and species frequently suggested as plastic-degrading marine bacteria [40,41].

Bacterial molecular characterization allows determination of the type of microorganism that plays a role in plastic polymer alteration. In turn, this alteration is controlled by bacteria colonization and subsequent EPS production.

3.2. In Vitro Colonization and Structural Changes of Polymers through FTIR

Plastic polymer colonization by bacteria has been reported, through formation of biofilm, at 7 days in coastal marine sediments where microorganism consortia such as several bacteria, diatoms, etc., are also involved [42,43]. Moreover despite microplastic colonization also depending on chemical parameters such as pH and salinity, the in vitro model system allows circumvention of these variables and studying of polymer colonization by bacteria under controlled conditions. Moreover the in vitro approach would unveil how a particular bacteria species works and what its role is in the alteration of plastic polymers. This would allow addressing whether these bacteria can release plastic-active exoenzymes, with potentially biotechnological benefits, that can successfully alter plastic polymers.

In this work, in vitro colonization of polymers by bacteria is assumed through EPS production (Table 2) and bacteria growth over plastic surface for 1 year (Table 3). Moreover in vitro colonization was performed with polypropylene and polystyrene spheres as most plastic fragments, isolated through saline gradient and analyzed with FTIR, were identified as such. This characterization is in accordance with a recent report concerning marine sand of drift line [44].

Table 2. Concentration of exopolysaccharides (EPSs, mg L^{-1}) produced by bacteria in in vitro assays (*, $p < 0.05$).

Genus Species	Phyla	EPS (mg L^{-1})
Bacillus thuringiensis	Firmicutes	4.5×10^{-2} (*)
Bacillus cereus		3.2×10^{-2} (*)
Bacillus sp.		1.1×10^{-1} (*)
Proteus penneri	Proteobacteria	1.8×10^{-3}
Alcaligenes faecalis		3.5×10^{-2} (*)
Myroides gitamensis	Bacteroidetes	2.9×10^{-3}

Table 3. Bacteria growth (in percentages, %) over whole and mechanically crushed polymer spheres made of polypropylene (PP) and polystyrene (PS) after one year in culture.

	Polypropylene (PP)		Polystyrene (PS)	
	Whole	Mechanically Crushed	Whole	Mechanically Crushed
Bacillus thuringiensis	157 (ç*)	162 (ç *)	134 (ç)	137
Bacillus cereus	130 (çΨ)	114	121 (ç)	122
Proteus penneri	137 (ç*Ψ)	153 (ç *)	125 (çΨ)	119
Bacillus sp.	118 (*)	112 (*)	134 (çΨ)	141 (ç)
Myroides gitamensis	115	104	106	96
Alcaligenes faecalis	124 (ç)	132 (ç *)	121 (ç*Ψ)	77

ç, significant differences compared to sphere weights in plates without bacteria (control); *, significant differences between respective spheres of PP and PS polymer; Ψ, significant differences between whole and mechanically crushed spheres of PP and PS polymer. Significant differences, $p < 0.05$. A value of 100% is for whole PP spheres 0.115 ± 0.020 g; for mechanically crushed PP, 0.094 ± 0.076 g; for whole PS spheres 0.133 ± 0.004 g; and for mechanically crushed PS, 0.131 ± 0.029 g.

With the exception of *Myroides gitamensis*, bacteria growth—over whole and mechanically crushed polymer spheres—can be inferred as a significant increment in the weight of polymer spheres was determined compared to those of control spheres (Table 3). Our results indicate that plastic spheres made of PP and PS are susceptible to colonization by bacteria (Table 3). Nonetheless *Bacillus thuringiensis* and *Proteus penneri* showed significative growth in the presence of PP compared to PS spheres (Table 3). Additionally, results also indicate that (i) there are significant differences in the weight of whole polypropylene

spheres compared to those mechanically crushed spheres for *Bacillus cereus* and *Proteus penneri* and (ii) these differences were also determined for *P. penneri* and *Alcaligenes faecalis* with polystyrene spheres (Table 3). With these results, we infer that specific taxonomic groups show preferences for certain plastic surfaces and that potential metabolic adaptation, such as differential attachment, to plastic may probably occur.

Bacteria growth over polymer spheres and EPS production are conducive to polymer alteration, while in turn, alteration could supply nutrient sources for bacteria growth. It is generally accepted that biofilm formation on the polymer surface is a prerequisite for microplastic alteration [42]. In this work, it seems to be confirmed that *M. gitamensis* both did not produce EPS (Table 2) nor did it grow over plastic spheres (Table 3). Likewise, no changes in FTIR spectra of *M. gitamensis* are observed as reported below. A definite challenge for this work is to unveil potentially plastic-altering bacteria regardless of environment variables, such as pH and salinity, that can be circumvented as they are under control in in vitro experiments.

Bioalteration has been related to changes in FTIR-spectral properties of polymers [15–17]. Three spectral regions are assumed as fingerprint regions for PP and PS, namely 1100–750 cm^{-1}, 1600–1000 cm^{-1} and 3100–500 cm^{-1}. Thus spectra of PP and PS spheres with inoculum were superimposed on spectra of polymer without inoculum (control) for analysis. Moreover, as an approach to value changes in spectra, area count reduction was used as a measure of the extent grade of polymer bioalteration and thus we would allow inferring the ability of bacteria to cause the polymer to deteriorate.

In this work, polymer bioalteration was characterized for flattening and splitting of peaks and a diminution of vibration bands below 50% (in term of relative area counts) in FTIR spectra through qualitative comparison between spectra of test spheres (with bacteria) and in absence of bacteria (control spheres). Spectra of PP whole spheres inoculated with *Alcaligenes faecalis* and *B. cereus* showed peak flattening in a vibration band between 3100 and 2500 cm^{-1} (Figure 1 A,B). Flattening in whole PP spectra was associated with a diminution in area counts around 80% and 81% for each bacteria respectively compared to area counts of control. Otherwise, *Alcaligenes faecalis*, *B. cereus* and *Proteus penneri* displayed a double peak around 1460 cm^{-1} (Figure 1C).

Significantly, only *A. faecalis* was able to alter spectra of mechanically crushed spheres of polypropylene as a diminution in vibration band, in area counts over 75%, was determined between 3100 and 2500 cm^{-1} (Figure 2A,B). Peak splitting, around 1460 cm^{-1} with three resulting peaks, was also observed (Figure 2C). In all PP spectra, flattening peak consisted of a band located at about 3000 cm^{-1} assignable to CH_2 as asymmetric stretching occurs [45]. Furthermore, double and triple peaks at 1000–1600 cm^{-1} are indicative of bioalteration of polypropylene as intermediate products, i.e., primary and secondary alcohols and formation of aldehydes and ketones, could have emerged [45]. In particular, a band around 1460 cm^{-1} reveals a bending deformation [46]. Despite other bacteria also growing over polymer spheres, both whole and crushed, spectra were similar, with each displaying vibration bands of polypropylene.

Concerning polystyrene, although bacteria growth over polymer spheres was also visible and an increment in sphere weight was also determined (Table 3), no changes were observed in FTIR spectra (Figures 3 and 4).

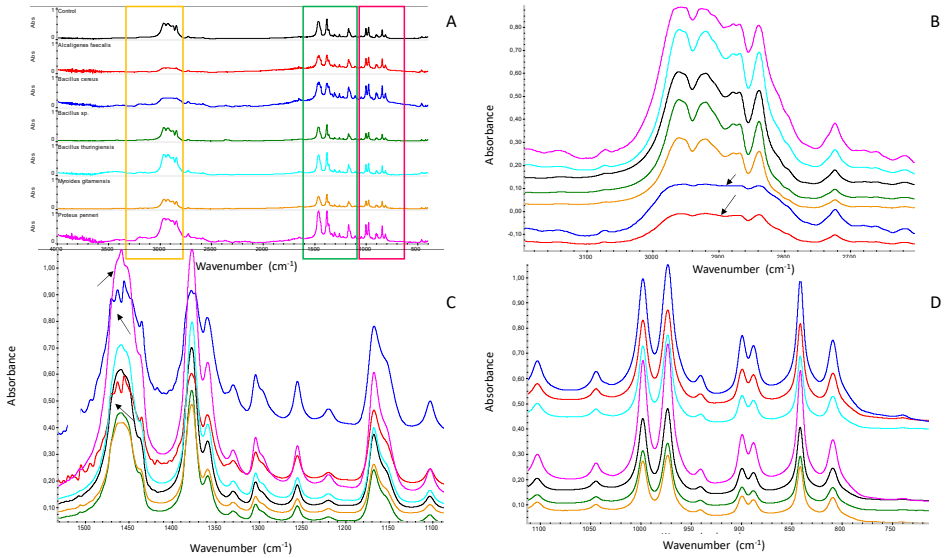

Figure 1. FTIR spectra of whole spheres of polypropylene (PP). (**A**) Spectra from polymers without inoculum (control) and with each one being bacteria inoculated; (**B**) Detail of spectra in vibration band between 3100 and 2500 cm^{-1}; (**C**) Detail of spectra in vibration band between 1600 and 1000 cm^{-1}; (**D**) Detail of spectra in vibration band between 1100 and 750 cm^{-1}. Red line corresponds to PP whole spheres inoculated with *Alcaligenes faecalis*, blue line with *Bacillus cereus*, green line with *Bacillus* sp., light blue line with *Bacillus thuringiensis*, orange line with *Myroides gitamensis*, pink line with *Proteus penneri* and black line corresponds to PP spheres without inoculum (control). Arrows are indicative of flattening peak (in **B**) and double peaks (in **C**).

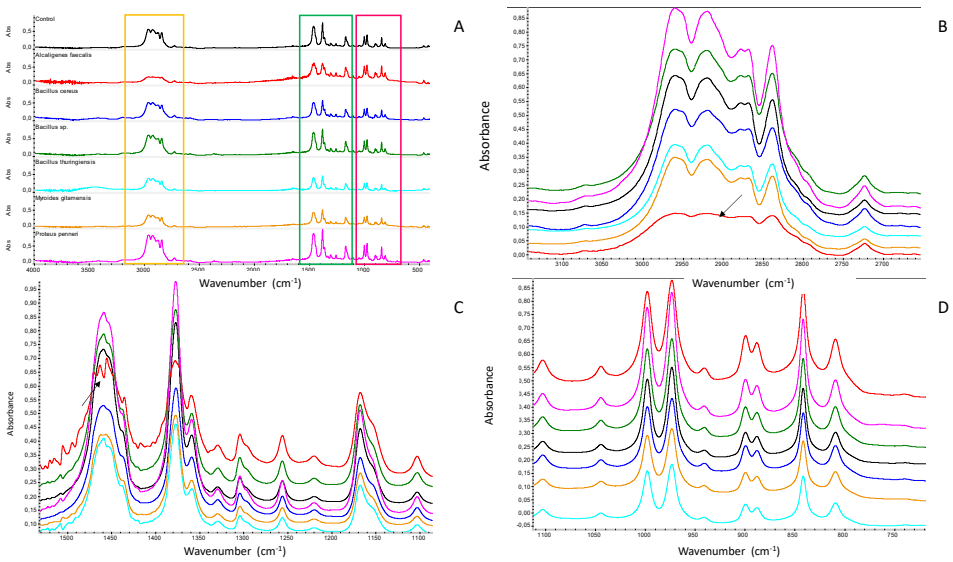

Figure 2. FTIR spectra of mechanically crushed spheres of polypropylene (PP). (**A**) spectra from polymers without inoculum (control) and with each one being bacteria inoculated; (**B**) Detail of spectra in vibration band between 3100 and 2500 cm^{-1}; (**C**) Detail of spectra in vibration band between 1600 and 1000 cm^{-1}; (**D**) Detail of spectra in vibration band between 1100 and 750 cm^{-1}. Red line corresponds to PP mechanically crushed spheres inoculated with *Alcaligenes faecalis*, blue line with *Bacillus cereus*, green line with *Bacillus* sp., light blue line with *Bacillus thuringiensis*, orange line with *Myroides gitamensis*, pink line with *Proteus penneri* and black line corresponds to PP spheres without inoculum (control). Arrows are indicative of flattening peak (in **B**) and triple peaks (in **C**).

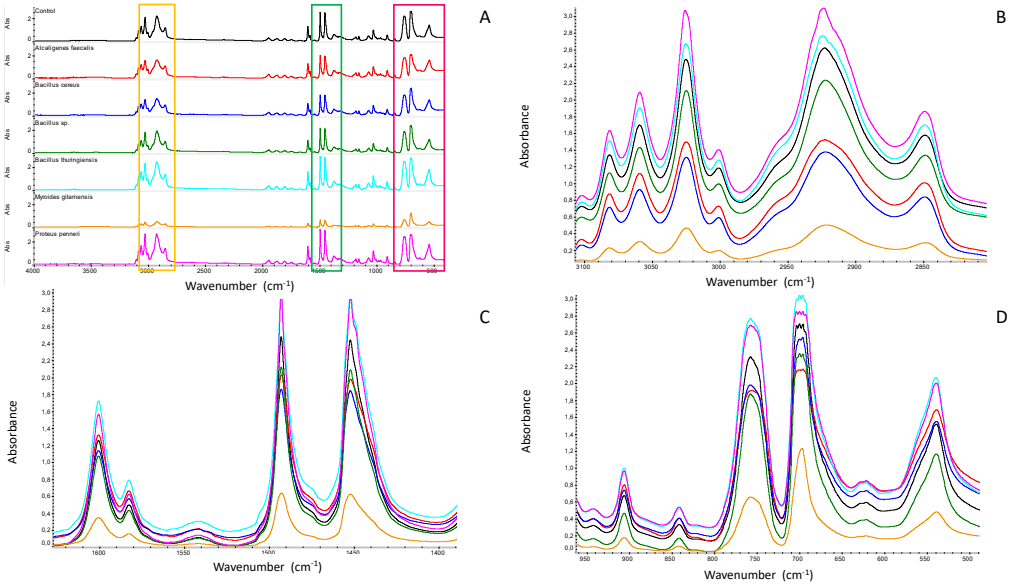

Figure 3. FTIR spectra of whole spheres of polystyrene (PS). (**A**) Spectra from polymers without inoculum (control) and with each one being bacteria inoculated; (**B**) Detail of spectra in vibration band between 3100 and 2500 cm^{-1}; (**C**) Detail of spectra in vibration band between 1600 and 1000 cm^{-1}; (**D**) Detail of spectra in vibration band between 1100 and 750 cm^{-1}. Red line corresponds to PS whole spheres inoculated with *Alcaligenes faecalis*, blue line with *Bacillus cereus*, green line with *Bacillus* sp., light blue line with *Bacillus thuringiensis*, orange line with *Myroides gitamensis*, pink line with *Proteus penneri* and black line corresponds to PP spheres without inoculum (control).

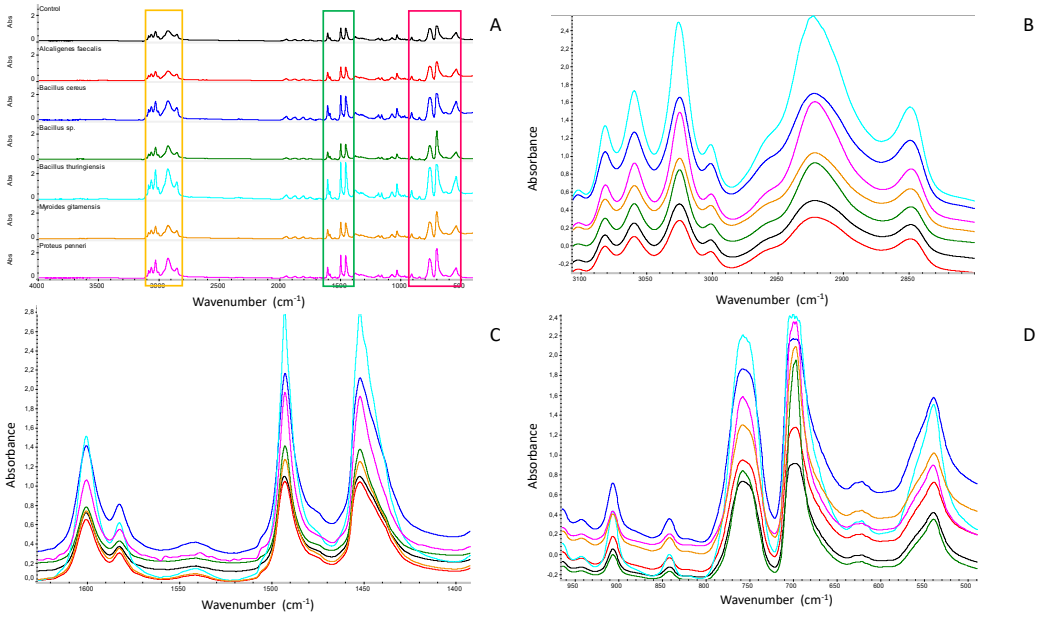

Figure 4. FTIR spectra of mechanically crushed spheres of polystyrene (PS). (**A**) Spectra from polymers without inoculum (control) and with each one being bacteria inoculated; (**B**) Detail of spectra in vibration band between 3100 and 2500 cm^{-1}; (**C**) Detail of spectra in vibration band between 1600 and 1000 cm^{-1}; (**D**) Detail of spectra in vibration band between 1100 and 750 cm^{-1}. Red line corresponds to PS mechanically crushed spheres inoculated with *Alcaligenes faecalis*, blue line with *Bacillus cereus*, green line with *Bacillus* sp., light blue line with *Bacillus thuringiensis*, orange line with *Myroides gitamensis*, pink line with *Proteus penneri* and black line corresponds to PP spheres without inoculum (control).

Results seem to suggest that the type of polymer, alongside metabolic adaptation of bacteria (i.e., polysaccharide production; [47,48]), may affect bacteria growth and their polymer attachment. In addition to chemical properties of plastic polymer spheres, differences between vibration bands of whole and mechanically crushed spheres might also be caused by grooves and pits inadvertently introduced during crushing [49].

In summary, type (PP vs. PS) and state (whole vs. mechanically crushed spheres) of plastic polymer would affect colonization and alteration of polymers by bacteria. By comparing all the spectra obtained from plastic spheres that were inoculated with different bacteria, it is concluded that bacteria successfully reduced the C-H stretches around 1460 cm^{-1} and C-H vibrations at 3000 cm^{-1}. These results open a door to further study if co-occurrence of bacteria (bacteria consortia) can favor changes in PS spectra and improve polymer bioalteration.

Significantly, bacteria associated with marine sand at the drift line can be investigated for plastic polymer alteration. This in vitro approach also establishes a framework to discern between colonizing and altering bacteria as bioalteration of microplastics should be accompanied by both bacteria colonization and changes in the FTIR spectra of polymers. The in vitro model could also be used to perform comparative analyses of gene transcripts of bacteria involved in altered and non-altered plastic surfaces. In particular, *A. faecalis* would be a candidate bacterium as bacteria species alter polyethylene under laboratory conditions. More research is also required in order to determine if altering bacteria and metabolites derived from bacteria consortia vary across different polymers. All in all, it is important to predict the functionality of bacteria based on genomic data and to gain insight into the principles that govern bacteria consortia interactions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app122111093/s1, Table S1: *Myroides* spp. strains included in phylogenetic analysis of 16S rRNA gene sequences retrieved from NCBI database. Table S2: *Proteus* spp. strains included in phylogenetic analysis of 16S rRNA gene sequences retrieved from NCBI database. Table S3: *Alcaligenes* spp. strains included in phylogenetic analysis of 16S rRNA gene sequences retrieved from NCBI database. Table S4: *Bacillus* spp. strains included in phylogenetic analysis of 16S rRNA gene sequences retrieved from NCBI database. Figure S1. Phylogenetic tree created with IQTREE v2.1.3. The evolutionary history was inferred from 16s rRNA sequences of *Myroides gitamensis* using the Maximum Likelihood method and the best-fit evolutionary model selected under the Bayesian information based on criteria TPM3E + F+ R2. Number in node represents values referring to sequence similarity. Bootstrap test was performed with 1000 replicates. LP indicates sequence analyzed in the present study The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (scale under figure). Figure S2. Phylogenetic tree created with IQTREE v2.1.3. The evolutionary history was inferred from 16s rRNA sequences of *Proteus penneri* using the Maximum Likelihood method and the best-fit evolutionary model selected under the Bayesian information based on criteria HKY + F + I. Number in node represents values referring to sequence similarity. Bootstrap test was performed with 1000 replicates. LP indicates sequence analyzed in the present study. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (scale under figure). Figure S3. Phylogenetic tree created with IQTREE v2.1.3. The evolutionary history was inferred from 16s rRNA sequences of *Alcaligenes faecalis* using the Maximum Likelihood method and the best-fit evolutionary model selected under the Bayesian information based on criteria HKY + F + I. Number in node represents values referring to sequence similarity. Bootstrap test was performed with 1000 replicates. LP indicates sequence analyzed in the present study The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (scale under figure). Figure S4. Phylogenetic tree created with IQTREE v2.1.3. The evolutionary history was inferred from 16s rRNA sequences of three sequences of Bacillus (namely *Bacillus thuringiensis*, *Bacillus cereus* and *Bacillus* sp.) using the Maximum Likelihood method and the best-fit evolutionary model selected under the Bayesian information based on criteria HKY + F. Node number represents values referring to sequence similarity. Bootstrap test was performed with 1000 replicates. LP indicates sequences analyzed in the present study The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (scale under figure).

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References

1. Oberbeckmann, S.; Löder, M.G.; Labrenz, M. Marine microplastic associated biofilms—A review. *Environ. Chem.* **2015**, *12*, 551–562. [CrossRef]
2. Stabnikova, O.; Stabnikov, V.; Marinin, A.; Klavins, M.; Klavins, L.; Vaseashta, A. Microbial life on the surface of microplastics in natural waters. *Appl. Sci.* **2021**, *11*, 11692. [CrossRef]
3. Arkatkar, A.; Arutchelvi, J.; Bhaduri, S.; Uppara, P.V.; Doble, M. Degradation of unpretreated and thermally pretreated polypropylene by soil consortia. *Int. Biodeterior. Biodegrad.* **2009**, *63*, 106–111. [CrossRef]
4. Auta, H.S.; Emenike, C.U.; Fauziah, S.H. Distribution and importance of microplastics in the marine environment: A review of the sources, fate, effects, and potential solutions. *Environ. Int.* **2017**, *102*, 165–176. [CrossRef] [PubMed]
5. Ru, J.; Huo, Y.; Yang, Y. Microbial degradation and valorization of plastic wastes. *Front. Microbiol.* **2020**, *11*, 442. [CrossRef]
6. Katsikogianni, M.; Missirlis, Y.F. Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *Eur. Cell Mater.* **2004**, *8*, 37–57. [CrossRef]
7. Fontanella, S.; Bonhomme, S.; Brusson, J.M.; Pitteri, S.; Samuel, G.; Pichon, G.; Lacoste, J.; Fromageot, D.; Lemaire, J.; Delort, A.M. Comparison of biodegradability of various polypropylene films containing pro-oxidant additives based on Mn, Mn/Fe or Co. *Polym. Degrad. Stab.* **2013**, *98*, 875–884. [CrossRef]
8. Aravinthan, A.; Arkatkar, A.; Juwarkar, A.A.; Doble, M. Synergistic growth of *Bacillus* and *Pseudomonas* and its degradation potential on pretreated polypropylene. *Prep. Biochem. Biotechnol.* **2016**, *46*, 109–115. [CrossRef]
9. Nhon, N.T.T.; Nguyen, N.T.; Hai, H.T.N.; Hien, T.T. Distribution of microplastics in beach sand on the Can Gio coast, Ho Chi Minh City, Vietnam. *Water* **2022**, *14*, 2779. [CrossRef]
10. Artham, T.; Sudhakar, M.; Venkatesan, R.; Nair, C.M.; Murty, K.V.G.K.; Doble, M. Biofouling and stability of synthetic polymers in sea water. *Int. Biodeterior. Biodegradation* **2009**, *63*, 884–890. [CrossRef]
11. Barnes, D.K.; Galgani, F.; Thompson, R.C.; Barlaz, M. Accumulation and fragmentation of plastic debris in global environments. *Phil. Trans. R. Soc. B* **2009**, *364*, 1985–1998. [CrossRef]
12. Umamaheswari, S.; Murali, M. FTIR spectroscopic study of fungal degradation of poly (ethylene terephthalate) and polystyrene foam. *Chem. Eng.* **2013**, *64*, 19159–19164.
13. Mooney, A.; Ward, P.G.; O'Connor, K.E. Microbial degradation of styrene: Biochemistry, molecular genetics, and perspectives for biotechnological applications. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 1–10. [CrossRef]
14. Matusinovic, Z.; Wilkie, C.A. Fire retardancy and morphology of layered double hydroxide nanocomposites: A review. *J. Mater. Chem.* **2012**, *22*, 18701–18704. [CrossRef]
15. Raddadi, N.; Fava, F. Biodegradation of oil-based plastics in the environment: Existing knowledge and needs of research and innovation. *Sci. Total Environ.* **2019**, *679*, 148–158. [CrossRef]
16. Lucas, N.; Bienaime, C.; Belloy, C.; Queneudec, M.; Silvestre, F.; Nava-Saucedo, J.E. Polymer biodegradation: Mechanisms and estimation techniques. *Chemosphere* **2008**, *73*, 429–442. [CrossRef]
17. Dussud, C.; Ghiglione, J.-F. Bacterial degradation of synthetic plastics. In *Marine Litter in the Mediterranean and Black Seas*; Briand, F., Ed.; CIESM Publisher: Paris, France, 2014; Volume 46, pp. 49–54.
18. Besley, A.; Vijver, M.G.; Behrens, P.; Bosker, T. A standardized method for sampling and extraction methods for quantifying microplastics in beach sand. *Mar. Pollut. Bull.* **2017**, *114*, 77–83. [CrossRef]
19. Murray, M.G.; Thompson, W.F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **1980**, *8*, 4321–4325. [CrossRef]
20. Lee, J.; Ren, X.; Yu, H.W.; Kim, S.J.; Kim, I.S. Membrane biofouling of seawater reverse osmosis initiated by sporogenic *Bacillus* strain. *Environ. Eng. Res.* **2010**, *15*, 141–147. [CrossRef]
21. Bernbom, N.; Ng, Y.Y.; Kjelleberg, S.; Harder, T.; Gram, L. Marine bacteria from Danish coastal waters show antifouling activity against the marine fouling bacterium *Pseudoalteromonas sp. strain S91* and zoospores of the green alga *Ulva australis* independent of bacteriocidal activity. *Appl. Environ. Microbiol.* **2011**, *77*, 8557–8567. [CrossRef]

22. Rozewicki, J.; Li, S.; Amada, K.M.; Standley, D.M.; Katoh, K. MAFFT-DASH: Integrated protein sequence and structural alignment. *Nucleic Acids Res.* **2019**, *47*, W5–W10. [CrossRef] [PubMed]
23. Okonechnikov, K.; Golosova, O.; Fursov, M. Unipro UGENE: A unified bioinformatics toolkit. *Bioinformatics* **2012**, *28*, 1166–1167. [CrossRef]
24. Trifinopoulos, J.; Nguyen, L.T.; von Haeseler, A.; Minh, B.Q. W-IQ-TREE: A fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res.* **2016**, *44*, W232–W235. [CrossRef] [PubMed]
25. Kalyaanamoorthy, S.; Minh, B.Q.; Wong, T.K.F.; von Haeseler, A.; Jermiin, L.S. ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat. Methods* **2017**, *14*, 587–589. [CrossRef] [PubMed]
26. Hoang, D.T.; Chernomor, O.; von Haeseler, A.; Minh, B.Q.; Vinh, L.S. UFBoot2: Improving the ultrafast bootstrap approximation. *Mol. Biol. Evol.* **2018**, *35*, 518–522. [CrossRef]
27. Hillis, D.M.; Bull, J.J. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. *Syst. Biol.* **1993**, *42*, 182–192. [CrossRef]
28. Dubois, M.; Gilles, K.; Hamilton, J.K.; Rebers, P.A.; Smith, F. A colorimetric method for the determination of sugars. *Nature* **1951**, *168*, 167. [CrossRef]
29. Lee, J.W.; Nam, J.H.; Kim, Y.H.; Lee, K.H.; Lee, D.H. Bacterial communities in the initial stage of marine biofilm formation on artificial surfaces. *J. Microbiol.* **2008**, *46*, 174–182. [CrossRef]
30. McCormick, A.R.; Hoellein, T.J.; London, M.G.; Hittie, J.; Scott, J.W.; Kelly, J.J. Microplastic in surface waters of urban rivers: Concentration, sources, and associated bacterial assemblages. *Ecosphere* **2016**, *7*, e01556. [CrossRef]
31. Cholewinska, P.; Moniuszko, H.; Wojnarowski, K.; Pokorny, P.; Szeligowska, N.; Dobicki, W.; Polechonski, R.; Górniak, W. The occurrence of microplastics and the formation of biofilms by pathogenic and opportunistic bacteria as threats in aquaculture. *Int. J. Environ. Res. Public Health* **2022**, *19*, 8137. [CrossRef]
32. Harrison, J.P.; Schratzberger, M.; Sapp, M.; Osborn, M.A. Rapid bacterial colonization of low-density polyethylene microplastics in coastal sediment microcosms. *BMC Microbiol.* **2014**, *14*, 232–247. [CrossRef]
33. Mazurkiewicz, M.; Martinez, P.S.; Konwent, W.; Deja, K.; Kotwicki, L.; Węsławski, J.M. Plastic contamination of sandy beaches along the southern Baltic–a one season field survey results. *Oceanologia* **2022**, *64*, 769–780. [CrossRef]
34. Costa, L.L.; Fanini, L.; Ben-Haddad, M.; Pinna, M.; Zalmon, I.R. Marine litter impact on sandy beach fauna: A review to obtain an indication of where research should contribute more. *Microplastics* **2022**, *1*, 554–571. [CrossRef]
35. Thiel, M.; Hinojosa, I.A.; Miranda, L.; Pantoja, J.F.; Rivadeneira, M.M.; Vásquez, N. Anthropogenic marine debris in the coastal environment: A multi-year comparison between coastal waters and local shores. *Marine pollution bulletin* **2013**, *71*, 307–316. [CrossRef]
36. Khambhaty, Y.; Plumb, J. Characterization of bacterial population associated with a brackish water desalination membrane. *Desalination* **2011**, *269*, 35–40. [CrossRef]
37. Ferguson, R.L.; Buckley, E.N.; Palumbo, A.V. Response of marine bacterioplankton to differential filtration and confinement. *Appl. Environ. Microbiol.* **1984**, *47*, 49–55. [CrossRef]
38. Pollock, T.J.; Armentrout, R.W. Planktonic/sessile dimorphism of polysaccharide-encapsulated sphingomonads. *J. Ind. Microbiol. Biotechnol.* **1999**, *23*, 436–441. [CrossRef] [PubMed]
39. Hasegawa, M.; Kishino, K.; Yano, T. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* **1985**, *22*, 160–174. [CrossRef]
40. Ettoumi, B.; Guesmi, A.; Brusetti, L.; Borin, S.; Najjari, A.; Boudabous, A.; Cherif, A. Microdiversity of deep-sea Bacillales isolated from Tyrrhenian sea sediments as revealed by ARISA, 16S rRNA gene sequencing and BOX-PCR fingerprinting. *Microbes Environ.* **2013**, *28*, ME13013. [CrossRef]
41. Khoramnejadian, S. Microbial degradation of starch based polypropylene. *J. Pure. Appl. Microbiol.* **2013**, *7*, 2857–2860.
42. Moore, C.J. Synthetic polymers in the marine environment: A rapidly increasing, long-term threat. *Environ. Res.* **2008**, *108*, 131–139. [CrossRef]
43. Lobelle, D.; Cunliffe, M. Early microbial biofilm formation on marine plastic debris. *Mar. Pollut. Bull.* **2011**, *62*, 197–200. [CrossRef] [PubMed]
44. Miri, S.; Saini, R.; Davoodi, S.M.; Pulicharla, R.; Brar, S.K.; Magdouli, S. Biodegradation of microplastics: Better late than never. *Chemosphere* **2022**, *286*, 131670. [CrossRef] [PubMed]
45. Käppler, A.; Fischer, D.; Oberbeckmann, S.; Schernewski, G.; Labrenz, M.; Eichhorn, K.J.; Voit, B. Analysis of environmental microplastics by vibrational microspectroscopy: FTIR, Raman or both? *Anal. Bioanal. Chem.* **2016**, *408*, 8377–8391. [CrossRef] [PubMed]
46. Muhonja, C.N.; Makonde, H.; Magoma, G.; Imbuga, M. Biodegradability of polyethylene by bacteria and fungi from Dandora dumpsite Nairobi-Kenya. *PLoS ONE* **2018**, *13*, e0198446. [CrossRef]
47. Sol, D.; Laca, A.; Laca, A.; Díaz, M. Approaching the environmental problem of microplastics: Importance of WWTP treatments. *Sci. Total Environ.* **2020**, 140016. [CrossRef]

48. Jaiswal, K.K.; Dutta, S.; Banerjee, I.; Pohrmen, C.B.; Singh, R.K.; Das, H.T.; Kumar, V. Impact of aquatic microplastics and nanoplastics pollution on ecological systems and sustainable remediation strategies of biodegradation and photodegradation. *Sci. Total Environ.* **2022**, *806*, 151358. [CrossRef]
49. Hossain, M.R.; Jiang, M.; Wei, Q.; Leff, L.G. Microplastic surface properties affect bacterial colonization in freshwater. *J. Basic Microbiol.* **2019**, *59*, 54–61. [CrossRef]