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Biofilm formation on polyethylene and polylactic acid microplastics in freshwater: Influence of environmental factors

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ABSTRACT

To overcome the environmental challenges posed by conventional plastics, new alternative plastic materials are being developed and increasingly used. However, their fate in the natural environment is still poorly understood. This study compared biofilm formation on polyethylene (PE) and polylactic acid (PLA) microplastics (MPs) under different conditions i.e. water regimes (static and moving conditions), nutrient levels (excess of nitrogen and phosphorus), light exposure (light and dark), and pollution levels (presence and absence of wastewater). Although PE and PLA differ in their composition and physico-chemical properties, the processes of aging and biofilm formation on both materials exhibited many similarities. Light availability had the strongest influence, as the formation of biofilm on both PE and PLA was suppressed in the dark, as shown by reduced biomass, chlorophyll *a* content, and extracellular polymeric substances (EPS). The availability of nutrients influenced the composition of the biofilm. High N:P ratios favoured photosynthetic microorganisms with increased chlorophyll *a* and EPS content, while phosphorus enrichment reduced their presence. Pollution, simulated by adding sterilized municipal wastewater, slightly reduced total biofilm mass but supported the growth of photosynthetic microorganisms. Static condition led to the formation of elongated microbial structures. Biofilm growth altered the density of MPs (increasing for PE, decreasing for PLA), reaching ~1.1 g/cm^3 for both, suggesting biofilm-induced sedimentation. Crystallinity of both PE and PLA decreased by 9–30 % due to the presence of biofilm, which has amorphous structure. The multivariate analysis confirmed environmental factors as primary drivers of biofilm development over polymer type.

1. Introduction

Microplastics (MPs), i.e. plastic particles from 1 μm to 1000 μm in size [1], are persistent pollutants that present challenges for environmental management due to their negative effects and long-term stability in the environment [2]. MPs, once introduced or formed, are dispersed across various ecosystems—including oceans, freshwater systems, soil, and even glaciers—through natural processes such as wind and water currents [3,4]. When MPs enter the aquatic environment, those with densities lower than water tend to accumulate on the water surface, whereas denser particles are subject to deposition and resuspension, leading to their accumulation in sediments or continued downstream transport [5] via rivers and streams. In coastal regions MPs can accumulate through tidal and current-driven deposition [6] and influence sediments through direct interference with microbial networks [7].

However, at the same time when MPs enter the aquatic environment, they are rapidly colonized by microorganisms, resulting in the formation of a biofilm that alters their physico-chemical properties [8,9]. Biofilm also facilitates the adsorption of pollutants and enhances the potential of MPs to act as vectors, transporting contaminants and associated microorganisms through aquatic systems [10]. Biofilm contains diverse microbial communities, including autotrophs, heterotrophs, predators, and symbionts [11]. The development of the biofilm is influenced by various factors, including the type of plastic substrate, environmental conditions, and colonization time [11]. Plastic properties such as polymer type, size, colour, shape, and surface characteristics contribute to

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the selective formation of initial microbial communities [12]. For instance, different bacterial species exhibit a preference for specific types of MPs, with polystyrene (PS) hosting *Pseudoalteromonas* and *Alteromonas*, polyethylene (PE) hosting *Faecalibacterium* and *Veillonella*, polypropylene (PP) hosting *Bdellovibrio* and *Lewinellafor*, and polyvinyl chloride (PVC) hosting *Bacillus* spp. and *Shewanella* [13-15].

In addition, MPs with functional groups, such as aromatic rings, can promote bacterial adhesion and biofilm formation [16]. Surface roughness facilitates microbial adhesion and provides additional sites for nutrient sorption [17], and the hydrophilicity of MPs has also been positively correlated with bacterial affinity [18]. Additionally, MPs colour influences bacterial community structure and biofilm functional diversity [19], whereas their size and shape appear to have a lesser impact on biofilm formation [13,16]. However, certain shapes, such as uniform spherical MPs, are less favourable for microbial attachment and subsequent biofilm formation [20,21].

It is also important to consider the environmental conditions as they may play an important role in the formation and development of the biofilm [22]. In fact, it has been estimated that environmental factors can contribute significantly to biofilm formation, accounting for up to 31 %, compared to the 16 % attributed to the properties of the MPs themselves [23]. Salinity is a major factor in marine environments, influencing biofilm formation [24,25]. However, in freshwater ecosystems, temperature, pH, light, and nutrient availability are also important. For instance, higher concentrations of phosphorus (P) and nitrogen (N) have been positively correlated with increased biofilm formation on MPs and leading to faster biofilm development and the emergence of specific microbial communities [26,23,27]. Additionally, temperature plays a significant role, with greater biomass and faster growth rates observed in summer compared to fall and winter [28]. Temperature also affects biofilm diversity, indicating its influence on biofilm community composition. In contrast, it is negatively correlated with biofilm density [29]. Similarly, light influences both the thickness and volume of biofilm. Increased light availability leads to surface thickening, elevated gross primary production, and enhanced P uptake. In contrast, under low-light conditions, biofilms exhibit increased expression of photosynthesis-related mRNA, indicating physiological adaptation to limited light. Additionally, light availability affects species composition, favouring phototrophic organisms under high-light conditions [30,31]. While the effects of environmental factors have been studied [23,24], a comprehensive evaluation of their individual impacts on biofilm formation remains lacking.

This study aims to address this gap by systematically investigating biofilm formation on different MPs substrates under varied conditions in stream water in a laboratory setting. Specifically, the formation of biofilm on conventional plastic material polyethylene (PE) and polylactic acid (PLA), which is used as biodegradable alternative under different conditions were compared: i) water regimes (static, moving), ii) nutrient levels (N, P), iii) light regimes (light, dark), and iv) levels of pollution (presence and absence of wastewater). We focused on the most influential environmental factors that determine the formation of biofilms on PE and PLA and how the biotic aging can alter their physico-chemical properties. Biofilm development was evaluated using several parameters, including biofilm mass, chlorophyll *a* content, extracellular polymeric substances (EPS) concentration, density and crystallinity, and changes in surface morphology. To our knowledge, this is one of the more comprehensive studies investigating the effects of a wide range of environmental factors on biofilm growth on MPs under controlled conditions. The results will improve our understanding of the aging processes of MPs and consequently the behaviour and fate of MPs made from conventional polymers such as PE and new alternatives such as PLA.

2. Materials and methods

2.1. Preparation of microplastics

Polyethylene (PE) MPs were prepared by grinding low-density PE pellets (Sigma Aldrich, USA). The pellets were ground at 8000 rpm on a Retsch ZM 300 centrifugal mill through a 1 mm stainless steel sieve. During grinding, the sample and the mill were cooled with dry ice. The particles were then sieved through a sieve with a mesh size of 800 μm and only particles smaller than the mesh size of a sieve were used in the study. Polylactic acid (PLA) MPs were produced from plastic cutlery by grinding and sieving in the same way as PE. Both MPs were characterized in terms of their chemical composition and particle size distribution.

The surface chemical composition of unaged MPs was determined by Fourier-transform infrared spectroscopy (FTIR) using a Lumos II FTIR imagining microscope (Bruker, USA) with an ATR mode and an MCT detector, cooled with liquid nitrogen. The wavenumber ranged from 4000 cm^{-1} to 599 cm^{-1} (resolution 4 cm^{-1}, 64 scans). FTIR analysis confirmed that the materials were PE and PLA, as stated. The FTIR spectra of both materials can be found in the supplementary information (Fig. S1).

The particle size distribution of unaged MPs (Fig. S2) was determined with the laser diffraction analyzer (Microtrac S3500 Bluewave, USA), whereby the measurement was repeated three times with the dry unit. The mean particle size was 184.0 ± 121.4 and 125.3 ± 65.9 μm for PE and PLA, respectively.

2.2. Experimental design

The simulation of the environmental aging of PE and PLA was carried out using natural stream water as a medium. The water samples were taken using the grab method at mid-depth of the Glinščica stream (46°03'03.5 "N, 14°28'07.1 "E). Immediately after sampling, the temperature, pH, and dissolved oxygen concentration were measured, then the water samples were stored at −18 ± 2 °C and subsequently other physico-chemical parameters were determined (details are given in subchapter 2.3.7).

Aging was carried out in 250 mL Erlenmeyer flasks covered with cellulose plugs, which prevented the introduction of impurities into the sample and allowed gas transfer. Each Erlenmeyer flask contained 200 mL of stream water and 1 g/L MPs. Five replicates were prepared for each experimental condition to ensure a sufficient amount of MPs for the final analysis. All MPs were aged simultaneously but under different conditions: the MPs were incubated under conventional aging conditions that has been used for aging of MPs in our previous studies [32,20,21]. Briefly, the incubation was conducted using laboratory shakers (Labnet Orbit 1900s, USA) set at 160 rpm, room temperature (22 ± 2 °C), and a light/dark cycle of 16/8 h, with a light intensity of approximately 1100 lx (PE-control and PLA-control). Samples were also incubated in the dark to evaluate effects of light on development of microbial community in biofilm. Flasks incubated in the dark were wrapped in aluminium foil to prevent light exposure (PE-dark and PLA-dark). To assess contribution of nutrients on biofilm development, N and P were added to the system. Stock solutions of N and P were prepared with concentrations of 1 g/L for each nutrient, the actual concentration during aging was 10 mg/L of P and N. KNO_3 (Sigma-Aldrich, USA) was used as the nitrogen source (PE-N and PLA-N), while KH_2PO_4 (Sigma-Aldrich, USA) served as the phosphorus source (PE-P and PLA-P). To simulate presence of co-contaminants during biofilm development, wastewater collected from central municipal wastewater treatment plant was used: briefly, wastewater was collected after the primary sedimentation tank. The plant primarily treats municipal wastewater, with approximately 11 % of its inflow coming from industries that discharge wastewater with a composition similar to municipal wastewater [33]. The wastewater was sterilized in an autoclave (Tuttnauer

2840EL steam sterilizer, Israel) and stored at −18 ± 2 °C until use. Then it was thawed to room temperature and added to the treatment (PE-polluted and PLA-polluted). Note, that in this study, we refer to these wastewater-amended samples as "polluted" to distinguish them from those for which only stream water was used. Although not all possible pollutants in the wastewater could be identified due to the complexity and variability of the wastewater composition, the sample can be considered representative municipal wastewater entering natural freshwater systems. The basic physico-chemical parameters were measured and are presented in Table S1. Finally, to evaluate effects of water movement, samples that were incubated without shaking (PE-static and PLA-static) were placed next to the shakers at the same height from the lamps as the other samples to ensure uniform light exposure. Experimental conditions for each treatment are listed in Table 1.

Preliminarily, the development of the biofilm on PE-control and PLA-control samples was monitored on days 14, 21, 26, and 28. During this time period, the stationary growth phase of the biofilm was assessed, and the experiment was terminated upon its attainment (after 4 weeks). Each week, the contents of all Erlenmeyer flasks were filtered with a cellulose filter (pore size 7–12 μm, Macherey-Nagel, Germany) and fresh stream water (with added N, P or wastewater) was replaced. At the end of the experiment, all five replicates of each aged MPs were filtered (cellulose nitrate filter, pore size 0.45 μm, Sartorius, Germany) and mixed into a single sample for further analysis.

2.3. Analysis

2.3.1. Surface characterization

The field emission scanning electron microscope (FE-SEM) Apreo 2 (Thermo Fischer Scientific, USA) at an acceleration voltage of 2 kV was used for the visualization of surface of unaged and aged MPs. Prior to FE-SEM analysis, all MPs samples were dried in a laboratory dryer at 40 ± 2 °C and then coated with a thin Au/Pd layer (approximately 10 nm).

2.3.2. Biofilm mass

The mass of biofilm developed on MPs was determined by calculating the difference between the weight of the aged MPs and the aged MPs after removal of the biofilm by Fenton oxidation [34]. The digestion was performed with four replicates containing approximately 40 mg of aged MPs each. 1 mL Fe^{2+} solution (15 g/L $FeSO_4{\cdot}7\ H_2O$ with 6 mL/L 97 % (*v/v*) H_2SO_4) and 1 mL 30 % (*w/w*) H_2O_2 were added, and oxidation was carried out overnight at room temperature (22 ± 2 °C). The samples were then filtered (cellulose nitrate filters, pore size 0.45 μm, Sartorius Stedim Biotech, Germany), dried to constant mass at room temperature (22 ± 2 °C) and weighed again. The mass of the biofilm that developed on the MPs was used to calculate the amount of biofilm (Eq. 1):

$$B = \frac{m_{aged\ MPs} - m_{MPs}}{m_{aged\ MPs}} \bullet 100 \quad (1)$$

where B is the amount of the biofilm on the aged MPs (%), $m_{aged\ MPs}$ is the mass of the aged MPs before digestion (mg), and m_{MPs} is the mass of the aged MPs after digestion (mg). The effect of Fenton oxidation on both types of pristine MPs was also determined. The weight of PE and PLA was minimally reduced (1.7 ± 2.1 % and 1.3 ± 3.9 %, respectively (n = 10)).

2.3.3. Chlorophyll a

The presence of photosynthetic microorganisms was determined through the measurement of chlorophyll *a* content in the biofilm. Chlorophyll *a* content was determined according to the method described in [20,21], using some modifications. Approximately 50 mg of aged MPs (three replicates) was homogenized with 1.8 mL of cold 95 % (*v/v*) ethanol (−18 ± 2 °C) in a mortar with a pestle. Absorbance of the supernatant was measured at 664 nm and 648 nm (BioTek Synergy LX multi-mode reader, Agilent Technologies, USA). The content of chlorophyll *a* was calculated according to [35], and expressed as mg of chlorophyll *a* per g of biofilm. To ensure that the MPs itself did not interfere with the measurement, the same procedure was applied to MPs without biofilm. The absorbance values were similar to the blank sample (ethanol only).

2.3.4. Extracellular polymeric substances

The biofilm was also evaluated by the presence of extracellular polymeric substances (EPS). EPS are high-molecular compounds that consist primarily of polysaccharides, proteins, lipids, and nucleic acids and are excreted by microorganisms [36]. They form a structural matrix that embeds cells, providing mechanical stability, protection from environmental stress, and facilitating nutrient retention and microbial communication [37]. Their concentration was determined according to [38,39] with some modifications: 3 mL of deionized water was added to approximately 50 mg of the dried aged MPs (three replicates). The samples were incubated for 30 min at 80 ± 1 °C and filtered after cooling to room temperature (0.45 μm pore size, LLG Labware, Germany). 2 mL of the filtrate was added to 1 mL of 6 % (*w/v*) phenol and 5 mL of H_2SO_4 (97 % (*v/v*)). After cooling to room temperature, the absorbance was measured at 490 nm (BioTek Synergy LX multi-mode reader, Agilent Technologies, USA). D-(+)-glucose was used to prepare the calibration curve, and the EPS content was expressed as mg EPS per g biofilm. The same procedure was applied to the MPs without biofilm, and the absorbance was similar to the blank sample (2 mL of deionized water, 1 mL of phenol and 5 mL of H_2SO_4).

2.3.5. Density

The density of aged MPs was determined using a pycnometer according to the standard method described in [40]. For this analysis, the MPs with biofilm were first dried in a laboratory dryer at 40 ± 2 °C. The measurements included weighing the empty pycnometer, the pycnometer containing approximately 100 mg of dried MPs, the pycnometer with both dried MPs and added ethanol (96 % (*v/v*)) and the pycnometer filled with ethanol only. The density was calculated based on the following equation (Eq. 2):

$$\rho_{aged\ MPs} = \frac{m_2 - m_1}{m_2 - m_1 - m_3 + m_4} \cdot \rho_{EtOH} \quad (2)$$

where $\rho_{aged\ MPs}$ is the density of aged MPs (g/cm^3), m_1 is the mass of the empty pycnometer (g), m_2 is the mass of the pycnometer and aged MPs (g), m_3 is the mass of the pycnometer with aged MPs and ethanol (g), m_4 is the mass of the pycnometer and ethanol (g), and ρ_{EtOH} is the density of

Table 1
List of samples with experimental conditions.

Sample	Sample	Light/dark [h]	Shaking [rpm]	Medium
PE-control	PLA-control	16/8	160	stream water
PE-dark	PLA-dark	**0/24**	160	stream water
PE-N	PLA-N	16/8	160	stream water + **10 mg/L N-NO_3^-**
PE-P	PLA-P	16/8	160	stream water + **10 mg/L P-PO_4^{3-}**
PE-polluted	PLA-polluted	16/8	160	stream water (90 %) + **wastewater (10 %)**
PE-static	PLA-static	16/8	**0**	stream water

ethanol (g/cm^3). The density of ethanol was also determined based on the density of deionised water [40]. The density determination was performed for two masses of each MPs and repeated five times for each mass. To assess the impact of biofilm on MPs density, the density of unaged PE and PLA was also determined for comparison.

2.3.6. Crystallinity

Crystallinity was assessed for pristine PE and PLA, as well as for aged PE and PLA samples with and without biofilm. Biofilm removal was carried out using the Fenton oxidation process, as described in Section 2.3.2. To ensure that the Fenton treatment itself did not alter the polymer crystallinity, additional measurements were conducted on pristine PE and PLA following the same Fenton oxidation procedure.

The melting enthalpy of samples was determined by differential scanning calorimetry (DSC) on a Mettler Toledo DSC1 instrument (Greifensee, Switzerland) coupled to a TC100 intracooler (Offenburg, Germany). The temperature range was from 25 °C to slightly above the melting points of the individual samples: 130 °C for PE and 190 °C for PLA. Experiments were performed at heating and cooling rates of 10 °C/min in a nitrogen atmosphere at a flow rate of 30 mL/min, using standard 40 μL aluminum crucibles with hermetically sealed lids. Four replicates were performed for each sample, with each replicate containing approximately 3 mg.

First, a three-minute conditioning phase was carried out, followed by an initial multi-stage heating cycle with a dynamic temperature increase up to the glass transition region of the polymer (50 °C for PE and 80 °C for PLA). During this phase, a two-minute isothermal conditioning was maintained. The temperature was then increased to the final target value to melt the sample and effectively extinguishing all previous thermal processes of the polymers. The sample was then cooled to 0 °C and then subjected to a second dynamic heating run.

The melting temperature was determined from the resulting second heating curve and the enthalpy of melting was extracted by integrating the peak area. This enthalpy value is directly related to the crystallinity of the material.

Degree of crystallisation, *Xc*, was determined using the equation (Eq. 3):

$$X_C = \frac{\Delta H_{m2}}{\Delta H_m^{\circ}} \quad (3)$$

where ΔH_{m2} is the enthalpy of melting from the second heating run (J/g), ΔH_m° is the enthalpy of melting of a 100 % crystalline PE or PLA (J/g), reported to be 293.6 J/g for PE [41] and 93.7 J/g for PLA [42].

2.3.7. Analysis of stream water and wastewater

Stream water and wastewater were analysed for their physico-chemical parameters. Temperature, pH (both measured with the FiveEasy Standard Benchtop Meter, Mettler Toledo, Switzerland) and concentration of dissolved oxygen (Seven2Go™, Mettler Toledo, Switzerland) were determined immediately when stream water was sampled. The temperature and concentration of dissolved oxygen were not determined for the wastewater as it was further processed before use (sterilisation, freezing, and thawing). After collection, the stream samples were frozen at −18 ± 2 °C until further analyses were performed. The dissolved organic carbon (DOC) was determined according to the standard method ISO 8245 [43] using the TOC multi N/C 3100 analyzer (Analytik Jena, Germany). The concentrations of N-NO_3^-, N-NO_2^-, P-PO_4^{3-}, and Cl^- were determined according to the standard methods [44] and the concentration of N-NH_4^+ according to ISO 7150–1 [45].

The fluctuation of the composition of the stream water was evaluated by measurement its composition every week (Table S1). No abnormal fluctuations in physico-chemical parameters were observed throughout the study period. Minor variations remained within the expected natural range for stream waters, indicating consistent environmental conditions across treatments.

2.3.8. Data evaluation

The differences in biofilm amount, chlorophyll *a* content, EPS content, density, and crystallinity were statistically analysed using OriginPro 2023 software (OriginLab Corp., USA). The Shapiro-Wilk test was used to assess the normality of the data, while the homogeneity of variances was tested using the Levene's test. As the data were not normally distributed for all parameters except crystallinity, non-parametric comparisons were performed using the Mann-Whitney *U* test. For crystallinity, parametric comparisons were performed using the Student's *t*-test. Statistical significance was set at $p < 0.05$.

Principal component analysis (PCA) is a multivariate statistical method that simplifies complex data sets by transforming correlated variables into a smaller number of uncorrelated components, thus highlighting patterns and groupings in the data [46]. It was performed separately for PE and PLA under all aged conditions, including data on the amount of biofilm, chlorophyll *a* content, EPS content, density, and crystallinity. The analysis was also performed using OriginPro 2023 software (OriginLab Corp., USA).

3. Results

3.1. Biofilm development and its effect on microplastics properties

Initially, biofilm development over time on PE and PLA MPs was monitored to determine the time point at which the stationary phase began (Fig. S3). The growth of biofilm from the beginning of the experiment was linear up to day 21. Afterwards, the amount of biofilm remained constant since day 26 and thus the experiment was terminated on day 28 (four weeks after starting the experiment). Consequently, the differences in biofilm development under different conditions were evaluated by measuring the amount of biofilm (B), chlorophyll *a* content (c(chlorophyll *a*)), EPS content (c(EPS)), and changes in the density and crystallinity (subchapters 3.2. and 3.3.).

3.2. Polyethylene microplastics

The surface morphology of PE MPs was examined by FE-SEM before and after aging (Fig. 1). For pristine PE (Fig. 1A), the surface was smooth with some irregularities. After aging, all PE samples (Figs. 1B, 1D – 1 G), with the exception of those aged in dark (PE-dark) (Fig. 1C), had visible microorganisms on their surface. The samples incubated in increased P concentration (PE-P) exhibited a thick biofilm accompanied by visible cracks caused by drying of the biofilm prior analysis (Fig. 1E). In addition, different forms of microorganisms were observed under different aging conditions (PE-control, PE-N, PE-polluted). Interestingly, samples incubated in static conditions (PE-static) (Fig. 1G) contained filamentous microorganisms.

The amount of biofilm on PE MPs (Fig. 2) showed a statistically significant difference only for PE-dark, which had a much lower amount of biofilm compared to the control. Other conditions, such as PE-N, PE-P, PE-polluted, and PE-static, were not statistically significantly different from the control, while PE-P showed a slight, although not statistically significant, increase (Fig. 2A). Regarding chlorophyll *a* content, all environmental conditions resulted in statistically significantly different values compared to the control. PE-dark and PE-P had a lower chlorophyll *a* content, while PE-N, PE-polluted, and PE-static had statistically significantly higher contents of chlorophyll *a* (Fig. 2B). All experimental conditions resulted in statistically significantly different levels of EPS compared to the control, with the contents generally being lower than those under the control condition (Fig. 2C).

Aging changed the densities of PE – only PE-static showed no statistically significant differences in density compared to pristine PE (Table S2 and S3), while all other samples showed significantly higher densities. The comparison with the control sample showed that the densities of PE-dark, PE-polluted and PE-static were statistically significantly lower, while the densities of PE-N and PE-P were comparable to

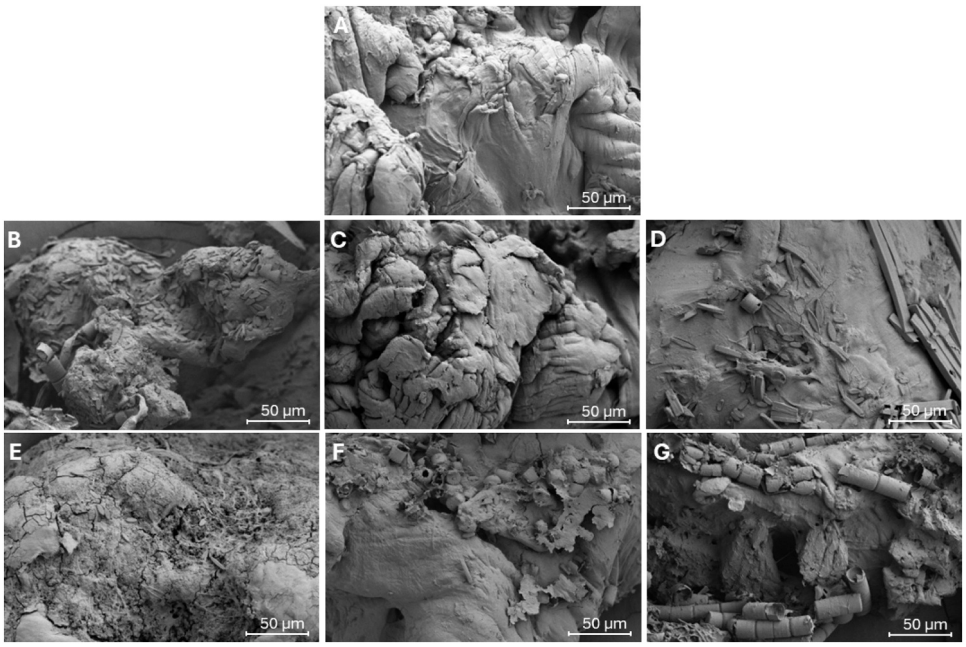

Fig. 1. Images from scanning electron microscope of polyethylene (PE): (A) pristine PE, PE aged (B) under common conditions (PE-control), (C) with absence of light (PE-dark), (D) with addition of nitrogen (PE-N), (E) with addition of phosphorus (PE-P), (F) in presence of pollutants from wastewater (PE-polluted), (G) under static condition (PE-static). All images were taken at 500x magnification.

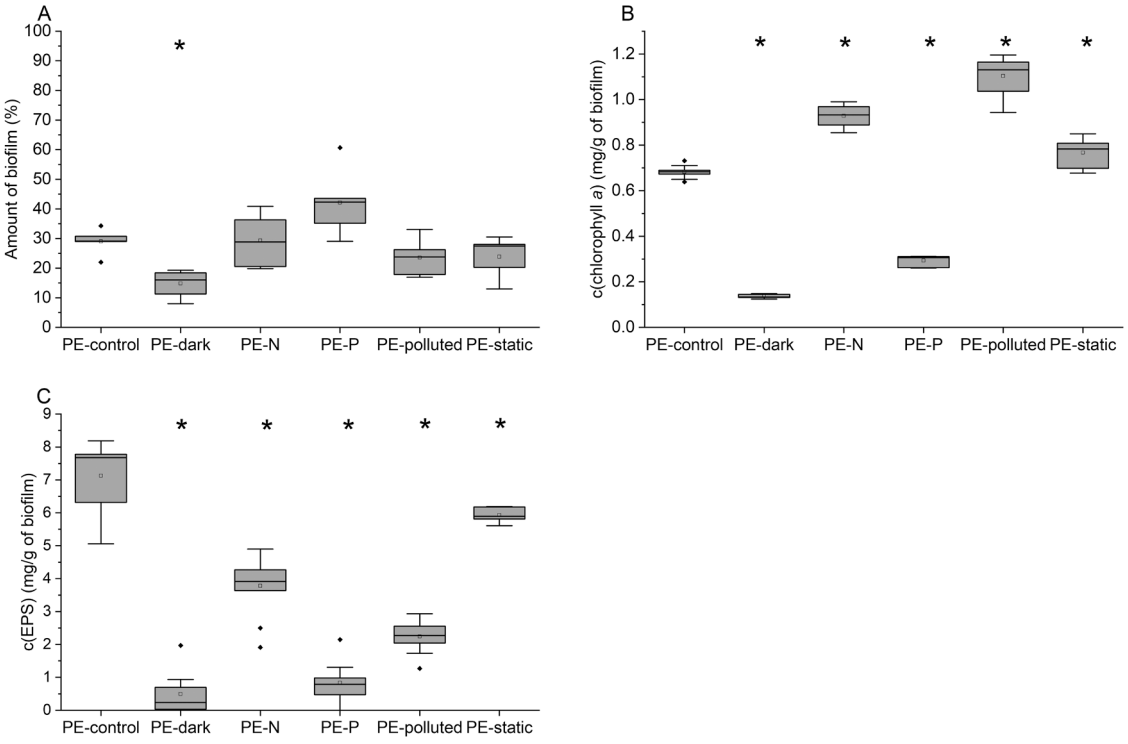

Fig. 2. Amount of biofilm (A), chlorophyll *a* content (B), and content of extracellular polymeric substances (EPS) (C) developed on polyethylene (PE) microplastics after aging under different environmental conditions: PE aged under common conditions (PE-control), with absence of light (PE-dark), with addition of nitrogen (PE-N), with addition of phosphorus (PE-P), in presence of pollutants from wastewater (PE-polluted), under static condition (PE-static).

those of the control sample (Fig. 3A).

Comparison of aged PE samples to pristine PE showed that all aged samples were significantly different from the pristine PE (Table S2 and S4), except for PE-static, which remained similar to pristine PE. Notably, PE-static was also the only sample that differed significantly from the PE-control (Fig. 3B). Additionally, crystallinity was evaluated in aged PE samples after biofilm removal (Figure S4). No statistically significant differences were observed among these samples; all showed crystallinity values comparable to pristine PE.

The results of biofilm formation and changes in MPs characteristics were further analysed using PCA (Fig. 4). The first two principal components (PC 1 and PC 2) explained a cumulative 65 % of the variance.

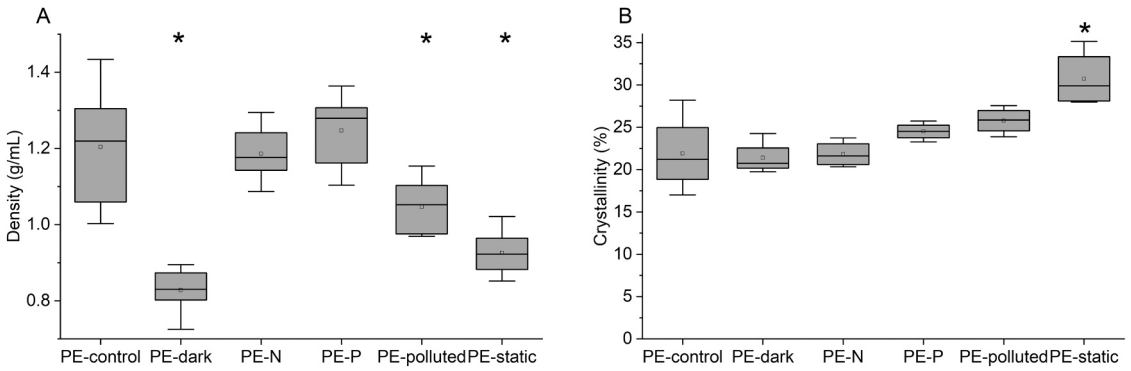

Fig. 3. Changes in density (A) and crystallinity (B) of polyethylene (PE) microplastics after aging under different environmental conditions: PE aged under common conditions (PE-control), with absence of light (PE-dark), with addition of nitrogen (PE-N), with addition of phosphorus (PE-P), in presence of pollutants from wastewater (PE-polluted), under static condition (PE-static).

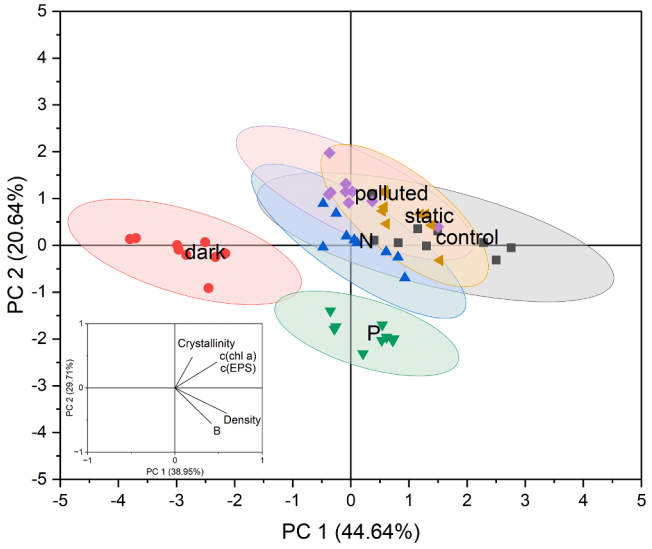

Fig. 4. Cluster graph from PCA analysis showing changes in biofilm formation and characteristics of polyethylene (PE) after aging under different environmental conditions: PE aged under common conditions (control), with absence of light (dark), with addition of nitrogen (N), with addition of phosphorus (P), in presence of pollutants from wastewater (polluted), under static condition (static). The inset graph displays the extracted eigenvectors for each variable.

For aged PE, the eigenvectors for density and biofilm quantity were close to each other. Chlorophyll *a* and EPS content were also closely related as their eigenvectors point in the same direction. The PCA cluster plot also showed that PE-polluted, PE-static, PE-control, and PE-N group together, while PE-dark and PE-P were clearly separated from them.

3.3. Polylactic acid microplastics

The surface of PLA was examined by FE-SEM. Pristine PLA (Fig. 5A) has a rougher surface, and after aging, biofilm formation was visible under all conditions except for samples incubated in the dark (PLA-dark), where only a few individual microorganisms were found on the surface (Fig. 5C). Similar to PE, the microbial communities on PLA differed under the different aging conditions, with the variable morphology of the microorganisms (PLA-control, PLA-P, PLA-N, PLA-polluted; Figs. 5B, 5D-F). In the PLA sample incubated under static conditions (PLA-static) (Fig. 5G), predominantly elongated forms of microorganisms were also found, reflecting similar growth patterns as on PE MPs.

Similar to PE, the amount of biofilm on PLA MPs was significantly lower only on the PLA-dark and also PLA-polluted compared to control (Fig. 6A). Chlorophyll *a* content in biofilm developed on PLA also exhibited statistically significant differences compared to the control in most conditions, except for the PLA-N (Fig. 6B). PLA-dark and PLA-P conditions led to lower chlorophyll *a* content, while PLA-polluted and PLA-static resulted in higher values, similar to the trends observed for PE MPs (Fig. 2B). All conditions resulted in significantly different EPS content compared to the control, with the exception of the PLA-N. Most of the experimental conditions showed lower EPS content compared to the control, except for the PLA-static, which had higher EPS levels (Fig. 6C).

The aging of PLA under all experimental conditions showed statistically significant differences in density compared to pristine PLA (Table S2 and S3), with all conditions showing lower densities. The comparison of the densities with the PLA-control showed a statistically significant difference only for PLA-dark (Fig. 7A). The analysis of crystallinity showed that aging under all experimental conditions resulted in statistically significant decrease of crystallinity compared to pristine PLA (Table S2 and S4). Compared to the PLA-control, only PLA-dark and PLA-static showed a statistically significantly lower crystallinity than the control sample (Fig. 7B). As with PE, crystallinity of aged PLA samples without biofilm was also evaluated. No statistically significant differences were found among these samples, and all values were comparable to pristine PLA (Figure S4).

As in the case of PE, the results of biofilm formation and changes in MPs characteristics for aged PLA were further examined using PCA. The first two principal components accounted for a cumulative 66 % of the variance. The PCA plot for aged PLA (Fig. 8) revealed distinct relationships between the variables. A similar trend to PE can be observed in the PCA analysis of the aged PLA samples. The cluster diagram shows that PLA-control, PLA-N, PLA-polluted and PLA-static group together, while PLA-P and PLA-dark are separated. The eigenvectors for density and biofilm amount in PLA samples point in opposite directions, indicating a negative correlation. In contrast to PE, where a positive correlation was observed, this indicates that an increase in biofilm quantity in PLA is accompanied by a decrease in density. The eigenvector for the crystallinity of PLA is closely linked to the amount of biofilm.

Finally, also all data for PE and PLA were analysed together and PCA showed comparable trend; only treatments incubated in dark were clustered together and separated from other treatments (Fig. S5).

4. Discussion

In this study we investigated the effects of environmental processes on the aging and biofilm development of conventional PE and biodegradable PLA MPs, in order to evaluate the key environmental parameters affecting this process, as well as the potential role of the polymer as a substrate for biofilm growth. Overall, the results (Table 2) indicated

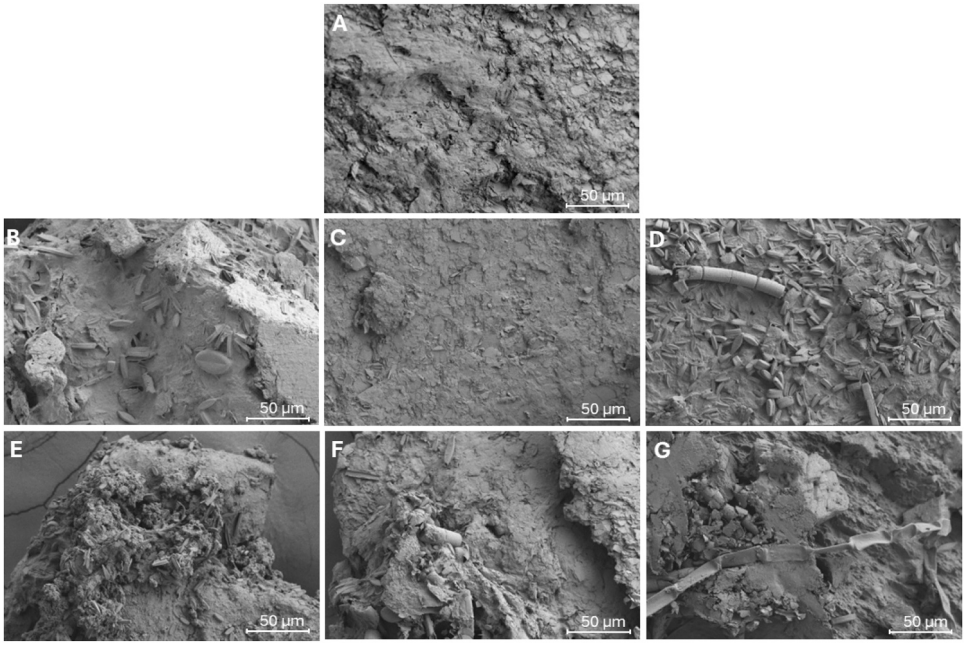

Fig. 5. Images from scanning electron microscope of polylactic acid (PLA): (A) pristine PLA, PLA aged (B) under common conditions (PLA-control), (C) with absence of light (PLA-dark), (D) with addition of nitrogen (PLA-N), (E) with addition of phosphorus (PLA-P), (F) in presence of pollutants from wastewater (PLA-polluted), (G) under static condition (PLA-static). All images were taken at 500x magnification.

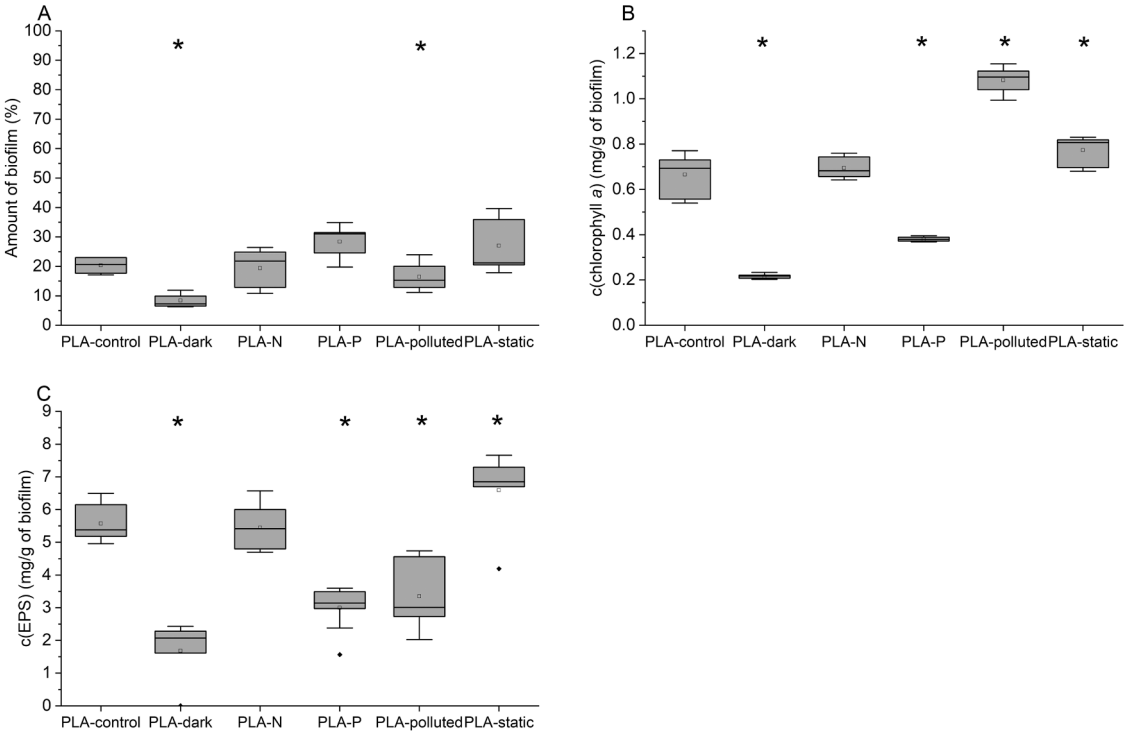

Fig. 6. Amount of biofilm (A), chlorophyll *a* content (B), and content of extracellular polymeric substances (EPS) (C) developed on polylactic acid (PLA) microplastics after aging under different environmental conditions: PLA aged under common conditions (PLA-control), with absence of light (PLA-dark), with addition of nitrogen (PLA-N), with addition of phosphorus (PLA-P), in presence of pollutants from wastewater (PLA-polluted), under static condition (PLA-static).

that environmental conditions can significantly influence biofilm growth and alter the properties of MPs. Notably, the observed patterns were highly similar for both PE and PLA, indicating that the influence of environmental factors outweighs the effect of polymer type. While previous studies have reported that environmental conditions play a more dominant role than substrate material in biofilm development [47, 48], this study provides a more detailed analysis of the specific environmental drivers involved.

The results showed that for both PE and PLA MPs the absence of light was the key environmental condition influencing biofilm development. This was also confirmed by the PCA analysis, in which PE and PLA MPs incubated in the dark clearly differentiated from the results obtained

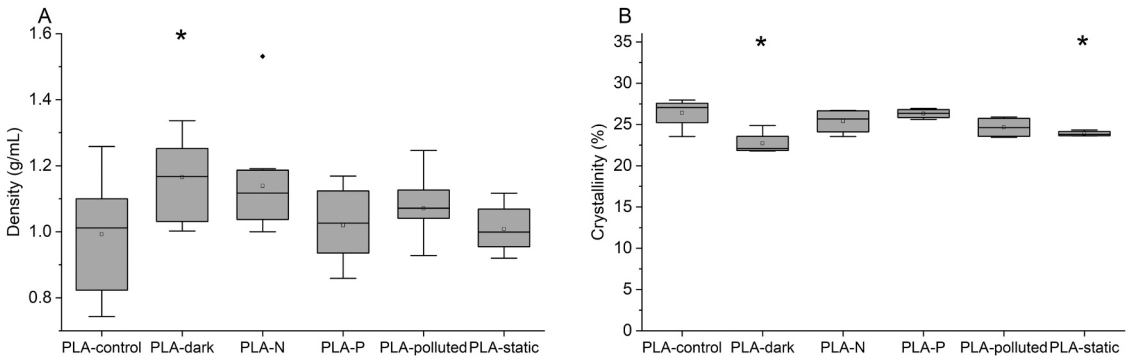

Fig. 7. Changes in density (A) and crystallinity (B) of polylactic acid (PLA) microplastics after aging under different environmental conditions: PLA aged under common conditions (PLA-control), with absence of light (PLA-dark), with addition of nitrogen (PLA-N), with addition of phosphorus (PLA-P), in presence of pollutants from wastewater (PLA-polluted), under static condition (PLA-static).

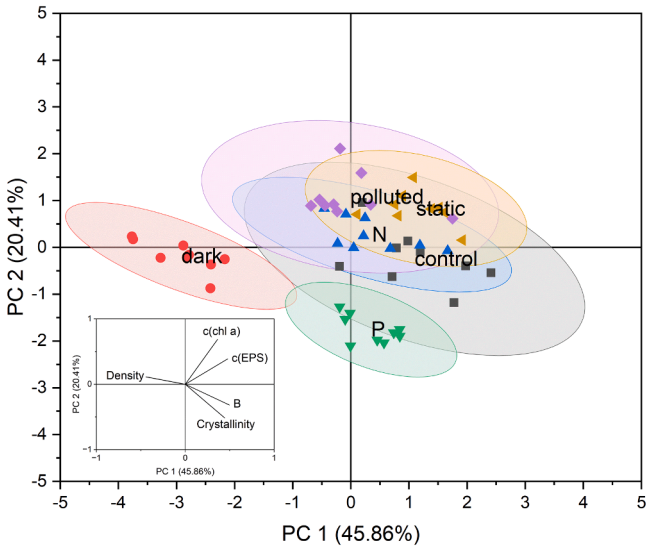

Fig. 8. Cluster graph from PCA analysis showing changes in biofilm formation and characteristics of polylactic acid (PLA) after aging under different environmental conditions: PLA aged under common conditions (control), with absence of light (dark), with addition of nitrogen (N), with addition of phosphorus (P), in presence of pollutants from wastewater (polluted), under static condition (static). The inset graph displays the extracted eigenvectors for each variable.

under other conditions. In general, the absence of light would affect the presence of photosynthetic microorganisms (for which the content of chlorophyll *a* is a specific marker, and whose content was significantly lower in both PE and PLA), but significant changes were also observed in the total mass of the biofilm and its EPS content (an important proxy of biofilm structure and community composition). This underlines the crucial role of light in promoting biofilm development and gives rise to an important consideration regarding the standard conditions under which general biodegradability tests are performed (e.g. all OECD ready biodegradability tests [49]). Since biodegradation in natural aquatic environments occurs under light exposure, conventional tests conducted in the dark may not accurately reflect microbial activity in such environment. Since the availability of light can promote the growth of photosynthetic microorganisms capable of enhancing biodegradation, for example by releasing exudates [50,51], future research should include light exposure as a factor in evaluating the biodegradation of plastics.

Nutrient availability and the N:P ratio also influenced biofilm development. The natural stream water used in this study had an N:P ratio of 33 at the first sampling, with values ranging from 11 to 19 at the next samplings, which is the typical N:P ratio of stream water [52,53]. The addition of P led to a significant reduction in the N:P ratio (between 0.03 and 0.12), resulting in biofilm formation comparable to the control, but with statistically significantly less chlorophyll *a* and EPS for both PE and PLA. This suggested that extreme deviations in the N:P ratio, especially towards P enrichment, may also negatively affect photosynthetic microorganisms [54]. Consequently, an imbalance in nutrient availability may have altered the biofilm composition, shifting the community towards heterotrophs rather than autotrophs. Since EPS plays a key role in the stability and protection of biofilms, its reduction indicates altered microbial metabolic strategies. The addition of N led to a significant increase in the N:P ratio (between 140 and 352) and promoted the formation of photosynthetic microorganisms [55]. Chlorophyll *a* content and EPS production were higher in N-enriched samples than in those with added P. In presence of high concentration of N, the chlorophyll *a* content in biofilm even exceeded that of the control, suggesting that a higher N:P ratio favours the growth of photosynthetic microorganisms. These results are consistent with previous studies showing that P and N availability affects photosynthetic efficiency, with extreme N:P ratios negatively impacting microalgal photochemical yield and light energy conversion efficiency [56,57].

In general, increased pollution of stream water (addition of 10 % sterilized municipal wastewater) reduced biofilm growth but promoted the growth of photosynthetic microorganisms on both PE and PLA MPs. This could be due to increased nutrient availability (NH_4^+, NO_3^-, PO_4^{3-}; Table S1) or specific compounds that could be present in the wastewater – such as vitamins, silica, and trace elements like Fe, Mg, Mn – which selectively favour autotrophic microbial communities [58-60]. Photosynthetic microorganisms rely less on EPS for biofilm integrity compared to heterotrophic bacteria [61]. This shift in microbial composition, driven by wastewater components that promote autotrophic growth, likely explains the reduced EPS production on both PE and PLA.

Under static conditions, the biofilm exhibited higher chlorophyll *a* level for both PE and PLA and higher EPS content in the case of PLA. Interestingly, both PE and PLA were extensively colonized by different microorganisms, with fibrous and elongated structures observed under static conditions. This can be attributed to nutrient concentration gradients, which occur when nutrient consumption exceeds the transport rate, causing slower diffusion compared to growth. Two-dimensional model simulations [62] demonstrated that limited nutrient transport results in highly porous or filamentous biofilms. The peaks of the biofilm receive more nutrients than the valleys, leading to locally accelerated growth and reinforcing the formation of elongated microbial structures. This could also lead to excessive production of EPS in case of PLA as increased EPS production likely compensates for the lack of strong adhesion forces and supports a three-dimensional matrix, maintaining biofilm integrity. EPS plays a crucial role in biofilm stability through

Table 2
Summary of experimental results for the aging of PE and PLA MPs under different environmental conditions: with absence of light (Dark), with addition of nitrogen (N), with addition of phosphorus (P), in presence of pollutants from wastewater (Polluted), under static condition (Static); comparing to the samples, aged under common conditions (control). Red arrows (↓) indicate a statistically significant decrease in the parameter value compared to the control, while green arrows (↑) indicate a statistically significant increase. The tilde symbol (~) denotes no statistically significant difference relative to the control.

	dark		N		P		polluted		static	
	PE	PLA	PE	PLA	PE	PLA	PE	PLA	PE	PLA
Amount of biofilm	↓	↓	~	~	~	~	~	↓	~	~
Chlorophyll *a* content	↓	↓	↑	~	↓	↓	↑	↑	↑	~
EPS content	↓	↓	↓	~	↓	↓	↓	↓	↓	↑
Density	↓	↑	~	~	~	~	↓	~	↓	~
Crystallinity	~	↓	~	~	~	~	~	~	↑	↓

non-covalent interactions [63,64], and lectin-like proteins may cross-link polysaccharides [65]. The higher EPS production likely facilitates biofilm cohesion in an environment relying on matrix self-organization rather than substrate attachment, potentially favouring microbial species adapted to a polymer-rich, self-sustaining environment. However, in case of PE, the increase of EPS content in biofilm was not observed.

The results showed that environmental conditions have a significant influence on MPs aging. Importantly, they not only affected the development of the biofilm, but also altered the physico-chemical properties of the MPs. In fact, with the exception of PE-dark, the density of all aged PE MPs was consistently higher than that of the pristine PE. This increase is attributed to the formation of biofilm, which can have density from 1.0 to 1.5 g/cm^3 [66,67]. Consequently, the density of PE MPs with biofilm shifts to values above 1 g/cm^3, which is consistent with our previous results [68]. The only exception was the PE MPs aged in the dark, where the density was close to that of the pristine particles. However, this slight change is probably due to the small amount of biofilm covering these particles. However, the trend was different for the PLA MPs; pristine particles had a density of almost 1.5 g/cm^3 and aging led to their decrease (again, except for dark-aged PLA), which is also consistent with previous analyses of biofilm on high-density MPs [32,68]. Finally, it is noteworthy that in most cases both PE and PLA MPs showed comparable density (1.1 g/cm^3) after aging, suggesting that prolonged exposure to environmental conditions leads to similar density changes regardless of polymer type. This result has important implications for understanding the transport of MPs in aquatic systems. It would be expected that pristine PLA sink in water and pristine PE will float, but biofilm formation changes the density of both materials, which may cause them to settle and occur in similar environmental compartments. This is consistent with monitoring studies where low-density MPs were found in the sediment [69,70]. The observed density shifts highlighted the pivotal role of biological interactions for the transport of MPs of all polymer types.

The results on crystallinity showed that the crystallinity of PE and PLA can also be influenced. Crystallinity would be expected to increase after aging and the development of a biofilm, as amorphous areas can be easily utilized by microorganisms [71,72]. However, the results showed that the crystallinity of PE and PLA MPs generally decreased. This may be related to the presence of a biofilm on MPs; biofilms are indeed amorphous [73,74], which reduces the overall crystallinity of the aged microplastic particles. This hypothesis was supported by results when the biofilm was removed prior to measurement: no statistically significant differences in crystallinity were observed between the aged and pristine samples for either PE or PLA, indicating that the presence of biofilm accounted for the apparent crystallinity reduction.

Finally, this study examined how individual environmental factors affect biofilm formation on microplastics under controlled conditions, allowing us to isolate specific influences. However, natural aquatic systems are much more complex. While no visible degradation and thus possible release of dissolved organic carbon (DOC) or assimilable organic carbon (AOC) is expected within the 4-week period being too short period for leaching [75,76], long-term or stressful conditions may show otherwise, particularly for materials like PLA. As the PLA particles were derived from commercial products, the possible role of undetected additives needs further exploration. Future research should also assess microbial community composition and incorporate long-term field studies to better understand biofilm dynamics under realistic environmental scenarios.

5. Conclusion

This study provides important new insights into how environmental conditions influence biofilm development and MP aging, contributing to the growing understanding of MP behaviour in aquatic systems. While it is well established that factors such as light, nutrient availability, and hydrodynamic conditions shape microbial colonization, our study integrates these parameters in a controlled setting to disentangle their individual contribution and effects on properties of investigated MPs.

While both PE and PLA supported biofilm growth, significant differences emerged under different aging conditions. Furthermore, biofilm formation affected the physical properties of MPs, particularly density and crystallinity. Although PE density increased and PLA density decreased, aging resulted in comparable densities above that of water, suggesting their tendency to settle and accumulate in sediments. Crystallinity was also reduced for both polymers due to biofilm presence. These findings highlight the importance of considering environmental heterogeneity when assessing MP aging and behaviour in aquatic ecosystems rather than emphasizing polymer type alone. Moreover, the application of controlled yet environmentally relevant setups—such as

those used in this study—can provide valuable mechanistic understanding to support field-based observations.

CRediT authorship contribution statement

Mark Starin: Writing – original draft, Methodology, Investigation. **Janja Novak:** Writing – original draft, Investigation. **Barbara Klun:** Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Gilberto Binda:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Kalcikova Gabriela:** Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Ula Putar:** Writing – original draft, Methodology, Investigation, Conceptualization. **Nataša Čelan Korošin:** Writing – original draft, Methodology, Investigation.

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2025.118689.

Data availability

Data will be made available on request.

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