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Biofilm formation and its influences on the properties of microplastics as affected by exposure time and depth in the seawater

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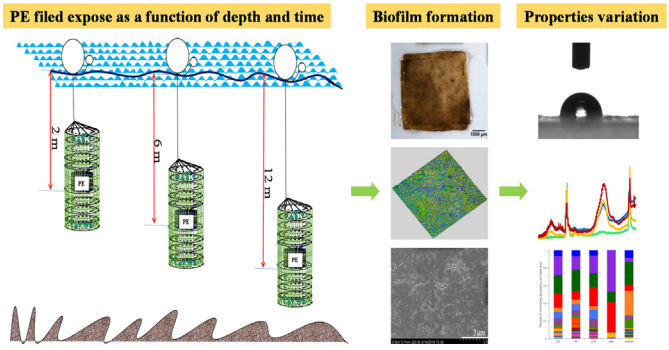
HIGHLIGHTS

- Biofilm formation and its influences on PE properties were investigated.
- The thickness of biofilms on PE increases with exposure time but decreases with depth.
- Biofilms could decrease the hydrophobicity and change the functional groups of PE.
- The dominant PE colonizing microbial community varies during the biofilm formation.

GRAPHICAL ABSTRACT



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ABSTRACT

The effects of microbial colonization and biofilm formation on microplastics in the marine and coastal environments have aroused global concern recently. However, the simultaneous influences of exposure time and depth on biofilm formation, and subsequently on the properties variations of microplastics is less studied. In this study, polyethylene (PE) film was exposed at three depths (2 m, 6 m, and 12 m) for three time periods (30 days, 75 days, and 135 days) in the coastal seawater of Yellow Sea, China. The results show that the total amount of biofilms markedly increased with exposure time, but decreased with water depth. Typical morphologies and compositions of biofilms such as coccus-, rod-, disc-shaped bacteria and filaments, as well as a dense layer of extracellular polymeric substances were observed on the surfaces of the PE microplastics. Biofilm formation could decrease the hydrophobicity of PE microplastics, and increase the abundances of hydrophilic C—O and C═O groups on the surface of PE. Alphaproteobacteria, Gammaproteobacteria and Bacteroidia were identified as the core microbiome of the PE associated biofilms, while the dominant bacteria families vary from the early to the late phases of the biofilm formation. Our results indicate that microplastics associated biofilms could affect the environmental processes and fates of microplastics in the marine and coastal environment.

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1. Introduction

Microplastics are defined as plastic particles or debris with a diameter of <5 mm (Thompson et al., 2004; Law and Thompson, 2014). Due to their high abundance, ubiquity, and threats to the marine environment and ecology, microplastics have recently become globally a popular research topic (Zettler and Amaral-Zettler, 2013; Auta et al., 2017; Kooi et al., 2017; Alimi et al., 2018; Zhou et al., 2018; Zhou et al., 2020). Many microorganisms, including bacteria, fungi, algae, and protists can easily colonize the surfaces of microplastics in the form of biofilms due to their large specific surface area (De Tender et al., 2015). Biofilm is mainly composed of diverse microbial consortium and their secreted extracellular polymeric substances (EPS) (Flemming and Wingender, 2010; Rummel et al., 2017). The formation of biofilms generally involves microbial attachment, secretion of EPS, and proliferation of microorganisms (Palmer et al., 2007; Zettler and Amaral-Zettler, 2013).

The biofilm formation may affect the physical and chemical properties of microplastics, such as surface micro-morphology and roughness, surface charge, specific surface area, and density, which may further affect the vertical migration, weathering, and adsorption-desorption of chemical pollutants and pathogens on microplastics in the marine environment (Dussud et al., 2018a; Johansen et al., 2018; Gong et al., 2019; Jacquin et al., 2019; Richard et al., 2019). Biofilm formation may also have biological effects on the microplastics such as the shifting of microbial community structure that inhabit the surface of the microplastics, affecting the bio-toxicity and bio-transferring within different trophic levels (Rummel et al., 2017). The rapid colonization and aggregation of microplastics by microalgae and microbes could possibly change buoyancy of the aggregates, leading to the different sedimentation (Lagarde et al., 2016; Chen et al., 2019). Weathering can lead to changes in the properties of microplastics such as topography, roughness, and chemical functional groups (Ter Halle et al., 2016). These changes are conducive to microbial adhesion and provide favorable conditions for biofilm formation. Conversely, the formation of biofilms may also affect the weathering rate and the vertical distribution of microplastics in the water column (Rummel et al., 2017). The biofilm could also serve as a source for microplastic-degrading bacteria, as proved by the coexistence of the grooves and cracks on the surface of microplastics and the perfectly embedded microbes (Zettler and Amaral-Zettler, 2013), or by the identification of several polymer-degrading bacterial species on the surface of microplastics (Yoshida et al., 2016; Delacuvellerie et al., 2019; Jacquin et al., 2019; Roager and Sonnenschein, 2019).

The driving factors affecting the microbial colonization and the community structure on the surface of microplastics mainly include substrate types and sizes, environmental factors (temperature, oxygen, light, pH, nutrients and salinity), as well as temporal and spatial effects (Oberbeckmann et al., 2014; De Tender et al., 2017; Rummel et al., 2017; Jiang et al., 2018; Gong et al., 2019; Hossain et al., 2019; Kesy et al., 2019; Kirstein et al., 2019; Li et al., 2019; Parrish and Fahrenfeld, 2019). For example, the microbial communities attached to the substrate surfaces were distinct among different polymer types, and were different from the non-plastic substrates and the ambient environment (Bryant et al., 2016; Dussud et al., 2018a, 2018b; Ogonowski et al., 2018; Miao et al., 2019). The microbial community structure within the microplastics biofilm also varies distinctly between different spatial sites including estuary, harbor, offshore, and pelagic seawater (Kesy et al., 2019; Li et al., 2019; Xu et al., 2019). In addition to the horizontal spatial effects, changes of water depth which are associated with the variation of temperature, salinity, nutrient, and dissolved oxygen among others, thus could affect the biofilm formation and development on the surface of microplastics in the seawater. However, to our knowledge, the effect of seawater exposure at different depths on the formation of biofilm on the surface of microplastics is still not well studied. Furthermore, the dynamic influences of the biofilm formation on the morphological, physical and chemical properties of microplastics under different exposure time and depth remains unclear.

In this study, PE film was selected as the test microplastics because its abundance dominated the plastics found in the Bohai Sea according to the previous surveys (Zhang et al., 2017; Zhou et al., 2018). The main aims of this study were to illustrate the dynamic processes of biofilm formation on the surface of the PE microplastics immerged at different depths for different times in the coastal seawater. In addition, the subsequent influences of biofilm formation on the surface morphology, physicochemical properties, and microbial community profiles on the PE microplastics were also investigated. The results may provide a new perspective on the interaction mechanisms of biofilm formation and microplastics fate in the marine and coastal environments.

2. Materials and methods

2.1. Design of the microplastics exposure device

The design concept of the microplastics exposure device (Fig. S1a) comes from the floating cultured lantern nets used for offshore scallop cultivation. These lantern nets (Fig. S1b) are woven with nylon thread with a pore diameter of 3 cm and length of 1.6 m. They have 10 layers separated by polyporus rubber discs on which scallops are cultivated. As for the PE incubation, a cylindrical stainless steel cage (Fig. S1c, diameter 20 cm, height 23 cm, mesh aperture 1 mm) was placed between the two rubber discs in the middle of the scallop cage, and six nylon net bags (Fig. S1d, size: 11 × 10 cm, pore diameter: 0.15 mm) containing microplastics were placed in the stainless steel cage. Each nylon bag contains 200 pieces of PE microplastics which were prepared by cutting from the commercial plastic film purchased from Yongmao Plastic Factory (Laizhou, China). The density of the PE film is 0.921 g cm^{-3}, and the thickness of the PE film is 8 μm. Those microplastics with a standard square shape and an average size of 4 ± 1 mm in side were selected for our study (Fig. S1e). The scallop cage was hung by reins on the horizontal rope with floats. Several stones were filled at the bottom of the lantern nets to assure its suspension to a certain depth in the water column. The advantages of the incubation device include low cost, ease of operation, and reusability.

2.2. In situ experiment setup and sample collecting

In order to expose the microplastics to seawater, an offshore aquaculture area in Yantai City, Shandong Province was selected for this experiment. The area has about 14 m water depth and the bottom sediment is silty (40% mud content, 60% sand content). The tidal current has an average flow rate of 0.5 m/s (Pan, 2009). The microplastics samples in the stainless steel cage were suspended in seawater at different depths (2 m, 6 m, and 12 m) by adjusting the length of the reins at the top of the lantern net. Samples were collected and analyzed after 30 days, 75 days, and 135 days of exposure. For each expose time and each depth triplicates were deployed and sampled. The basic water parameters were measured and recorded at each sampling time for all depth (Table S1).

2.3. Quantitative determination of biofilm content

The surface morphology of the submerged PE microplastics was observed using a stereomicroscope. Microplastics samples were carefully cleaned 3 times with sterile seawater and placed in a clean glass dish with the nitrocellulose filter (Whatman AE 98, Germany). They were photographed and recorded under a stereomicroscope (Nikon SMZ25, Japan).

Crystal violet staining was used to quantify the total amount of biofilms formed on the surface of microplastics after submerging exposure (Lobelle and Cunliffe, 2011). Four pieces of PE film were placed into a sterile petri dish and carefully cleaned 3 times with 2 mL of sterilized seawater. They were allowed to dry for 45 min; then 0.5 mL of 1% crystal violet solution was added into the dish and left for another 45 min at

room temperature. The excess dye solution was carefully discarded, the PE microplastics were washed 3 times with 5 mL of sterilized seawater, and samples were dried for 45 min at room temperature. They were then placed in a 2 mL centrifuge tube and 1 mL of 95% ethanol solution was added and allowed to decolorize them for 10 min. The decolorizing solution was transferred to a cuvette, and the absorbance was measured at 595 nm, which represents the amount of biofilm formed on the surface of the PE microplastics. Treatment with only the decolorizing solution alone served as a blank control. Commercial virgin PE pieces also served as a control group, and each experiment was set in 3 parallels.

2.4. Morphological observation of biofilm

Scanning electron microscopy (SEM, Hitachi S-4800, Japan) was used to observe the morphology of the biofilm on the surfaces of the microplastics (Zhou et al., 2018). The microplastics were washed 3 times with sterile seawater in the clean bench to remove impurities attached to the surface. Then the samples were placed in a fixing solution containing 2.5% glutaraldehyde at room temperature for 2–4 h, washed three times with 0.1 mol/L phosphate buffer saline (PBS), and gradually dehydrated in 10%, 30%, 50%, 70%, and 90% ethanol each for 10 min and finally twice in 100% ethanol solution for 15 min allowing for complete dehydration. Platinum was sprayed on the surfaces of the samples using an ion sputter coater for 100 s, and then sample stage was placed into the SEM vacuum chamber with an accelerating voltage of 3 kV.

2.5. Steric composition of biofilm

The steric composition of the biofilm formed on the PE surface was observed by confocal laser scanning microscopy (CLSM). Six films from each depth at each sampling time were collected and washed three times with sterile seawater. Then the microplastics were stained with the following fluorescent dyes for 30 min in the dark: 3.34 μmol/L SYTO9 (Thermo Fisher, USA), staining living cells in green, 20 μmol/L PI (Thermo Fisher, USA), staining dead cells in red, and 0.125 mg/mL Concanavalin A (Con A) (Thermo Fisher, USA), staining the extracellular polymer in blue. The residual dyes were removed and the PE microplastics were rinsed with sterile water 3 times before placing on a coverslip for CLSM observation (Olympus FV1000, Japan). Three sets of lasers were used for the excitation of SYTO 9, PI, and Con A at the wavelengths of 488, 559, and 633 nm, respectively. Three areas were randomly selected on each microplastics surface for imaging scanning. The size of each scanning area was 635 μm × 635 μm. Z stack scanning was performed from the surface to the bottom of the microplastics with a slice thickness of 1.0 μm. Fluorescent images were obtained and merged with the Olympus FV10-ASW software. The thickness of the biofilm was derived from the distance in *Z*-axis between the appearance and the disappearance of the fluorescent signals.

2.6. Determination of hydrophobicity of the PE film

The contact angle measuring device (DataPhysics OCA50, Germany) was used to evaluate the change in hydrophobicity of the PE microplastics caused by the growth of biofilm. Briefly, 3 pieces of PE from each depths (2 m, 6 m, and 12 m) and exposure time (30 days, 75 days, and 135 days) were washed three times with deionized water and placed in a 6-well plate to dry on air. The dried PE film was pasted on the surface of the slide in the light path of the contact angle meter. A droplet of water (about 2 μL) was dropped using a micro syringe, held in contact with the film for 10 s, and then photographed. The static contact angle of water droplets on the surface of the PE film was fitted using SCA20 software (Version 2). Virgin PE films were also tested as a control.

2.7. Determination of functional groups on the PE film

Fourier transform infrared spectroscopy (FTIR) analysis provided information on the composition of microplastics and it also provides information on the weathering or the oxidation of microplastics. For this reason, it is commonly used in studies involving the identification of microplastics and assessments of their weathering characteristics. In this study, FTIR (Nicolet iS10, Thermo Fisher, US) was used to characterize the changes of functional chemical groups on the PE surface caused by biofilm formation. The PE microplastics were rinsed 3 times in ultrapure water, and then processed with ultra-sonication twice for 30 min each to remove the attached biofilm. Virgin PE pieces were also set as a control. The PE samples were air dried and scanned by FTIR with a resolution of 4 cm^{-1} and a mid-IR range of 650–4000 cm^{-1} at a rate of 32 scans per analysis.

2.8. DNA extraction and 16S rRNA sequencing

Total genomic DNA from the microplastics associated biofilm was extracted from 10 pieces of the PE according to the standard operating procedures of the MP FastDNA® kit for soil. The V4 hypervariable region of 16S rRNA gene was amplified by PCR using the primers 515FmodF (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806RmodR (5′-GGAC TACNVGGGTWTCTAAT-3′). Qualified DNA samples were sent to high-throughput DNA sequencing by the Illumina HiSeq and MiSeq platforms at Novogene Bioinformatics Technology Co. Ltd. (Beijing, China) and Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China), respectively.

2.9. Data analysis and statistics

Quality control and bioinformatics analysis of the DNA sequencing data were performed using the free online platform of Majorbio Cloud Platform (www.majorbio.com). All the data analysis and statistics were performed using Origin 8.0 and Microsoft Excel 2010. The differential significance analysis among different treatments was performed by one-way analysis of variance (ANOVA) with Duncan's multiple range test from the SPSS 20.0 software.

3. Results and discussion

3.1. Dynamic quantitative analysis of the biofilm formation

The dynamic biofilm formation on the PE surfaces at different depths within seawater was initially recorded by a digital camera and observed by naked eyes. All the PE microplastics exposed to seawater showed obvious color variation (Fig. S2a–i) compared with the virgin PE film (Fig. S2j), which indicates different extent of biofouling and biofilm formation on the PE surfaces. The level of biofouling on the PE surfaces decreased markedly with greater exposure depths and increased significantly with greater exposure time.

The total amount of biofilms formed on the PE surfaces was further quantitatively determined by crystal violet staining (Fig. 1). The total amount of PE biofilm at all three water depths increased over time within the first 75 days, although there is no significant difference ($P > 0.05$). Harrison et al. (2014) reported that microorganisms could colonize on the surface of microplastics in offshore environments within a few hours, and the biofilms formation could be finished within the following 14 days. This supports strongly our findings, that at the first sampling time (30 day), the biofilm on the surface of PE has been almost completely formed.

Moreover, the total amounts of the biofilm at PE exposed to the seawater at depth of 2 m and 6 m were significantly higher than those from the depth of 12 m within the first 75 days ($P < 0.05$). This indicated that the development of biofilm is closely related to the immersing depth of the microplastics. Changes of expose depth will result in the variation of

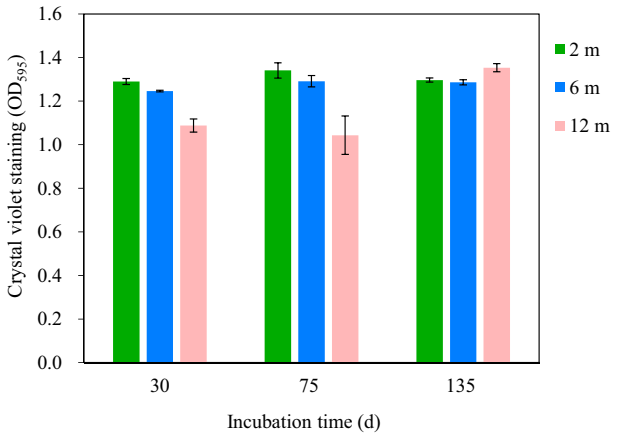

Fig. 1. Variations of the microplastics biofilm biomasses for different exposure times and water depths. Different letters indicate significant differences ($P < 0.05$).

temperature, light intensity, organic matter, nutrient, as well as other environmental factors like dissolved oxygen, pH etc., which may consequently affect the rate of microbial colonization and the biofilm formation on the surface of microplastics (De Tender et al., 2017). In this study, the rate of the biofilm development by microorganisms in shallow water (2 m and 6 m) is significantly higher than that at greater depths (12 m). However, at the end of the 135 day exposure, the amount of biofilm at 12 m depth was significantly increased to the comparable level with those at 2 m and 6 m. This could be due to the nutrient and biota exchange between the microplastics immerged in the deep layer (12 m) and the re-suspended sediments at the bottom (14 m) of the study area (Fig. S3a).

3.2. Dynamics of biofilm morphology and steric composition

According to the SEM images microbial colonization and biofilm formation has been determined at all the PE samples exposed at different depth and 3 time points (Fig. 2). A large variety of biofilm morphological types were observed on the surface of the PE microplastics, including coccus- (Fig. 2a), rod- (Fig. 2g) and disc-shaped bacterial cells (Fig. 2b), intertwined filaments (Fig. 2d, e, h), as well as a dense layer of EPS (Fig. 2c, f, i). In general, the density of the biofilm on the surface of the PE increased with the length of exposure and decreased as exposure depth increased. Similarly, Zettler and Amaral-Zettler (2013) found a rich bio-community on the surface of plastic marine debris, including diatoms, cyanobacteria, infusorian and bacteria. However, neither the microalgae nor the protozoa were found on the surface of the PE in this study, which was probably due to the protection of the lantern nets and nylon bags where the microplastics were incubated in. The mesh size of the nylon bag of 0.15 mm could exclude most of the alga and protozoa (Fig. S3b), but allow the bacteria and fungi to pass freely and to colonize the PE surface. Although the blocking effects caused by the small mesh size nylon bags may reduce the biomass and biodiversity of residents in the "plastisphere", it focus our attention on the features and effects of the biofilm formed by microplastic colonizing microbes only.

Steric composition of the biofilm was investigated by CLSM (Fig. 3). Different colors of fluorescence indicate different composition of the biofilm. At the first and second sampling time point (day 30 and day 75), the biofilm mainly consisted of living cells (green) and their

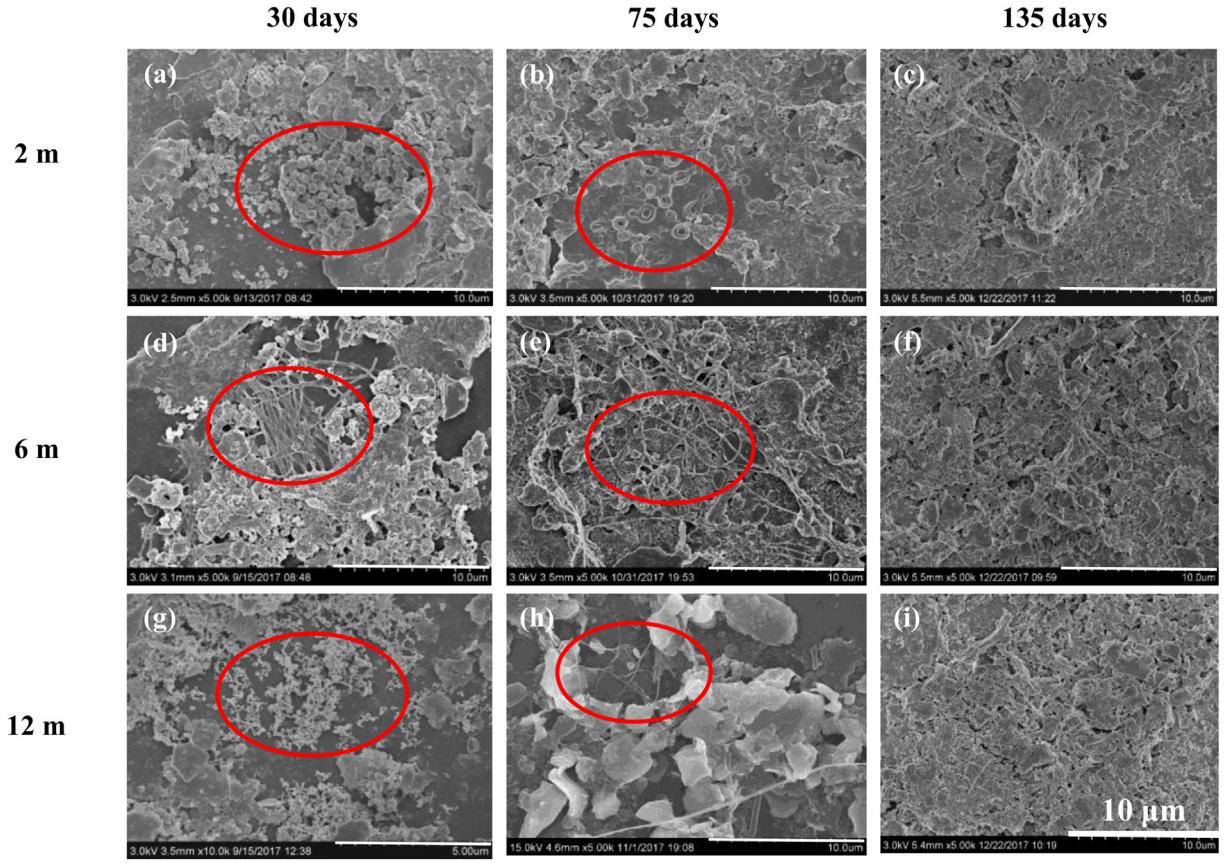

Fig. 2. SEM images of the PE associated biofilms morphology for different exposure times and water depths. The red circles stand for the typical microplastics colonizing microbes with coccus- (a), disc- (b), rod-shaped cells (g), and intertwined filaments (d, e, h), while (c), (f), (i) stand for a dense layer of EPS.

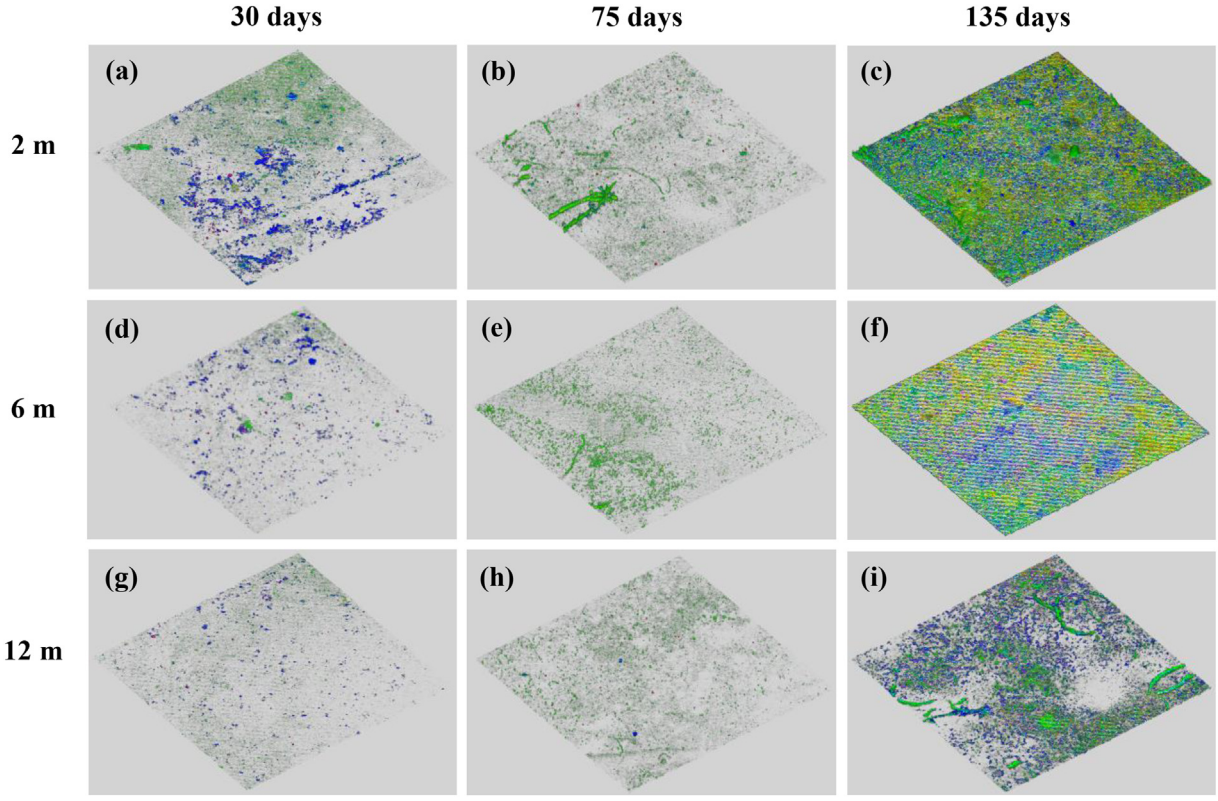

Fig. 3. Merged fluorescent CLSM images of microplastics biofilms for different exposure times and water depths (green: living cells; red: dead cells; blue: EPS).

extracellular polysaccharide (blue) (Fig. 3a, d, g for day 30, and Fig. 3b, e, h for day 75). As of the third sampling (day 135), the steric composition of the biofilm has shifted to a large amount of living cells and extracellular polysaccharides, as well as a small amounts of dead cells (red) (Fig. 3c, f, i). Moreover, the thickness of the biofilm increased significantly over time, but decayed with depth (Fig. 4).

Temporal dynamics of the biofilm formation on the surfaces of different microplastics were investigated using CLSM (Harrison et al., 2018; Michels et al., 2018). A considerable number of bacteria and microalgae were found at the early stage of the biofilm formation within the first 8–12 days, while a pronounced biofilm formed by bacteria and microalgae, as well as polysaccharides and DNA was found within a few weeks (Michels et al., 2018). The temporal dynamics of the biofilm formation pattern on the PE surface in this study were almost in accordance with previous research. The EPS component is essential in facilitating the initial stages of the bacterial colonization and the early biofilm formation (Webb et al., 2008; Harrison et al., 2018). With the time increasing and the bacterial proliferation intensifying, biofilms continue to grow, manifested in the planar expansion and stereo thickening. The mature biofilm showed different features than the early and developed biofilm in coverage, thickness, and composition (De Tender et al., 2017). This is consistent with the results of naked eye observation, crystal violet staining, and the SEM. Furthermore, here we have also found a distinct depth gradient of the biomass of the PE biofilm. This could be well explained by the depth-decay theory that with increased water depth, bottom waters are generally colder, are more saline and acidic but contain less oxygen and are less exposed to light (Gong et al., 2015). This will undoubtedly have an impact on the community structure and diversity of microorganisms in the bottom seawater, and ultimately on the formation of biofilm on the PE microplastics surface.

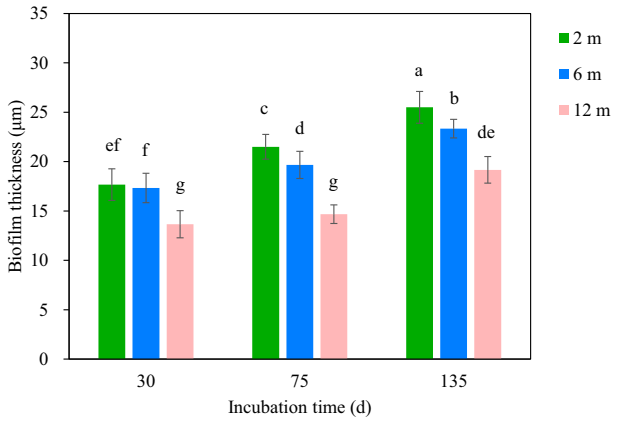

Fig. 4. Variation of the microplastics biofilms thickness for different exposure times and water depths. Different letters indicate significant differences ($P < 0.05$).

3.3. Influences of biofilm formation on the PE hydrophobicity

Water contact angle is widely used as an indicator for the hydrophobicity of different materials. A greater contact angle indicates a stronger hydrophobicity of the tested material. Fig. 5 shows that the hydrophobicity of the PE decreased with the exposure time increasing at each depth; while there was no significant difference among the different depths at a given sampling time except for the samples taken after 30 days. Moreover, all the PE surfaces with biofilm formation showed significantly lower hydrophobicity than the virgin PE microplastics (control 105.8 ± 0.7°).

Some studies have shown that microbial attachment and biofilm formation can alter the hydrophilicity of the microplastics surfaces (Nauendorf et al., 2016), but studies on the effects of different exposure depths on the hydrophobicity of microplastics are still very limited. Lobelle and Cunliffe (2011) exposed PE film in the harbor at a depth of 2 m, and found that the hydrophobicity and the buoyancy of the film decreased with increasing exposure time. Our study showed that

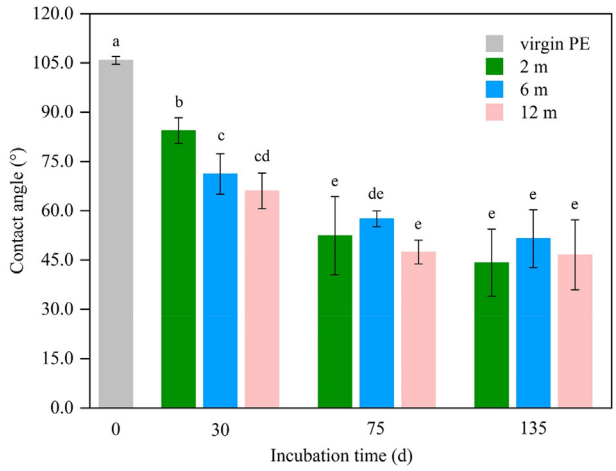

Fig. 5. Changes in the microplastics hydrophobicity as indicated by the droplet contact angle on the surface of PE film for different exposure times and water depths. Different letters indicate significant differences ($n = 3, P < 0.05$).

the hydrophobicity of the PE surfaces gradually decreased with the increasing exposure time, which is consistent with the dynamic characteristics of the biofilm formation (Fig. 1). It may be that the surfaces of microplastics exposed to seawater easily adsorb organic and inorganic nutrients from seawater within a few hours. This forms a conditioning film, which can quickly attract microorganisms and facilitate their utilization of those nutrients on said surfaces (Oberbeckmann et al., 2015). More microorganisms adhere, colonize, and aggregate on the surfaces of microplastics as exposure continues. This may cause changes in the micro-morphology, decrease the hydrophobicity, and increase the density of microplastics, which may cause vertical displacement of microplastics between different water depths (Kaiser et al., 2017; Kooi et al., 2017). Our study was the first to compare hydrophobicity of the PE microplastics at three different exposure depths. Results show that at the early stage of the biofilm formation (day 30), the hydrophobicity of the PE films decreases with the increasing water depth, which is in consistent with the biomass (Fig. 1) and thickness (Fig. 4) of biofilm formed on the PE surface. However, during the mid to late stage of the exposure time (day 75 and 135), the hydrophobicity of PE films did not significantly varied between different depths. This is not fully consistent with the results of the biomass and the thickness of biofilms, which indicate that the biofilm formation is not the only factor affecting the hydrophobicity of microplastics exposed to seawater. Chemical properties of seawater and the environmental factors may also impact on the hydrophobicity of microplastics.

3.4. Influences of the biofilm formation on the PE functional groups

The FTIR spectra of the PE surfaces were determined to investigate the influences of biofilm formation on the shift of chemical functional groups (Fig. 6). The five peaks of the virgin PE microplastics (control group) on the infrared spectrum corresponded to the different vibration modes of the methylene group: 2914 cm^{-1} corresponds to the symmetric contraction peak of $-CH_2-$, and 2847 cm^{-1} corresponds to the antisymmetric contraction peak of $-CH_2-$, 1472 cm^{-1} corresponds to the $-CH_2-$ shear bending vibration peak, and 730 cm^{-1} and 718 cm^{-1} correspond to the rocking vibration peaks of $-CH_2-$. After 30 days, the PE surfaces showed a new peak at 1000 cm^{-1} at all three depths, which

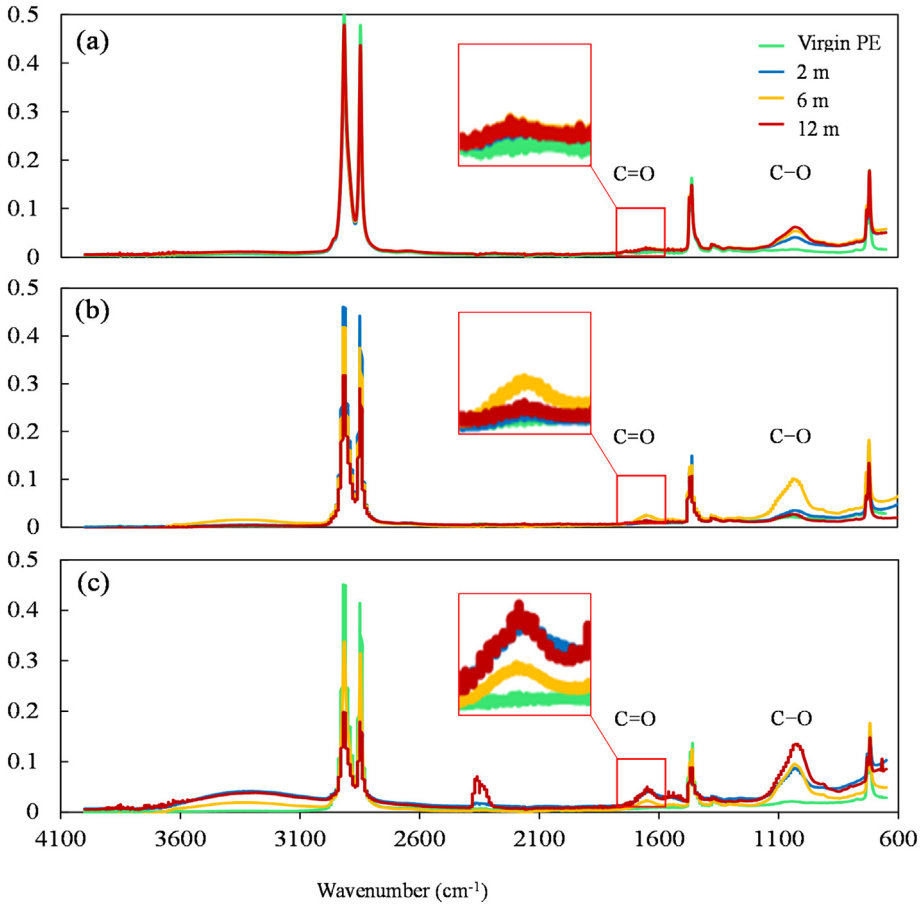

Fig. 6. Changes in the chemical groups on the PE surfaces as indicated by FTIR for different water depths and exposure times (a) 30 d; (b) 75 d; (c) 135 d.

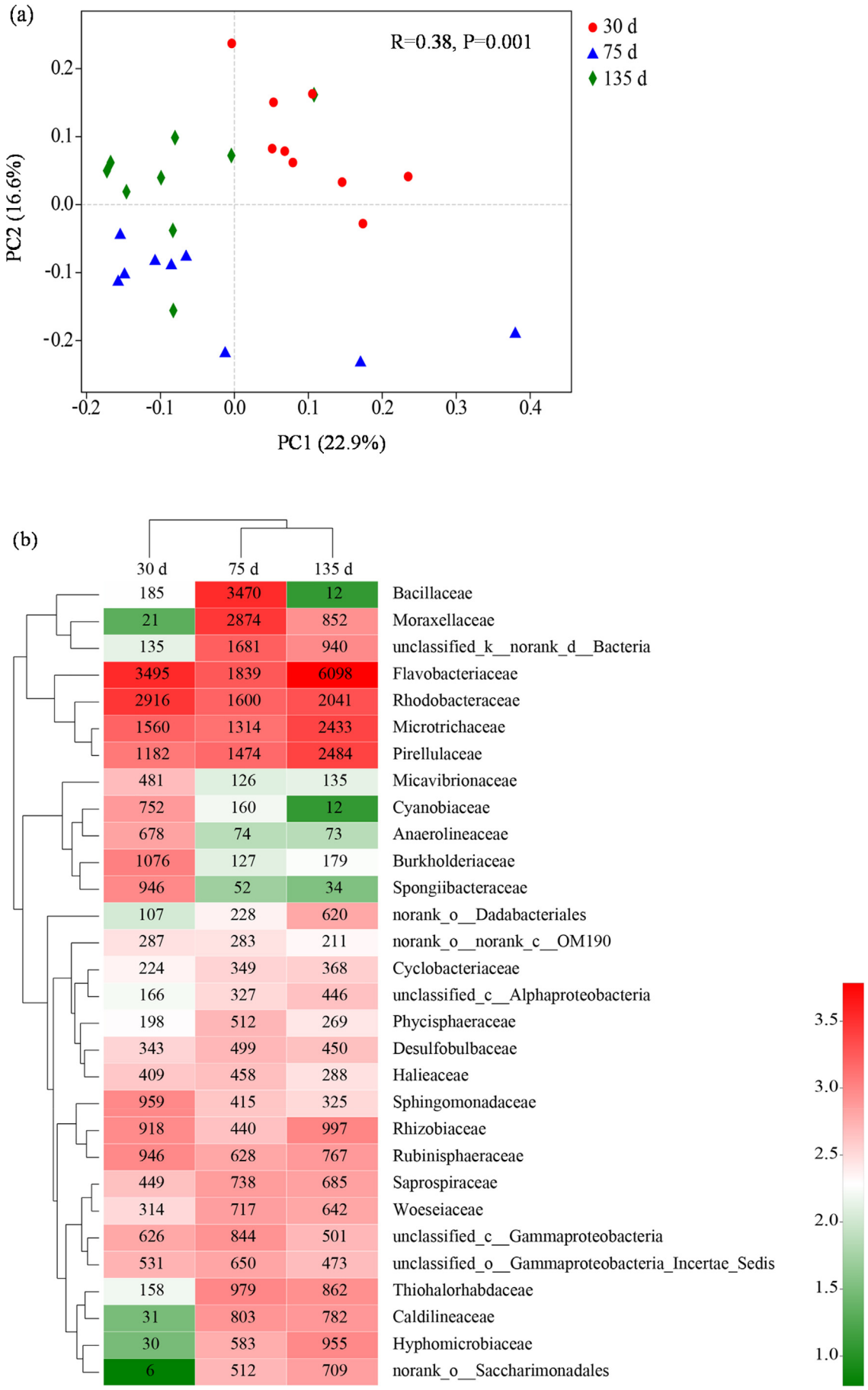

Fig. 7. Changes in the diversity of the microbial community structure for the PE associated biofilms: (a) PCoA analysis, (b) heat map of operational taxonomic units (OTU) on Family level.

corresponds to the vibration peak of the C—O bond. At exposure day 75 and 135, the PE surfaces showed additional peaks at both 1000 cm^{-1} and 1700 cm^{-1} at all three depths. The peak at wavenumber of 1700 cm^{-1} corresponds to the vibration peak of the C═O bond. Furthermore, the intensity of the C═O peak from samples taken at day 135 was significantly greater than at day 75.

Biofilms may affect the weathering and the degradation processes of microplastics (Rummel et al., 2017). The continuous colonization of microorganisms on the plastic surface may form a protective layer on the surfaces of plastics reducing the effects of the irradiation by ultraviolet light (Rummel et al., 2017). However, colonization by microorganisms may increase the probability of microplastics being biodegraded (Weinstein et al., 2016). In our study, the biofilm on the PE microplastics surfaces was gently removed. The effects of different environmental conditions including different exposure times and depth on the chemical functional groups on the PE surface were analyzed by FTIR (Fig. 6). The results show that the PE surface at each depth has a vibration peak of C—O bond at 1000 cm^{-1}, which was not found in the virgin plastic, suggesting the production of aliphatic and aromatic compounds. The PE film at each depth showed an additional vibration peak of C═O at 1700 cm^{-1} after 75 days of exposure, suggesting possible biodegradation effect occurring on the PE surfaces. The intensity of the vibrational peaks of C—O and C═O were significantly higher after 135 days exposure (compared to 75 days), suggesting that the biodegradation of the PE increased with exposure time. Yang et al. (2014) found that the PE film inoculated with PE degrading bacteria *Bacillus* sp. YP1 and *Enterobacter asburiae* YT1 showed new carbonyl peaks at 1700 cm^{-1} by using XPS and micro-ART/FTIR. Paço et al. (2017) reported that the PE film degraded by the marine fungus *Zalerion maritimum* showed new peaks at 3700–3000 cm^{-1} (—OH), 1700–1500 cm^{-1} (C═O) and 1200–950 cm^{-1} (C═C). The results of our study are consistent with those studies cited above, confirming that C═O can be used as a marker for biodegradation of the PE.

3.5. Microbial community structure of the PE associated biofilms

The principal coordinates analysis (PCoA) showed that the microbial community structure from the PE associated biofilms exhibit a significant difference ($P = 0.001$) among different exposure times (Fig. 7a), but does not significantly ($P = 0.670$) differ with depths (Fig. S4a), despite recognizable differences among different depths at each sampling time (Fig. S4b—d). This indicated that, compared with exposure depths, exposure times had a greater effect on the variation of microbial community structure of the PE associated biofilms. Fig. S5 shows the percentage of bacterial abundance on Class level in the PE associated biofilms at three sampling time points, as well as from the surrounding seawater and sediments. The PE associated biofilms exhibited significantly different microbial community profiles in comparison to those from the ambient seawater and sediment. This indicated that microplastics, as a new marine microbial habitat, can selectively provide a niche for the colonization of marine microbes and the formation of biofilms (Zettler and Amaral-Zettler, 2013; Dussud et al., 2018a, 2018b; Frère et al., 2018).

The dominant microbial colonizers on the PE microplastics belong to the Class Alphaproteobacteria, Gammaproteobacteria and Bacteroidia, which contribute over 50% of the total microbial communities (Fig. S5). Moreover, the percentage of the dominant microbes in the biofilm was shifting along with the exposure time. Fig. 7b shows the heat map of microbes from the top 30 families among different exposure time. Bacteria from the Flavobacteriaceae (Bacteroidia), Rhodobacteraceae (Alphaproteobacteria), and Microtrichaceae (Acidimicrobiia) families were the dominant colonizers on the PE surface at the early phase of the biofilm formation (30 days). At the middle phase of the biofilm formation (75 days), the top dominant colonizers shifted to Bacillaceae (Bacilli) and Moraxellaceae (Gammaproteobacteria), while microbes from Flavobacteriaceae (Bacteroidia) and Rhodobacteraceae (Alphaproteobacteria) remain the core species of the PE associated biofilms. At the late phase of the biofilm formation (135 days), the most dominant family of the PE colonizers has shifted to the Flavobacteriaceae (Bacteroidia) again, while microbes from the Rhodobacteraceae (Alphaproteobacteria), Microtrichaceae (Acidimicrobiia), and Pirellulaceae (Planctomycetes) increased significantly compared to the initial and middle phase. Previous studies also suggested that microbes from the Phylum Proteobacteria and Bacteroidetes were the most important microbial species initially colonizing the surfaces of plastics in marine ecosystems (Zettler and Amaral-Zettler, 2013; Keswani et al., 2016; Frère et al., 2018; Ogonowski et al., 2018). Alphaproteobacteria and Gammaproteobacteria were the primary colonizing groups in the biofilms on the microplastics surfaces, while Lactobacillus made up a secondary colonization group (Lee et al., 2008; Oberbeckmann et al., 2015). These results indicated that the core microbial community changes dynamically with the formation stages of the biofilm. These may affect the environmental processes and fate of microplastics such as weathering and degradation in the marine and coastal environments.

4. Conclusions

Microplastics have been regarded as a novel habitat for microbes in the terrestrial and aquatic ecosystems. Development of the microplastics associated biofilms are influenced by many biotic and abiotic factors. Biofilms formed on the microplastics surface play a vital role in the environmental processes and fate of microplastics. In this study, we found for the first time that biofilm formation on the PE film surface increases with exposure time but decreases with water depth of a natural coastal environment. Biofilms formed on the PE surface significantly reduced the hydrophobicity, and increased the abundance of hydrophilic groups such as C—O and C═O. Furthermore, the core microbiome and dominant bacterial indicators for early, middle, and late phases of the biofilm formation were changing along with exposure time. Results from this study strongly indicate that biofilms and the dominant functional microbes attached to the microplastics surface will affect the migration, sinking, weathering, and degradation of microplastics in the environment. Future studies should focus on the combined toxic effects of microplastics and the adsorbed environmental contaminants as well as pathogens, which could be induced by the formation of biofilms.

CRediT authorship contribution statement

Chen Tu: Methodology, Formal analysis, Writing - original draft.**Tao Chen:**Investigation, Writing - original draft.**Qian Zhou:**Investigation, Writing - original draft.**Ying Liu:**Methodology, Investigation.**Jing Wei:** Visualization, Methodology.**Joanna J. Waniek:**Writing - review & editing, Funding acquisition.**Yongming Luo:**Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

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. Supplementary data

Parameters of the seawater, design of the coastal exposure device for the PE microplastics immersion, biofouling and biofilm formation dynamics on the PE surfaces for different exposure times and seawater depths, re-suspended sediment and the invertebrate attached on the outer surface of the nylon bags, PCoA of microbial community structure for different treatments, abundance profiles of bacterial communities on Class level. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.139237.

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