microplastics

Communication

Promoting Biofilm Formation by *Serratia marcescens* on Three Types of Artificially Aged Microplastics Under Marine Conditions

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Abstract

Bacterial biofilms on different types of microplastics in aquatic environments have become an increasing ecological and public health concern. In this context, this study investigated biofilm formation on virgin and aged microplastics under marine conditions. *Serratia marcescens* biofilm formation was observed on both virgin and aged polyethylene particles after 7 days, with no significant changes by day 14. Concerning polypropylene microplastics, biofilms developed on aged particles but were not detectable on virgin particles, likely due to interference from the polypropylene red color matching *S. marcescens* cells. In contrast, expanded polystyrene spheres showed an initial biofilm formation that dissipated by day 14, potentially due to toxic residues from photooxidation, including potential styrene monomers and other chemical additives, inhibiting biofilm persistence. These findings indicate differences in biofilm formation across microplastics types, which may influence microplastic buoyancy and ecological impacts. Thus, microplastic color and additives should be considered in future studies on microplastics biofilm formation and biofouling.

Keywords: polyethylene; polypropylene; expanded polystyrene; bacteria; emerging pollutant; biofouling

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1. Introduction

Plastic production has grown exponentially worldwide, generating exorbitant amounts of waste in both coastal and oceanic environments [1]. These include microplastics (MPs), comprising plastic particles which range from 1 µm to 5 mm in size [2]. These are categorized as primary microplastics when originating from industrial or domestic products (i.e., pellets, microbeads, glitters, and personal care products), or secondary microplastics following plastic degradation by abiotic and/or biotic factors [3].

These compounds originate from different sources (e.g., airborne dust, landfill leachate, food waste, and effluents from water/wastewater treatment plants) [3] and exhibit different transport behaviors and aquatic organism effects [4] following size reductions under environmental conditions, reaching humans mainly through the consumption of contaminated seafood [5]. Several types of MPs are released into the marine environment and can be transported for long distances, especially those that display floating properties, such as polyethylene (PE), polypropylene (PP), and polystyrene (PES), while polyvinyl chloride (PVC), nylons (polyamide), and polyethylene terephthalate (PET) are most likely to sink in marine environments [6].

The physicochemical characteristics of MPs, such as hydrophobicity, density, size, functional groups, surface area, and roughness, can be altered by the formation of surface microorganism biofilms [7]. Biofilms formed on MP particles are, in fact, one of the main factors responsible for their transport dynamics and the sinking of small particles, as well as the assimilation of pollutants, such as organic pollutants, metals, and pathogenic organisms [7,8], in both marine and freshwater environments [9].

Biofilms are formed by a set of cells, usually bacterial, that produce extracellular polymeric substances (EPS), encompassing a macromolecular complex that allows for the adhesion of other microorganisms (e.g., algae and fungi), microfauna, and contaminants, that together establish a biofouling process capable of considerably increasing plastic particle biomasses, forming the so-called plastisphere [10,11]. The aging of microplastics can directly affect biofilm formation [12,13], where aged MPs exhibit modified physicochemical characteristics (particle size, surface area, crystallinity, chemical structure, hydrophobicity) [12]. Such changes caused by aging generate the formation of microcracks on their surface (promoted by weathering) and increased oxidation in some C-C and C-H bonds (caused by photooxidation) [14], thus increasing interactions (hydrogen bonds, electrostatic interactions, among others) with hydrophilic organic compounds and metals and the adhesion of biofilm-forming microorganisms [9,15].

In this sense, this study employed virgin and aged PE, PP, and expanded polystyrene (exPS) MP particles under different weathering conditions to assess biofilm formation in direct contact with *Serratia marcescens*, an opportunistic biofilm-forming enterobacterium [16,17].

2. Materials and Methods

2.1. Materials

The following materials were employed herein: 1 mm PE MPs; 4.95 mm PP (Cospheric LLC, Santa Barbara, CA, USA); 2–4 mm exPS; filtered seawater (filtered through 20 µm filters); thermometer; UV lamp (UV-A 340 nm); 100 mL glass beakers; sterile seawater and non-sterile seawater obtained from the Atlantic Ocean (Caxias, Oeiras, Portugal). A bacterial suspension of *S. marcenscens*, isolated from a sample collected in the Azores (Portugal) as previously reported [18], was used at ca. 4 g cells L^{-1}. The strain is stored and maintained at the iBB-Institute for Bioengineering and Biosciences, IST, Universidade de Lisboa, Portugal.

2.2. Microplastic Aging Assay

An MP aging assay was carried out according to Andrade et al. (2019) [19] for 60 days employing an aging chamber to promote physicochemical MP changes mimicking natural environmental aging conditions in seawater employing virgin MPs. Briefly, a 340 nm UV-A lamp was placed in a 25 L glass reservoir, with a cardboard lid, and all sides were covered with layers of aluminum foil (Figure A1). A 12/12 h photoperiod was set using a lamp (Figure A1) for 60 days. The plastics were weighed before and after the aging period through exposure to seawater for 60 days.

2.3. Biofilm Formation Assay

After the aging period, the MPs were dried, weighed, and placed in 100 mL beakers, with a working volume of 80 mL in different seawater compositions (sterile, fresh, and alongside a medium culture containing *S. marcescens*). Solution temperatures were measured daily, and the experiments were monitored for 14 days. The MP samples were observed by microscopy under a brightfield and fluorescence light employing an Olympus CX40 microscope, equipped with an Olympus U-RFL-T burner and a U-MWB mirror cube unit (excitation filter: BP450-480; barrier filter: BA515) at days 0, 7, and 14. Images were captured by an Evolution^{TM} MP5.1 CCD color camera employing the software Image-Pro Plus v5.1, both from Media Cybernetics, Inc. (Rockville, MD, USA).

To assess biofilm formation, the three types of plastics were exposed to sterile seawater, non-sterile seawater, and a *Serratia marcescens* bacterial cell suspension at 4 g L^{-1} for 14 days at room temperature, mixing 80 mL of filtered seawater and MPs in 100 mL graduated cylinder beakers, as follows: 300 mg of PE spheres (density 0.96 $g.cc^{-1}$); 5 mg of PP (density 0.9 $g.cc^{-1}$), and 100 mg of 1–2 mm exPS spheres. All assays were performed in triplicate (Figure A2). Each cylinder beaker was filled with 80 mL of each solution and covered with food wrap film. The MPs were observed by brightfield and fluorescence microscopy to assess biofilm formation on the 7th and 14th experimental days.

2.4. Statistical Analyses

The statistical analyses were carried out using Excel 365 Office 365 Microsoft® software. A contingency table and Chi-square test ($p < 0.05$) were applied to the experimental data. The control with seawater was not considered in the statistical tests. The Chi-square test was conducted to test the following hypotheses:

H_0: *The presence and absence of biofilm in microplastics are independent of the presence of Serratia marcescens.*

H_1: *The presence and absence of biofilm in microplastics are not independent of the presence of Serratia marcescens.*

3. Results

The microplastic aging test took place over 60 days in the seawater. The three types of tested MPs showed weight gain after the aging period, with a PE weight increase of 30.82%, a PP increase of 2.03%, and an exPS increase of 209% (Table 1). In particular, exPS showed a significant increase, probably related to aging. The exPS used in this study is a commercial product used as a thermal insulator and resistant to compressive stress due to its physical characteristics (lightness, relative strength, and thermal and electrical insulation). These MPs can exhibit effects when exposed to sunlight. In experiments with three types of exPS (gray polystyrene, white polystyrene, and exPS), gray PS and exPS were noted as absorbing water (44% and 60% weight gain, respectively) [20]. Furthermore, UV radiation has been shown to reduce molecular mass by breaking the polymeric chain and increasing crystallinity. Hardening of the surface layer causes surface degradation, creating small cracks and brittleness, and these damaged areas can then absorb water more easily through capillary action [21].

Table 1. Weight values of the MPs employed herein at the beginning and end of the UV-A aging process.

Microplastics	Before Aging (g)	After Aging (g)
PE	4.12	5.39
PP	2.93	2.99
exPS	0.53	1.64

All MPs floated from the beginning to the end of the analysis. *Serratia marcescens* bacterial cells formed a biofilm on the surface of the MP particles, mainly on virgin and aged PE, after the first 7 days (Table 2). Biofilm formation was also noted on the PP and exPS virgin and aged spheres (Table 2).

Table 2. Virgin and aged polyethylene, polypropylene, and expanded polystyrene microplastics observed under brightfield (BF) and fluorescence (FL) microscopy after 7 days of *Serratia marcescens* exposure.

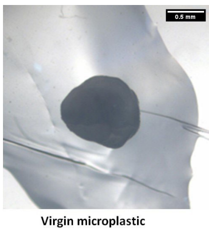
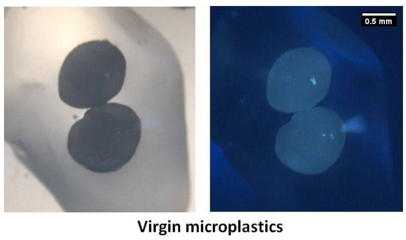
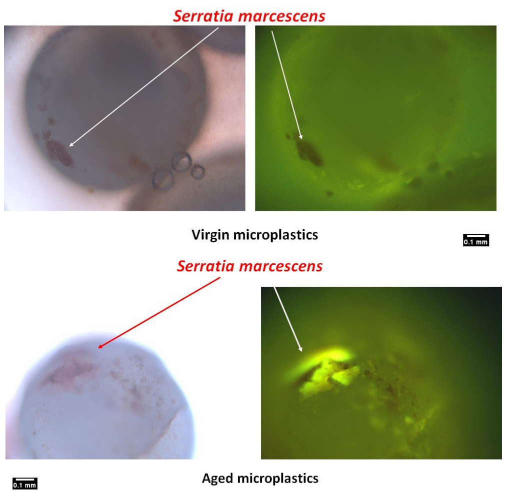
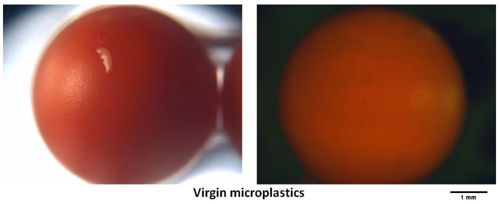
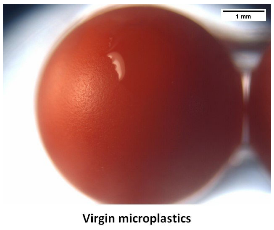
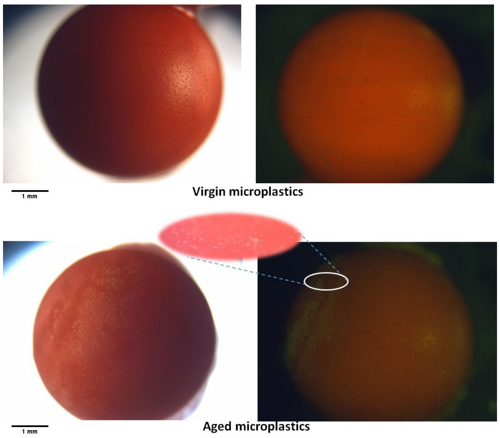
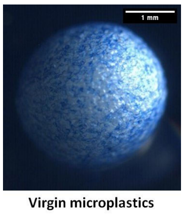
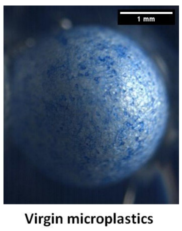
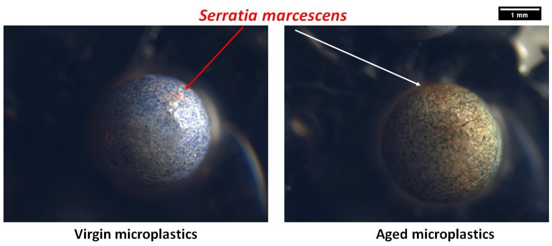
Microplastic Type	Images	Notes
Polyethylene (Control)		No biofilm formation; BF; amplification: 600×
Polyethylene (Control seawater)		No biofilm formation; BF and FL; amplification: 600×
Polyethylene (*Serratia marcescens*)		Biofilm formation; BF and FL; amplification: 1500×

Table 2. *Cont.*

Microplastic Type	Images	Notes
Polypropylene (Control)		No biofilm formation; BF and FL; amplification: 600×
Polypropylene (Control Seawater)		No biofilm formation; BF; amplification: 600×
Polypropylene (*Serratia marcencens*)		Slight biofilm formation; BF and FL; amplification: 600×
Expanded Polystyrene (Control, virgin)		No biofilm formation; BF; amplification: 600×
Expanded Polystyrene (Control Seawater, virgin)		No biofilm formation; BF; amplification: 600×
Expanded Polystyrene (exPS) (*Serratia marcescens*)		Biofilm formation; BF; amplification: 600×

Several changes were observed after 14 days. The PE beads exhibited bacterial biofilm formation at a rate similar to that of the first 7 days. The virgin and aged PP particles showed biofilms detectable under fluorescence microscopy, while the exPS spheres did not present biofilms detectable by fluorescence microscopy on both virgin and aged particles (Table 3).

Table 3. Virgin and aged polyethylene, polypropylene, and expanded polystyrene microplastics observed under brightfield (BF) and fluorescence (FL) microscopy after 14 days of *Serratia marcescens* exposure.

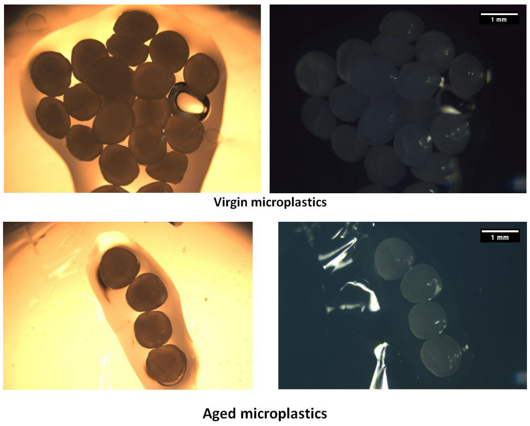
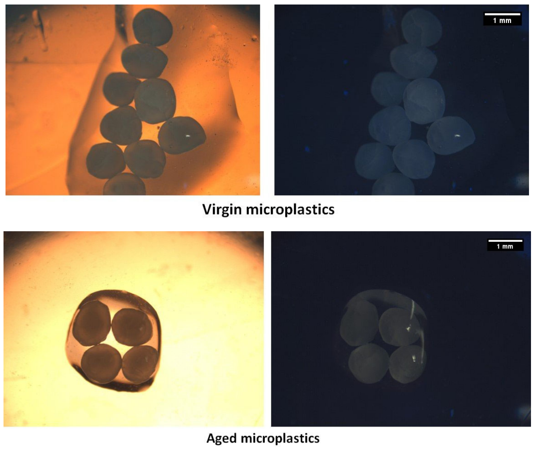
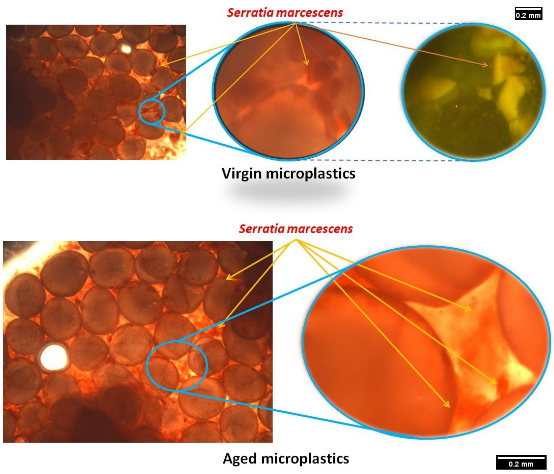
Microplastic Type	Images	Notes
Polyethylene (Control)		No biofilm formation; BF and FL; amplification: 600×
Polyethylene (Control Seawater)		No biofilm formation; BF and FL; amplification: 600×
Polyethylene (*Serratia marcencens*)		Biofilm formation; BF and FL; amplification: 600 and 1500×

Table 3. *Cont.*

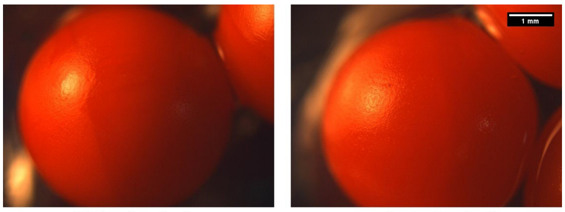
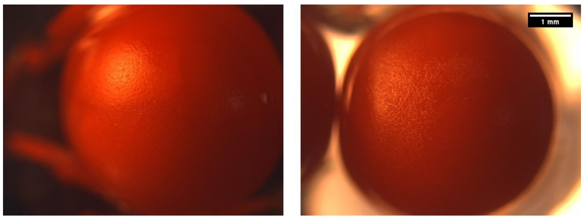
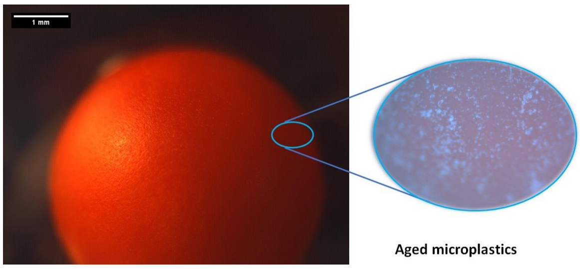
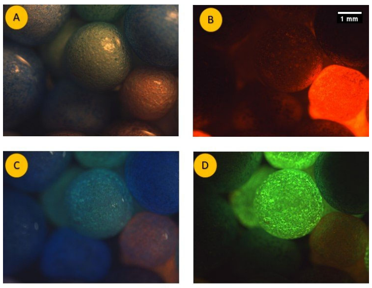
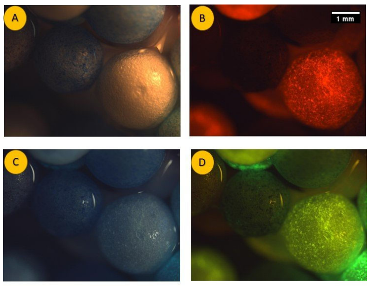
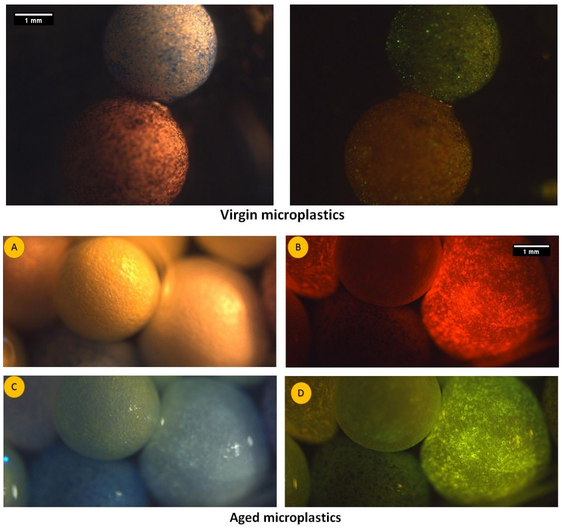
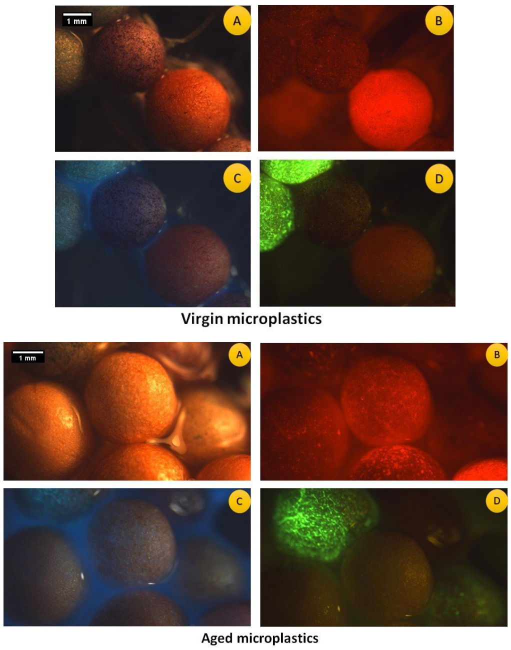
Microplastic Type	Images	Notes
Polypropylene (Control)	 Virgin microplastics Aged microplastics	No biofilm formation; BF; amplification: 600×
Polypropylene (Control Seawater)	 Virgin microplastics Aged microplastics	No biofilm formation; BF; amplification: 600×
Polypropylene (*Serratia marcencens*)	 Aged microplastics	Slight biofilm formation; BF and FL; amplification: 600 and 1500×
Expanded Polystyrene (Control)	 Virgin microplastics  Aged microplastics	No biofilm formation; BF (A) and FL (B, C and D); amplification 300×

Table 3. *Cont.*

Microplastic Type	Images	Notes
Expanded Polystyrene (Control seawater)		No biofilm formation; BF (A) and FL (B, C and D); amplification 300×
Expanded Polystyrene (*Serratia marcencens*)		No biofilm formation; BF (A) and FL (B, C and D); amplification 300×

For PE and PP microplastics, biofilm formation was observed, while for exPS this association could only be observed during the first 7 days (Table 4).

Table 4. Chi-square values for each of the microplastic types evaluated herein.

Type of Microplastic	One Week (7 Days)		*p*-Value *	Df
Virgin	Control	Polyethylene	0.014306	4
		Polypropylene	0.014306	4
		Expanded Polystyrene	0.014306	4
Aged	Control	Polyethylene	0.014306	4
		Polypropylene	0.014306	4
		Expanded Polystyrene	0.014306	4

Table 4. *Cont.*

Microplastics	Two weeks (14 days)		*p*-Value *	Df
Virgin	Control	Polyethylene	0.014306	4
		Polypropylene	0.014306	4
		Expanded Polystyrene	n.d.	4
Aged	Control	Polyethylene	0.014306	4
		Polypropylene	0.014306	4
		Expanded Polystyrene	n.d.	4

* When *p*-value < 0.05, the null hypothesis was rejected, indicating a statistically significant association between the presence and absence of biofilm. Df: degrees of freedom; n.d.: not determined.

4. Discussion

The surface of MPs serves as a support for the colonization of biofilm-forming microorganisms [22]. However, the level of MP weathering through abrasion, chemical oxidation, light radiation, and biodegradation processes [23], as well as abiotic and biotic factors, all play a role in the level of biofilm formation, as weathering leads to changes in particle size, crystallinity, thermodynamic properties, and functional surface chemical groups [14], making MPs more susceptible to organic and inorganic pollutant adsorption and further biodegradation [24]. In this sense, artificial aging methodologies conducted in laboratory conditions reproducing environmental factors responsible for aging, such as mechanical fragmentation, UV photo-oxidation, mechanical abrasion, hydrolysis, and biodegradation [14], can aid in assessing long-term behavior under weathering conditions [25].

Bacterial biofilms are formed in all environments, especially in aquatic systems [26], and are affected by polymer type, roughness, hydrophobicity, and surface area, and environmental factors such as salinity, temperature, nutrients, and geographic location [27]. Initially, microorganisms adhere to the surface of MPs, followed by the release of EPS, which aid in bacterial adhesion and protection from abiotic factors, leading to bacterial proliferation [7]. Herein, a low-cost system was applied to MP weathering [19], considering both hydrolytic and photooxidative processes employing *S. marcescens* cells, known to form biofilms [28]. Fluorescence microscopy is highly reproducible for quantifying MPs in sediments and water, both with and without the aid of fluorescent dyes such as Nile red [29–31]. In this case, dye-free biofilm identification was carried out, employing only natural cell fluorescence on MP particles.

Microplastic shapes can not only affect ecotoxicological MP characteristics but also biofilm formation and organismic interactions [22]. In this sense, the sphere format is less efficient concerning biofilm formation, although heightened EPS production is noted when compared to other MP forms, such as fragments and films [22]. Concerning the PE spheres employed herein, these are hydrophobic, opaque, and white with no fluorescence emission. A bacterial biofilm was visible after 7 days and was maintained for up to 14 days, and all particles remained floating during the experiments. In contrast, one study on high-density PE particles subjected to abiotic weathering alongside *Pseudomonas* sp. and *Lysinibacillus* sp. strains reported low biofilm formation after 24 h of exposure, but high EPS production [32]. In another study, plastics from PE bags were exposed to seawater for three weeks to assess biofilm formation and plastic fluctuation [33]. After three weeks, the plastic began to sink, and biofilms were formed, decreasing MP hydrophobicity [33].

The PP MPs employed herein are red, hindering microscopic observations, as *S. marcescens* cells exhibit the same color. Even so, *S. marcescens* biofilms were observed in aged PP MPs on day 7 and on day 14. Similarly, Candlen et al. [34] carried out a 16-week experiment to observe biofilm growth on PP particles and their association with plastic degradation in three situations, employing filtered water, freshwater, and seawater. The seawater samples contained Proteobacteria (identified by 16S rRNA sequencing analyses), thus forming biofilms more quickly and degrading the analyzed plastic, as confirmed by the detection of polyolefin on the MP surfaces [34]. In another study, Shan et al. [13] exposed virgin and aged PP fragments to river, estuarine, and marine environments for six weeks. Aged MPs showed greater biofilm growth during the study period. In addition, river water samples showed higher biofilm biomass than the estuarine and marine water samples. The authors also reported an increase in pathogenic bacteria and bacteria involved in geochemical nutrient cycling in aged MPs [13]. This indicates the importance of aged MPs as a vector for potentially pathogenic and zoonotic bacteria [35,36]. Finally, in another study, PP pellets were exposed to different environmental conditions, and MPs were exposed to UV-B from 0.5 to 1.5 years, presenting more surface cracks and greater production of carbonyl and hydroxyl chemical groups, promoting biofilm formation [37].

The exPS MPs employed herein are widely used in food, shipping, and electrical appliance packaging [38] and are a commercial product, containing additives. Herein, both virgin and aged exPS MP spheres exhibited a visible *S. marcescens* biofilm after 7 days, which disappeared after 14 days. Unlike the other MPs (PE and PP), the exPS MPs were of different colors (white, pink, blue, and purple), hindering fluorescence assessments. Despite this issue, higher biofilm formation was observed in blue spheres in the first week (7 days), and a change in sphere color was noted in aged MPs, due to photooxidation [39]. On day 14, no biofilms were detected, indicating that the surfaces of the analyzed exPS MPs, both virgin and aged, are not a suitable substrate for bacterial development, possibly due to the production of the styrene monomer, which is cytotoxic [40]. Furthermore, as the employed exPS is a commercial product, photooxidation residues and exPS additives may be released during the photooxidation process, such as flame retardants, plasticizers, UV stabilizers, antioxidants, and other toxic chemicals [41], inhibiting biofilm formation. Chemical residues from the photooxidation (UV-A and UV-B) of exPS were, in fact, found to be cytotoxic to human keratinocytes (HaCaT) in 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assays [42].

5. Conclusions

The three employed MPs analyzed herein remained floating during the entire experimental period, indicating unaltered floatability even following biofilm formation. Variability in microbial colonization was observed across MP types, where biofilms formed readily on PE and PP, with a stronger presence on aged particles, while exPS, a commercial product, showed an initial biofilm formation that disappeared by day 14. The observed biofilm inhibition on exPS may be due to the release of cytotoxic compounds, such as styrene monomers and other photooxidation byproducts, indicating that exPS has unique chemical properties that impact biofilm persistence, suggesting that biofilm formation is influenced not only by MP type and aging processes but also by the release of potentially toxic additives. Future studies aiming to monitor how biofilms affect MP buoyancy should assess both floating MPs, such as PE, PP, and PS, as well as MPs with lower buoyancy (e.g., polyvinyl chloride (PVC) and polyethylene terephthalate (PET). Additionally, the ecotoxicological effects of these MPs should also be investigated, especially concerning biofilm impacts on MP bioaccumulation in aquatic organisms, particularly filter feeders like marine bivalves.

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Appendix A

Figure A1. Apparatus employed to age microplastics. (**A**)—aging chamber containing UV-A 340 lamp; (**B**)—internal view of the chamber and the 340 nm UV-A lamp; (**C**)—view of the open chamber in operation.

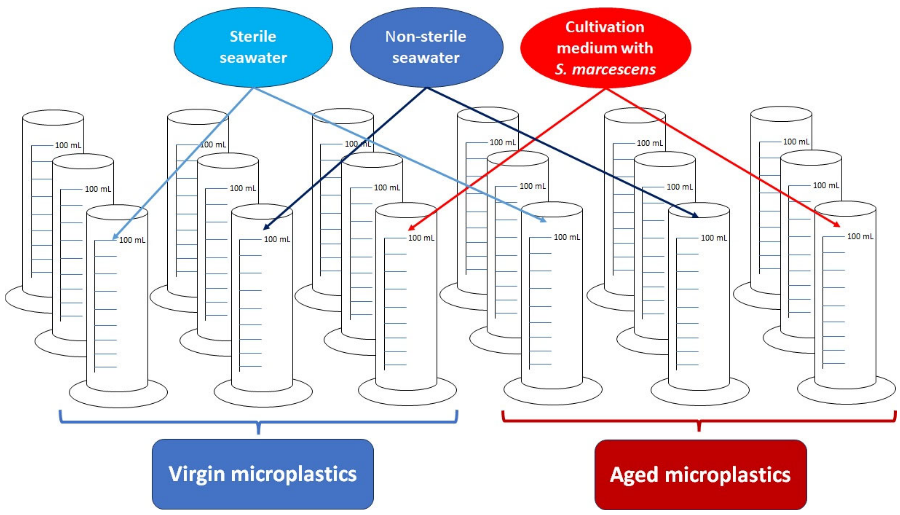

Figure A2. Experimental design employed in the polyethylene, polypropylene, and expanded polystyrene aging assay in seawater.

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