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Colonization Characteristics of Bacterial Communities on Plastic Debris Influenced by Environmental Factors and Polymer Types in the Haihe Estuary of Bohai Bay, China

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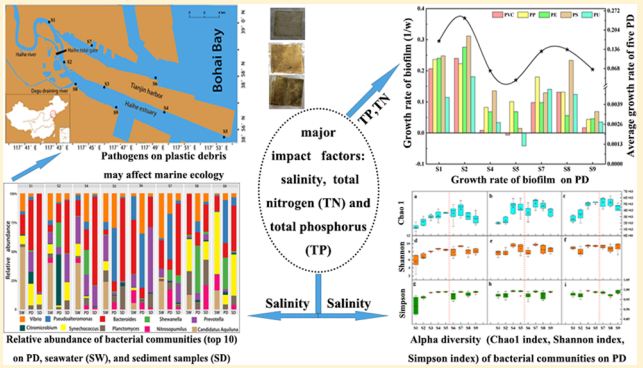
Supporting Information

ABSTRACT: The colonization characteristics of bacterial communities on microplastics or plastic debris (PD) have generated great concern in recent years. However, the influence of environmental factors and polymer types on the formation of bacterial communities on PD in estuarine areas is less studied. To gain additional insights, five types of PD (polyvinyl chloride, polypropylene, polyethylene, polystyrene, and polyurethane) were exposed for three-time periods (two weeks, four weeks, and six weeks) in the Haihe Estuary. 16S rRNA gene sequencing was used to identify the bacterial communities on PD, in seawater, and in sediment samples. The results indicate that the average growth rate of a biofilm is affected by nutrients (total nitrogen and total phosphorus) and salinity. Furthermore, salinity is the primary factor affecting bacterial diversity of the colonies on PD. In addition, genera of bacteria show selectivity toward the PD polymer type and tend to colonize their preferred substrate. Compared with seawater and sediment, PD could be carriers for enrichment of *Vibrio* in the estuarine environment with salinity ≥26 (± 2‰), which might increase the ecological risk of PD in marine environments.



1. INTRODUCTION

Global plastic production reached 335 million tons in 2016, and this value is exhibiting a continuing upward trend.[1] This production inevitably leads to large amounts of plastic waste discharged into the environment, which has been a pervasive feature, ranging from coastal estuaries to oceanic gyres and down to the abyssal seafloor.[2−4] Jambeck et al. predicted that unless the waste management infrastructure is improved, the cumulative quantity of plastic waste available to enter the ocean from land will increase by an order of magnitude in 2025.[5] Over time, a culmination of physical, biological, and chemical processes could reduce the structural integrity of plastic products,[6−10] resulting in plastic debris, including macroplastics,[11] microplastics (with the diameter of ≤5 mm), and nanoplastics (≤100 nm). With the properties of chemical stability and low bioavailability, plastic debris (PD) could exist for a long time in the ocean, thus affecting the marine environment.[12]

As a unique substrate, PD can adsorb microorganisms in the ocean and form the "Plastisphere".[13] Microbial communities on PD exist in the form of biofilms,[14] which were investigated by sampling or in situ experiments (Figure S1). Many studies have shown that the composition of microbial communities on PD are significantly different from that of the surrounding environments (water and sediment) or other natural media, both in marine and fresh water ecosystems,[15−17] and are obviously affected by location, time, and plastic type.[16−22] In addition, biofilms attached to PD had unique metabolic functions that were different from those of the surrounding environments. Bryant et al. pointed out that the overexpression of the *Che* gene, a secretory system gene, and the *nifH* gene in the biofilm on PD reflected the obvious enrichment chemotaxis, frequent intercellular interactions, and nitrogen fixation compared with the free bacterial communities in seawater.[23] Dussud et al. found that transportation across the cellular membranes, cell motility, and biodegradation of metabolites in the PD biofilms from the Mediterranean were significantly higher than those of planktonic bacterial communities in seawater.[19] Furthermore, PD could also act as a carrier in transport of microorganisms, including pathogenic bacteria, plastic-degrading bacteria, and harmful algae species.[24,25] In 2003, Masó et al. first proposed that marine microplastics could be used as migration tools for *Ostreopsis* sp. and *Coolia*

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sp.[24] Viršek et al. identified a fish pathogen (*Aeromonas salmonicida*) on plastic debris for the first time in the Italian North Adriatic Sea.[25] Moreover, the conditional pathogen *Vibrio* was found on PD in the North Atlantic,[13] Scottish beaches,[26] the North Sea, and the Baltic Sea.[27] Previous studies have also shown that there were some potential plastic-degrading bacteria including *Pseudomonas* sp., *Arthrobacter* sp.,[28] *Kocuria palustris*,[29] *Bacillus cereus*,[30] *Bacillus gottheilii*,[31] and *Rhodococcus* sp.[32] that settled on PD (Table S1).

Although the colonization characteristics of bacterial communities on plastic debris have been investigated in the sea, few studies have illustrated the key factors in determining the growth rate of biofilms and bacterial composition in estuarine areas. In addition, whether PD could provide a suitable living environment and improve the survival strategy of bacterial communities from the river to the sea remains unknown.

The Haihe Estuary, located in Bohai Bay, has many functional zones (including the Tianjin Port, the estuary of the Dagu River, a water park, and an industrial park), resulting in a natural experimental field for investigations of the environmental effects on the bacterial communities of PD. Moreover, Bohai Bay is a semi-enclosed bay, and the pollution of microplastics in Bohai Bay is more severe than in other bays of China.[33] Previous studies have utilized two strategies for studying bacterial communities on PD: sampling and in situ experiments. In sampling experiments, PD was randomly sampled in the field, and the source of PD was unclear because of the migration of PD in the environment.[17,20,34] Consequently, to avoid the influence of random migration of PD on bacterial communities, we established an in situ exposure experiment in the Haihe Estuary for three time periods (two weeks, four weeks, and six weeks) to elucidate the colonization characteristics of bacterial communities on PD that are affected by local environmental factors and polymer types.

There are four objectives for this study. (1) Investigate the spatial and temporal influences affecting the growth rate of biofilms and the composition of bacterial communities on PD in the Haihe Estuary. (2) Determine the main environmental factors influencing biofilm formation and composition of PD bacterial communities. (3) Explore the influence of polymer types on the composition and metabolic function of bacterial communities from the Haihe River to the Bohai Bay. (4) Compare the abundance of the pathogenic bacteria colonized on PD to seawater (SW) and sediment (SD) samples in estuarine areas.

2. MATERIALS AND METHODS

2.1. Selection of In Situ Sites. Due to the strong metabolic activity of microorganisms during the summer, the experiment was conducted from July 2018 to September 2018. A total of nine in situ sites were selected for this experiment (Figure 1). S1, S2, S3, S4, and S5, defined as the interaction zones between fresh water and seawater (F-S zones), were selected to explore the effects of environmental factors on bacterial communities attached to PD from the Haihe River to the Bohai Bay. The statistical results showed that the annual average precipitation in the Haihe River Basin was 500−600 mm. The annual average surface runoff is 60−70 mm, and the runoff coefficient is 0.10−0.15.[35,36] S1 is in front of the Haihe tidal gate, and S2 is behind the Haihe tidal gate, which is often open in summer. Therefore, the two sites are the most obvious areas of freshwater and seawater mixing. S3, S4, and S5 are in situ sites downstream of the Haihe Estuary, and S5 is almost seawater and less influenced by anthropogenic activities. The bacterial communities on PD from S6, S7, S8, and S9 are also investigated since these in situ sites are special zones (Sp zones) deeply influenced by anthropogenic activities. S6 is located at the Tianjin Port coal terminal, and S7 is at the Tianjin Port passenger station. S8 is located at the Dagu draining river estuary where all the treated wastewater from the southern part of Tianjin City enters into the Bohai Bay. S9 is located at the Lingang petroleum industrial zone. The location coordinates and environmental parameters (temperature, dissolved oxygen, salinity, pH, total nitrogen (TN), and total phosphorus (TP)) of each in situ site were collected and are summarized in Table S2.

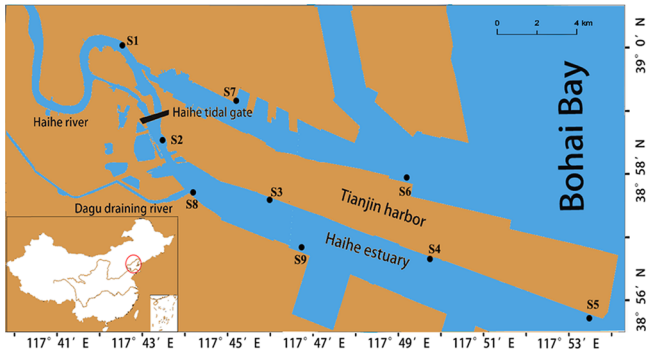

Figure 1. In situ experimental sites in the Haihe Estuary. S1, S2, S3, S4, and S5 are the interaction zones between fresh water and seawater (F-S zones). S6, S7, S8, and S9 are the special zones (Sp zones) deeply influenced by anthropogenic activities: S6 is located at the Tianjin Port coal terminal. S7 is located at the Tianjin Port passenger station. S8 is located at the estuary of Dagu draining river. S9 is located at the Lingang petroleum industrial zone.

2.2. In Situ Experimental Process. Previous studies have shown that polyethylene, polypropylene, and polystyrene are the main types of plastic debris in water samples and sediment of Bohai Bay.[33,37,38] Therefore, the samples used in this experiment included polyethylene (PE), polypropylene (PP), and polystyrene (PS) as well as polyvinyl chloride (PVC) and polyurethane (PU), which were purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). PVC, PE, PP, and PU are transparent sheets, while PS is white foam. Additionally, the sizes of PD are all 50.0 mm × 50.0 mm × 1.0 mm. For each type of PD, 10 pieces of PD were packed in 40-mesh nylon filter bags (200 mm × 200 mm), which also contained several green nylon cords to distinguish plastic types. Five nylon filter bags containing five different types of PD (PE, PP, PS, PVC, and PU) were bundled into one group and were bound with weights (3 kg) to ensure that the samples could be located on the interface between seawater and sediment. At each in situ site, there were three groups, one of which was harvested at two weeks, four weeks, and six weeks. Each obtained PD was packed in a labeled sterile vacuum bag. In addition, three replicate seawater samples were collected at each in situ site. Per replicate, 2 L of seawater samples were collected with a water sampler at the depth of 30 cm beneath the surface.[17,19] Potential microplastics and other impurities in the water samples were removed using a quantitative membrane (80−120 μm). Then, the filtered water samples were passed through a 0.22 μm Millipore polycarbonate membrane (Merck Millipore, Billerica, U.S.A.). At each in situ site, three replicate sediment samples from the upper sediment

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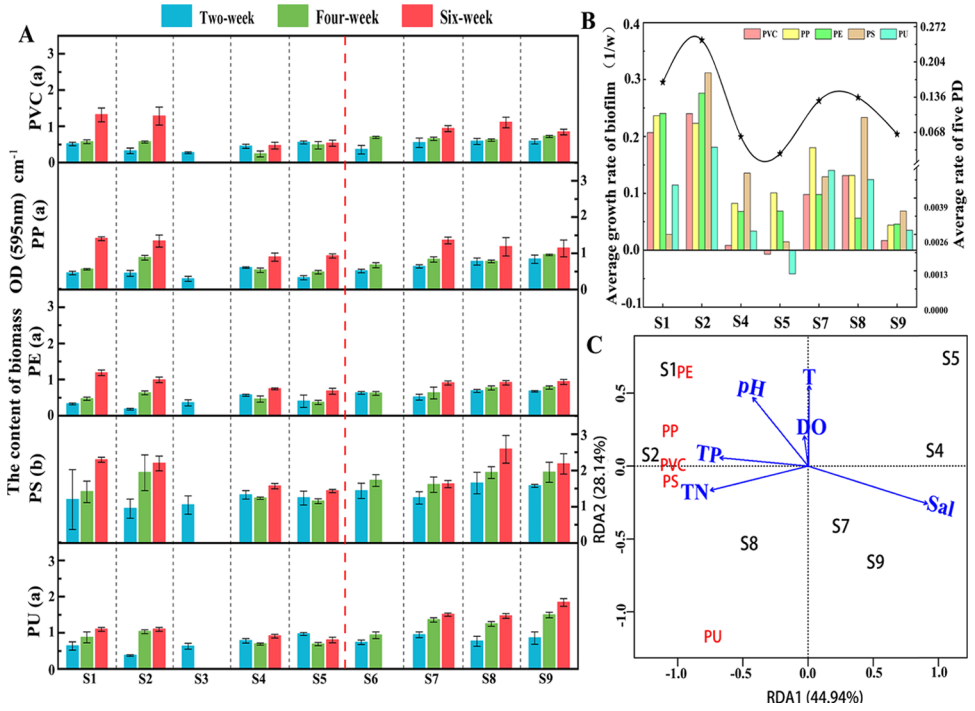

Figure 2. (A) Histogram of the optical density after crystal violet staining of PD. Different letters (a and b) indicate significant differences among the contents of biomass after the six-week exposure on five types of PD ($p < 0.05$) according to the one-way ANOVA test followed by Tukey's post-hoc tests. (B) Average growth rate of biofilms at different sites. Each histogram represents the average growth rate of biomass on PD from two weeks to six weeks. Each data point above the histogram represents the average growth rate on five PD types at each site. (C) Redundancy analysis (RDA) on the correlation between biofilm growth rates and environmental factors, including T (temperature), Sal (salinity), DO (dissolved oxygen), pH, TN (total nitrogen) and TP (total phosphorus).

layer (0−5 cm) were collected using a Van Veen Grab Sampler.[17] Approximately 100 g of grab-collected sediment was sampled in a sterile 100 mL falcon tube. The collected seawater, sediment, and PD samples were transported to the laboratory in ice bags within 2 h. Three types of travel blank samples (sterilized DI water, blank quartz sand, and virgin plastic debris (PVC, PP, PE, PS, and PU)) were used as controls during the sample collection. The filter paper, sediments, and PD were stored at −20 °C in the laboratory without any treatment.

2.3. Determination of Biofilm Content. The principle of biofilm content determination is that optical density is proportional to the quantity of biofilms on the surface of PD.[16,39] Following a previous study,[16] each PD was rinsed three times with sterilized DI water, and then three triplicate samples were placed in sterilized culture dishes. Next, each PD was air-dried at room temperature for at least 45 min and stained with crystal violet (1% w/v) for 45 min.[16] The stained PD was further washed three times with DI water and air-dried at room temperature for another 45 min. Each PD was cut into four pieces of similar size (25.0 ± 1.0 mm × 25.0 ± 1.0 mm × 1.0 mm) and placed into a 50 mL sterilized centrifuge tube (diameter = 30.0 mm) to which 10 mL of ethanol (95% v/v) was added. Finally, the ethanol solution was transferred to a quartz colorimetric dish (1 cm) to measure the optical density at 595 nm using a UV−vis spectrophotometer (DR6000, HACH Company, U.S.A.).

The average growth rate of biofilms was calculated using the following equation. OD represents optical density. OD (2) represents the biomass on each type of PD at each in situ site after a two-week exposure. OD (4) represents the biomass on each type of PD at each in situ site after a four-week exposure. OD (6) represents the biomass on each type of PD at each in situ site after a six-week exposure; ν represents the average growth rate of biofilms with the unit of "1/w" (w represents week), and t represents exposure time.

$$\nu = \frac{\Delta OD}{\Delta t} = \frac{OD(6) - OD(2)}{4}$$

2.4. DNA Extraction and 16S rRNA Sequencing. Each PD was rinsed three times with sterilized DI water. For each type of PD, three replicates were cut with scissors into small pieces less than 7.5 mm and then placed in 10 mL sterilized centrifugal tubes (diameter = 15.0 mm). The total DNA of bacterial communities on PD and sediment samples was extracted using the Power Soil DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA). For the seawater samples, the total DNA of bacterial communities on filter paper was extracted by the Power Water DNA Isolation Kit (Mobio Laboratories, Carlsbad, CA) according to the instructions. The extracted DNA of all samples was stored at −20 °C prior to sequencing.

2.5. Sequence Processing and Data Analysis. Bacterial communities on PD were detected by next-generation amplicon sequencing of 16S rRNA genes. PCR was applied to amplify the V4 hypervariable region of bacterial and archaeal 16S rRNA genes by using the primer 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and the reverse primer 806R (5′- GGACTACHVGGGTWTCTAAT-3′). The 50 μL reaction system contained 25 μL of the PCR master mix with a HF buffer, 3 μL of each primer, 10 μL of gDNA, and 6 μL of DI H_2O. The PCR amplification conditions were as follows: initial denaturation of 30 s at 98 °C, 25 cycles of 98 °C, 15 s;

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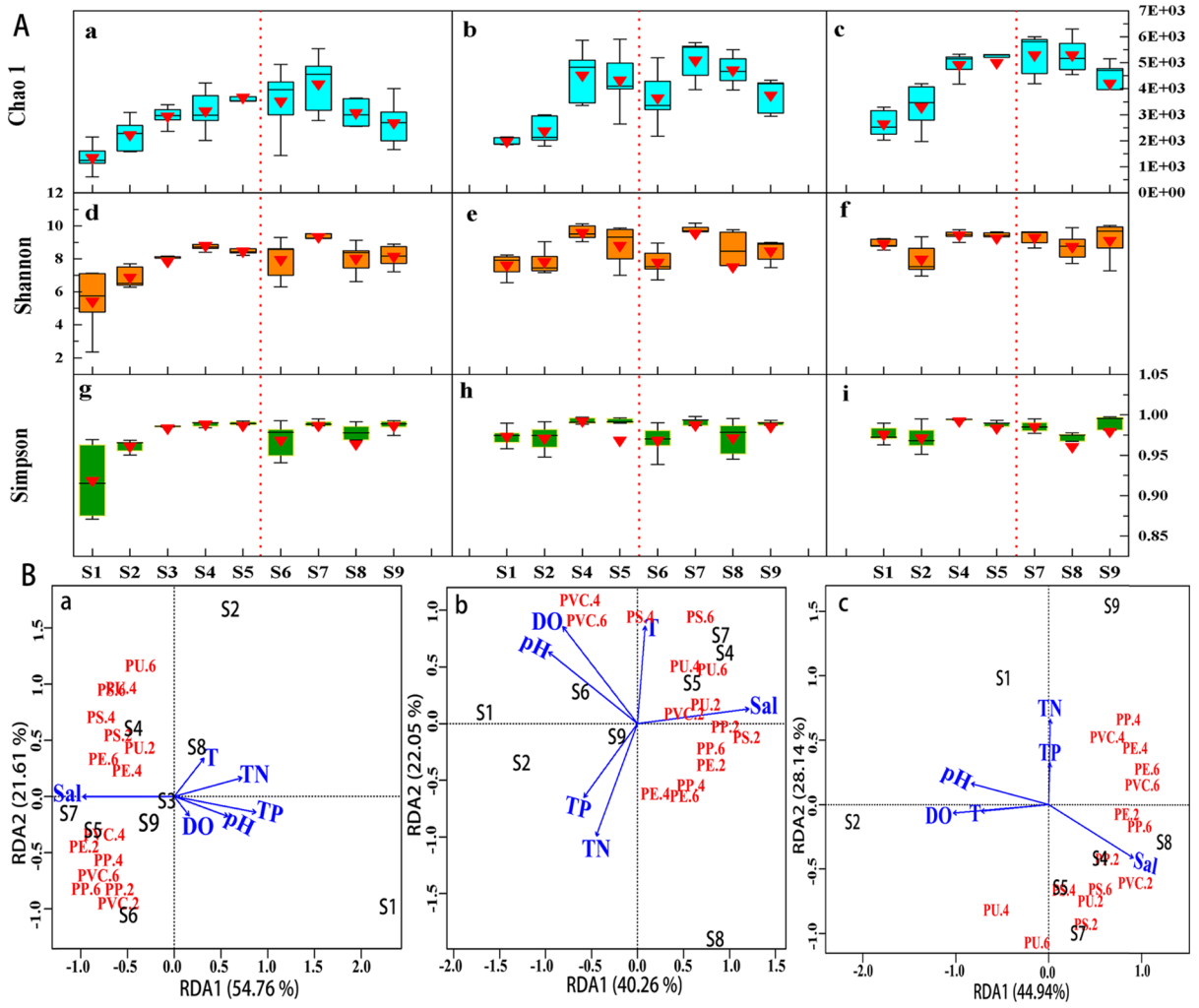

Figure 3. (A) Alpha diversity (Chao1, Shannon, and Simpson indexes) of bacterial communities on PD (n = 5) at different in situ sites. The red triangle represents the mean value, and the black solid line represents the median line. The box represents the first and third quartiles. (a) Chao 1 index after the two-week exposure, (b) Chao 1 index after the four-week exposure, (c) Chao 1 index after the six-week exposure, (d) Shannon index after the two-week exposure, (e) Shannon index after the four-week exposure, (f) Shannon index after the six-week exposure, (g) Simpson index after the two-week exposure, (h) Simpson index after the four-week exposure, and (i) Simpson index after the six-week exposure. (B) Redundancy analysis (RDA) on the correlation between alpha diversity (Chao1, Shannon, and Simpson indexes) and environmental factors, including T (temperature), Sal (salinity), DO (dissolved oxygen), pH, TN (total nitrogen), and TP (total phosphorus). Number 2 represents the Chao1 index, number 4 represents the Simpson index, and number 6 represents the Shannon index.

58 °C, 15 s; and 72 °C, 15 s, and a final extension step at 72 °C for 1 min. PCR products were purified using an agarose gel DNA purification kit (Qiagen, Chatsworth, CA) and then were subjected to an Illumina HiSeq4000 platform at GUHE Info Technology Co., Ltd. (Hangzhou, China).

2.6. Data Analysis and Statistics. The data for each sample were split from the original data according to the barcode and primer sequences based on the standard operation procedure. Vsearch v2.4.4 was used to stitch the reads of each sample to obtain the original Tags data (Raw Tags).[40] The sequences that were shorter than 150 bp and of low quality (quality score of <20) were removed from the raw sequence data. Operational taxonomic units (OTUs) were defined as clusters sharing 97% sequence identity. The OTU statistics and graphical output were conducted by Vsearch v2.4.4. The representative OTU sequence was annotated with species based on the SILVA128 database.[41] Alpha diversity was estimated by Chao 1, Simpson, and Shannon indexes using QIIME software, which was used to compare OTU abundance and evenness among samples, and then ranked abundance curves and dilution curves were generated. In addition, the relative abundance data for bacterial communities in seawater (SW), plastic debris (PD), and sediment (SD) were standardized by Z-score standardization. The differences between bacterial communities from all in situ sites and the three substrates (SW, PD, and SD) were compared by principal component analysis (PCA) and principal coordinates analysis (PCoA) based on Bray−Curtis similarities or weighed UniFrac distances.[42−44] PCA and PCoA were all carried out in R with the vegan package. Correlations between bacterial composition (average growth rate of biofilms and alpha diversity) and environmental factors were explored by redundancy analysis (RDA). RDA was carried out in R with the vegan 2.3-5 package. Meanwhile, based on R Package Vegan, PERMANOVA (Permutational multivariate analysis of variance) was used to distinguish markers for the composition of microbial communities among samples.[45] Furthermore, LEfSe analysis was used to screen marker species with different classification

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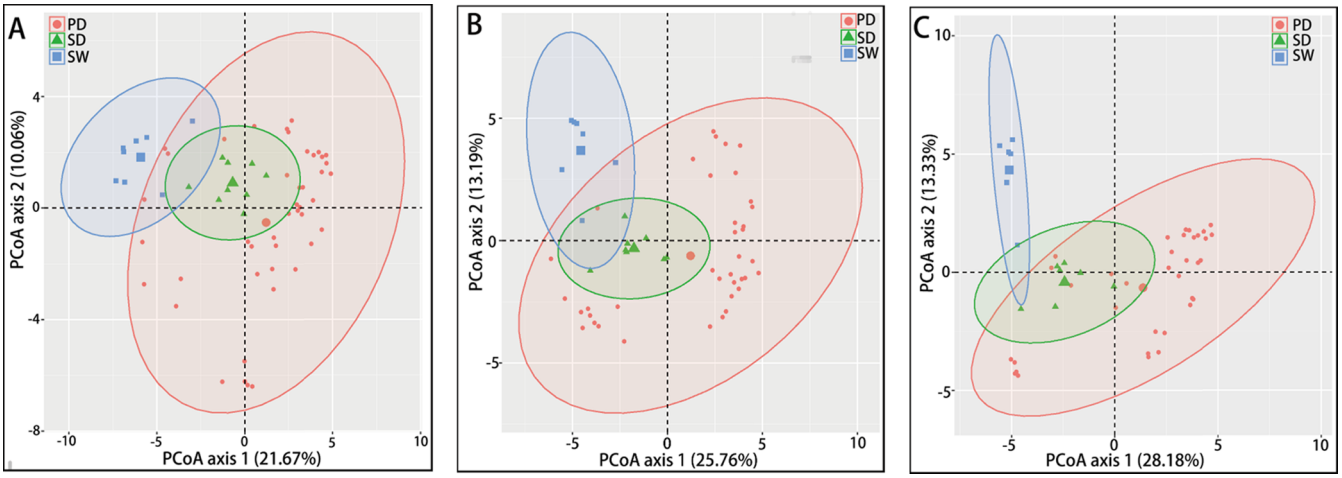

Figure 4. PCoA profile of pairwise community dissimilarity indices (Bray−Curtis), calculated from the OTU table of the bacterial communities on PD (n = 120, pink circles), seawater (n = 9, blue squares), and sediment samples (n = 9, green triangles). Ovals indicate the 95% confidence intervals for each sample type. (A) PCoA profile after the two-week exposure, (B) PCoA profile after the four-week exposure, and (C) PCoA profile after the six-week exposure.

levels. This method combined linear discriminant analysis with the nonparametric Kruskal−Wallis test (package "stats" version 3.2.0) and the Wilcoxon rank sum test. In addition, the bacterial community function was predicted by FAPROTAX and Bugbase software.[46,47] Var test, one-way ANOVA, and two-way ANOVA were used to evaluate the significant differences among samples. The post hoc analysis was followed by Tukey's post-hoc tests. $p < 0.05$ indicated that the factors were significantly different.

3. RESULTS AND DISCUSSION

3.1. Effect of Environmental Factors on the Biofilm Attached to PD. The PCR product concentration of the travel blank samples (sterilized DI water, blank quartz sand, and virgin plastic debris (PVC, PP, PE, PS, and PU)) was low, and all DNA was used for sequencing. For each travel blank sample, the number of reads was <100, which ensured that the experimental samples were not polluted during transportation.[16] Due to the water current, some of the PD experimental samples were irretrievable, including 10 PD samples (S3) for the fourth and sixth week and 5 PD samples (S6) for the sixth week. A total of 120 experimental PD samples were obtained, and their photos are displayed in Table S3. It could be seen that the biomass on the surface of PD increased with exposure time (ANOVA, $p < 0.05$) (Figure 2A). It was also observed that after the six-week exposure, the biomass on PD decreased by 41% from S1 to S5 (ANOVA, $p < 0.05$) (Figure 2A) where S1 is located at the upstream of the Haihe Estuary and S5 is closest to the Bohai Sea. Therefore, the growth of the biofilm may have been affected by the co-contribution of fresh water and seawater. In addition, the biomass on PS was significantly higher than on other polymer types (ANOVA, $p < 0.01$), indicating that the polymer type also influenced the biofilm formation (Table S4A). The average growth rate of the biofilm on PD was also calculated and is shown in Figure 2B, which indicated that the average growth rates of the biofilm on PD (from two weeks to six weeks) in S1 and S2 were significantly higher than those in S4 and S5 (ANOVA, $p < 0.01$) (Table S4B). Combining the results of RDA (Figure 2C), we found that nutrients (TN and TP) and salinity were the main factors influencing the average growth rate of the biofilm. The nutrients (TN and TP) showed a positive correlation with the average growth rate of the biofilm, while salinity showed a negative correlation with the average growth rate of the biofilm. Consequently, the samples in S1 and S2 showed faster growth rates (Figure 2B).

3.2. Effects of Environmental Factors on the Composition of Bacterial Communities Attached to PD. The taxonomic composition of bacterial communities was identified by 16S rRNA sequencing. The samples of PD, SW, and SD presented rarefaction curves with a stationary phase indicating the sufficient depth of sequencing to account for most of the taxa amplified, ensuring the accuracy of sequencing.[48] All p values of differential analysis are shown in Table S5. Alpha diversity analyses including Chao 1, Shannon, and Simpson indexes at different in situ sites were conducted and are shown in Figure 3A. As depicted in Figure 3A, the Chao 1 index increased from S1 to S3 (ANOVA, $p < 0.05$), suggesting that the increase in bacterial species on PD from S1 to S3 could be attributed to the increase in salinity (Table S2).[49] Notably, both the Shannon index and Simpson index did not show significant differences (ANOVA, $p > 0.05$) after a six-week exposure. In addition, RDA was conducted to analyze the correlation between the bacterial diversities on PD and environmental factors. As depicted in Figure 3B, the diversities of bacterial communities on PD are all positively correlated with salinity, verifying that salinity is a main factor affecting the diversity of bacterial communities colonizing PD.

In addition to salinity, the results of previous studies[17,18] suggested that bacterial diversity on PD might also be related to nutrients, temperature, or oxygen content. An in situ experiment at an estuary in the Baltic Sea (two weeks) indicated that bacterial diversity was positively correlated with salinity and temperature but negatively correlated with nutrient concentrations.[18] The results of RDA (Figure 3B) in our study also illustrate that the bacterial diversity is influenced by temperature, dissolved oxygen, and nutrients (TN and TP) at different exposure stages, but it is not obvious compared with salinity. Moreover, the variations in the Chao 1 index in the Sp zones (S6, S7, S8, and S9), which could be ascribed to the similar salinity at these sites, were not significant (ANOVA, $p > 0.05$) compared with the variation from S1 to S5 (Figure 2A).

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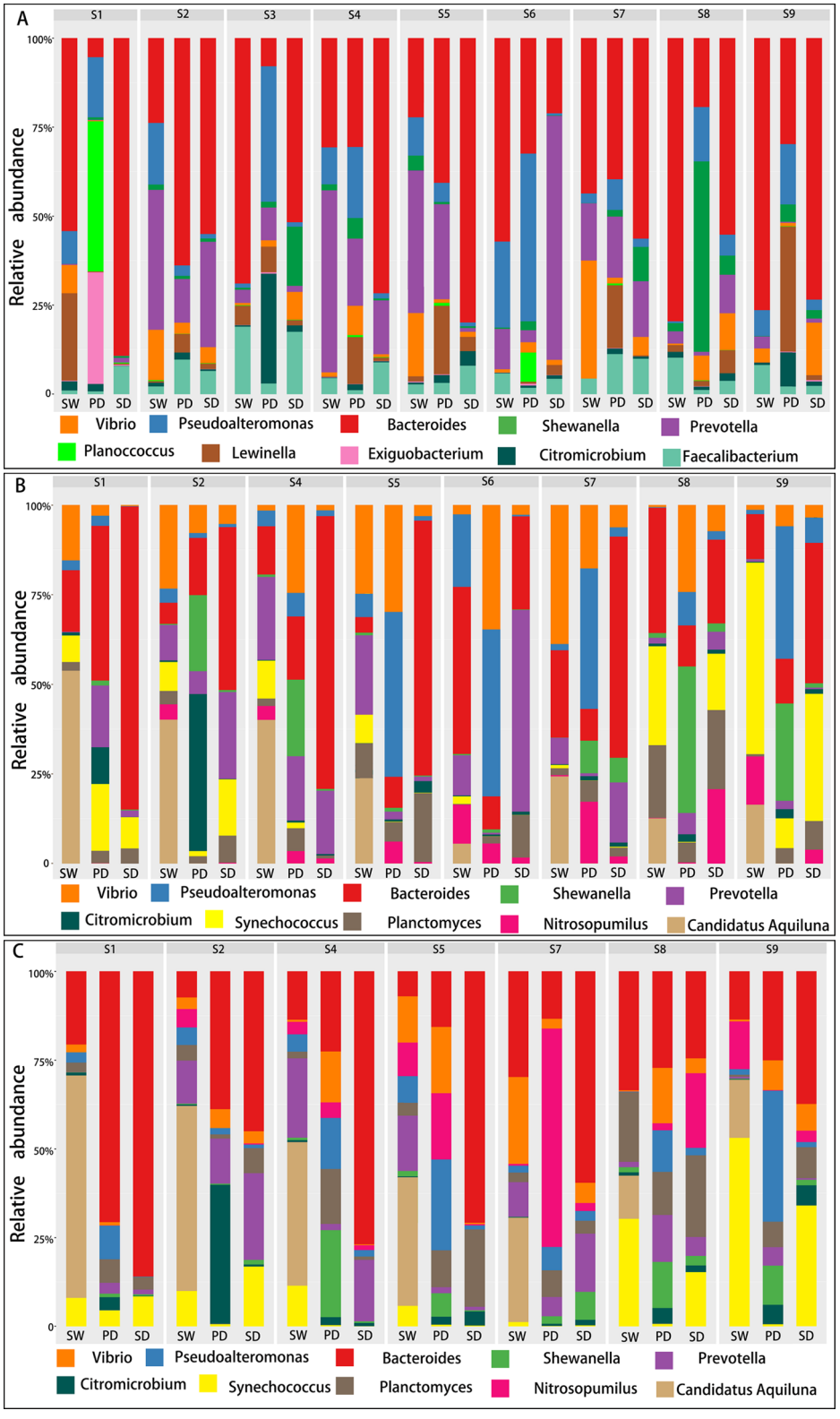

Figure 5. Relative abundance profiles of bacterial communities (top 10) on PD, seawater (SW), and sediment (SD) samples; the histogram of PD represents the sum of the bacterial communities on PD (n = 5) at the same experimental sites. (A) Relative abundance profiles of bacterial communities after the two-week exposure. (B) Relative abundance profiles of bacterial communities after the four-week exposure. (C) Relative abundance profiles of bacterial communities after the six-week exposure.

Although the Sp zones are deeply influenced by anthropogenic activities reflected by the different environmental parameters (e.g., TN, TP, T, and DO), it seems that salinity still plays a key role in the bacterial diversity on PD. Consequently, salinity should be the primary factor affecting the bacterial diversity, while temperature, dissolved oxygen, and nutrients (TN and TP) may have a secondary effect under similar salinity conditions in the Haihe Estuary of Bohai Bay.

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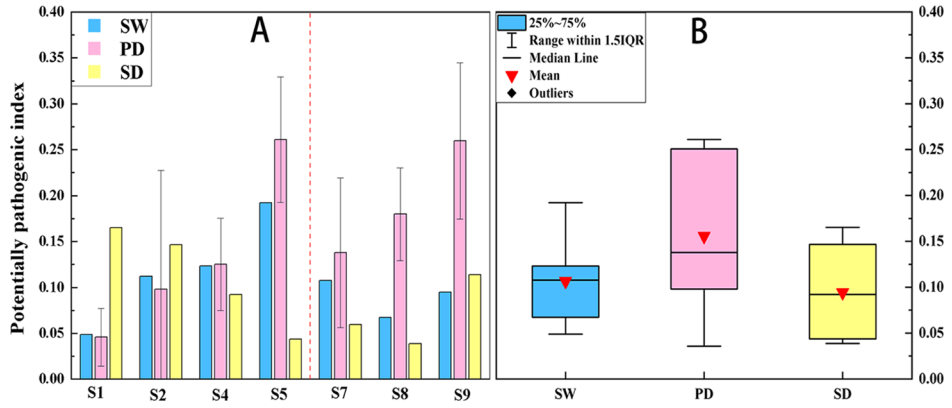

Figure 6. (A) Potentially pathogenic indexes of seawater (SW) (n = 1), plastic debris (PD) (n = 5), and sediment (SD) (n = 1) at each in situ site after six weeks based on Bugbase software. (B) Potentially pathogenic indexes of seawater (SW) (n = 7), plastic debris (PD) (n = 35), and sediment (SD) (n = 7) of all in situ sites after six weeks based on Bugbase software.

Aside from the taxonomic analysis, the PCA of the main genera was conducted to analyze the dynamics of bacterial colonization for different exposure times among different sites. As depicted in Figure S2A, there were no significant differences (ANOVA, $p > 0.05$) among the different sites after two weeks according to the PCA (Figure S2A). However, as exposure time increased, S1 and S2 separated from the other sites after the four-week and six-week exposure periods. Although S8 separated from the other sites after the four-week exposure, it partially overlapped with the other sites (except for S1 and S2) after the six-week exposure. Consequently, the results of PCA also indicated that the composition of bacterial communities might be influenced by salinity and other environmental parameters (e.g., TN, TP, T, and DO) in the estuarine areas. That is, S1 had the lowest salinity with the value of approximately 10‰, and the average salinity of S2 was approximately 17‰. However, the salinities of the other sites were all between 27 and 31‰. Consequently, S1 and S2 were separated from the other sites in the PCA after the four-week and six-week exposure periods, respectively (Figure S2B). Similar to S1 and S2, S8 was a special site, located in the estuary of the Dagu River. Since the environmental parameters (e.g., TN, TP, T, and DO) in S8 were different from the other sites (Table S2), the separation of S8 after a four-week exposure could be understandable. However, the salinity of S8 was approximately 27‰ (Table S2), which was similar to the salinities of the other sites (expect for S1 and S2); thus, S8 partially overlapped with the other sites again after longer exposure (six weeks). These combined results further verify that salinity plays a key role in the bacterial diversity on PD.

The detailed composition of bacterial communities at each in situ site was further revealed using heat maps. In Figure S3, the marker genera from each in situ site are listed. After the two-week exposure, each in situ site had a unique fingerprint, reflected by different abundances of the marker genera. Meanwhile, the marker genera of each in situ site varied as exposure time increased. Since the samples in S3 and S6 were not available after the six-week exposure, the composition of bacterial communities on PD from S1, S2, S4, S5, S7, S8, and S9 are discussed. In S1, several genera including *Nitrosopumilus*,[50] *Exiguobacterium*,[51] *Planococcus*,[52,53] *Marivita*,[54] and *Mycobacterium*[55] could be found with high abundance after the two-week exposure. According to the phylogenic tree, there was a close relationship among these genera, and some species could utilize various kinds of matter including sugar and carbohydrate polymers as a source of carbon. After the four-week exposure, a new fingerprint occurred in S1 including *Polaribacter*, *Fusibacter*, and *Bacillus*. After the six-week exposure, there was an increase in abundance (ANOVA, $p < 0.05$) of *Phascolarctobacterium*[56] and *Desulfococcus*,[57] which could utilize special materials (e.g., sulfate) as electron acceptors. In S2, *Streptococcus*,[58] *Blautia*,[59] *Lachnospira*,[60] *Faecalibacterium*,[61] and *Coprococcus*[62] were the main genera after the two-week exposure. Some species from these genera can be found in the human and animal feces. After the four-week exposure, a large number of bacteria, including *Erythrobacter*[13,23] and *Citromicrobium*,[63] which had photosynthetic capabilities, emerged. Many bacteria (e.g., *Bacteroides*, *Prevotella*, and *Sutterella*) were found after the six-week exposure. Since S4 and S5 had similar environmental parameters (except for TN), the fingerprints of S4 and S5 during the six-week exposure were similar. In S7, a significant fingerprint indicated that *Nitrosopumilus* and *Nitrospina* became the dominant genera (ANOVA, $p < 0.05$) after the six-week exposure. *Pseudoalteromonas*, a potential pathogen, showed high abundance in S8 (ANOVA, $p < 0.05$) and could produce neurotoxin and tetradotoxin leading to fish death.[19] In S9, *Citromicrobium*, *Lewinella*, *Polaribacter*, *Tenacibaculum*, and *Flavobacterium* were the dominant genera after the two-week exposure. After the four-week exposure, the abundance of *Phascolarctobacterium* and *Parabacteroides* increased (ANOVA, $p < 0.05$). The number of *Erythrobacter*,[13,23] which can conduct photosynthesis, was increased (ANOVA, $p < 0.05$) after the six-week exposure. Combining the results stated above, it was found that the bacterial communities on PD in different environments showed unique fingerprints, and the variation of their composition was time-dependent.

The top 10 bacterial genera with high abundance are also summarized and shown in Figure S4. *Bacteroides* and *Pseudoalteromonas*, which could survive in the complicated environment, were the dominant genera in most of the in situ sites after the two-week exposure. The amount of *Vibrio* in all in situ sites was increased after the four-week exposure (ANOVA, $p < 0.05$). Meanwhile, *Shewanella*, which belongs to the Vibrionaceae family, became one of the top three genera with high abundance. After the six-week exposure, it was found that the abundance of *Nitrosopumilus* and *Nitrospina* increased (ANOVA, $p < 0.05$) in many in situ sites.

Combining the results of Figures S3 and S4, the general characteristics of bacterial colonization on PD among all the in

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Environ. Sci. Technol. 2019, 53, 10763−10773

Table 1. Summary

section	summary of the results section
3.1 Effects of Environmental Factors on the Biofilm attached to PD	(1) The biomass on PD decreased from freshwater to marine environments (from S1 to S5) (ANOVA, $p < 0.05$). (2) Polymer types influence the formation of biofilms. (3) Nutrients (TN and TP) and salinity are the main factors affecting the average growth rate of biofilms.
3.2 Effects of Environmental Factors on the Composition of Bacterial Communities Attached to PD	(1) The number of bacterial species on PD increased as the distance between the in situ experimental sites and the Bohai Bay decreased. (2) Salinity is the primary factor affecting the bacterial diversity, while temperature, dissolved oxygen, and nutrients (TN and TP) also influence the bacterial diversity with similar salinity conditions in the Haihe Estuary of Bohai Bay.
3.3 Effects of Polymer types on the Composition of Bacterial Communities Attached to PD	(1) Polymer types have fewer effects on the diversity of bacterial communities on PD.
3.4 Comparison of the Composition of Bacterial Communities Colonized on Plastic Debris, Seawater, and Sediment	(1) The relative abundance of major genera in SW, PD, and SD was significantly different (ANOVA, $p < 0.01$). (2) The relative abundance of potentially pathogenic bacteria (e.g., *Vibrio*) on PD was 2−10 times higher than that in SW and SD in the estuarine environment. (3) Enrichment of *Vibrio* colonizing PD is positively correlated with salinity.

situ sites can be summarized as follows. (1) The bacteria that could utilize various matters as carbon sources and survive in the complicated environment (e.g., F-S zones) colonized on PD first. (2) Some pathogenic bacteria accumulated on PD after prolonged exposure (4−6 weeks). (3) The amount of photoautotrophic and chemoautotrophic bacteria increased at the late stage of the in situ experiment.

3.3. Effects of Polymer Types on the Composition of Bacterial Communities Attached to PD. Five types of PD (PVC, PP, PE, PS, and PU) were utilized at all in situ sites, and the Chao 1 index of each type of PD from the different in situ sites was summarized. As depicted in Figure S5, the Chao 1 index of each type of PD increased with exposure time. However, there was no significant difference in the Chao 1 index (ANOVA, $p > 0.05$) among the five types of PD for the same exposure time, indicating that the polymer type had fewer effects on the diversity of bacterial communities on PD in the Haihe Estuary of Bohai Bay.

The major genera of bacterial communities (top 10) on the different types of PD during the six-week exposure are summarized and shown in Figure S6. *Bacteriodes* and *Pseudoalteromonas* occupied the top two positions of relative abundance among the five types of PD. Moreover, the photoautotrophic and chemoautotrophic bacteria (e.g., *Citromicrobium* and *Nitrosopumilus*) also showed high abundances on the five types of PD during the six-week exposure. Combining the above results, it was found that the distribution of bacterial genera on each type of PD followed the trends described in Section 3.2.

In addition to the major genera of bacterial communities colonized on PD, the marker genera on each type of PD were distinguished. In Figure S7, all the bacterial genera on the same type of PD from the different in situ sites are summarized so that the special genera that reflected the polymer type of PD were probed. There were two criteria used to screen the marker genera: (1) the genera on each type of PD had a relatively high abundance, and (2) the genera reflecting the polymer type of PD could be detected during the six-week in situ experiments. According to our study, the marker genera on each type of PD (ANOVA, $p < 0.05$) were *Bacillus* and *Shewanella* for PVC, *Bdellovibrio* and *Lewinella* for PP, *Faecalibacterium* and *Veillonella* for PE, *Pseudoalteromonas* and *Alteromonas* for PS, and *Alcanivorax* for PU. Although the bacteria could settle on different types of PD, the marker genera indicated that the bacteria had selectivity toward the polymer type of PD and tended to colonize their preferred substrate. In addition, the colonization of some potential pathogens (e.g., *Pseudoalteromonas*[19]) on a specific PD (e.g., PS) could increase the risk on PD.

3.4. Comparison of the Composition of Bacterial Communities Colonized on Plastic Debris, Seawater, and Sediment. The differences in bacterial communities among three substrates (SW, SD, and PD) were detected via PCoA based on the OTU tables of the bacterial communities. According to the PCoA (Figure 4), it was clearly seen that the confidence ellipse of bacterial communities in SW deviated significantly from that of PD and SD (PERMANOVA, $p < 0.05$), while the confidence ellipse of bacterial communities on SD almost covered that of PD (PERMANOVA, $p > 0.05$) during the six-week exposure, indicating similarity between bacterial communities on PD and SD. As depicted in Figure 5, *Bacteriodes* and *Pseudoalteromonas* were the major genera on the three substrates (SW, SD, and PD) in most of the in situ

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Environ. Sci. Technol. 2019, 53, 10763−10773

sites after the two-week exposure, possibly due to the wide distribution and adaptability of these bacteria. After the four-week exposure, the relative abundance of *Vibrio* increased. Furthermore, the number of autotrophic bacteria (e.g., *Planoctomyces* and *Nitrosopumilus*) increased after the six-week exposure. The growth pattern of bacterial communities on the three substrates was also consistent with the results of Section 3.2. Figure 5 also showed that the relative abundance of *Pseudoalteromonas* on PD was higher than in SW and SD (ANOVA, $p < 0.05$) in most of the in situ sites during the six-week exposure, indicating that PD may serve as a novel habitat able to accumulate and enrich some special bacteria. Meanwhile, *Vibrio* as a potentially pathogenic bacteria could also colonize on PD and had a higher relative abundance compared with that on SW and SD in S4, S5, S6, S8, and S9 after the four-week exposure (ANOVA, $p < 0.05$), further suggesting the accumulation effect and the potential risk on PD.

Although previous studies have shown that PD could enhance the pathogenicity of bacterial communities,[19,24,25,27,48] the effect of environmental factors on the pathogenicity of bacterial communities has not been well studied. Some published studies indicated that temperature and salinity were the main environmental factors that affect the conditioned pathogenic *Vibrio* in seawater and sediment.[64−68] Vezzulli et al. found that the occurrence of *Vibrio* spp. on the seasonal scale was correlated with temperature,[67] and Thompson et al. suggested that temperature was a good predictor for monitoring the occurrence of *Vibrio* in North Atlantic Coastal areas.[66] However, Martinez-Urtaza et al. indicated that salinity was the primary factor governing the temporal and spatial distribution of *Vibrio parahaemolyticus*, whereas seawater temperature had a secondary effect and only modulated the abundance of *Vibrio* in periods and areas of reduced salinities.[65] Furthermore, Pezzati et al. carried out a 16-month in situ study to investigate the ecology of pathogenic *Vibrio* spp. in coastal sediment in the Mediterranean Sea, which indicated that, in the pelagic environment, the total variance of cultivable *Vibrio* data was explained by sea surface temperature (40%), salinity (13%), and organic matter concentration (7%).[68] In our study, the RDA analysis (Figure S8) showed a positive correlation between salinity and the enrichment of *Vibrio* colonizing PD in the Haihe River to the Bohai Bay. Briefly, in the estuarine environment (S3, S4, S5, S6, S8, and S9) with salinity of ≥26 (± 2‰), the relative abundance of *Vibrio* on PD (at 4 and 6 weeks) was 2−10 times higher than that of SW and SD (ANOVA, $p < 0.05$) (Figure 5B,C); in the mixed system of fresh water and seawater (S1 and S2) with salinity of ≤18 (± 2‰), the enrichment of *Vibrio* colonizing PD was not obvious (ANOVA, $p > 0.05$) (Figure 5B,C). In addition, Bugbase software (Figure 6) was used to predict potentially pathogenic indexes of PD, SD, and SW. As shown in Figure 6B, potentially pathogenic indexes of PD were higher than those of SW and SD (ANOVA, $p < 0.05$), indicating the potentially pathogenic risk on PD in the marine environments.

Moreover, nitrification and hydrocarbon degradation on PD (after six weeks) were higher than those in SW and SD (ANOVA, $p < 0.05$) (Figure S9). Previous studies have demonstrated that nitrogen and phosphorus were the limiting factors affecting growth of hydrocarbon-degrading bacteria and the rate of petroleum hydrocarbon degradation in relatively nutrient-deficient marine environments.[69] Therefore, we inferred that the strong nitrification on PD supplied sufficient nitrogen sources for the growth of hydrocarbon-degrading bacteria. In addition, potential plastic-degrading bacteria such as *Pseudomonas*,[28] *Bacillus*,[31,32] and *Arcobacter*[29] were found on PD.

Taking the Haihe Estuary of Bohai Bay as an example, this study mainly discussed the effects of environmental factors and polymer types on the average growth rate of biofilm and the composition of bacterial communities. The main results are as follows (Table 1). First, nutrients (TN and TP) show a positive correlation with the average growth rate of biofilms, while salinity shows a negative correlation with the average growth rate of biofilms. Second, salinity is the primary factor affecting the bacterial diversity on PD while temperature, dissolved oxygen, and nutrients (TN and TP) may have a secondary effect under similar salinity conditions. Third, although the polymer types have fewer effects on the diversity of bacterial communities on PD, some genera in the bacterial communities show selectivity for the polymer type of PD and tend to colonize their preferred substrate. Fourth, the relative abundance of major genera in SW, PD, and SD is significantly different. The salinity is a main factor altering the enrichment of conditioned pathogenic *Vibrio* on PD in estuarine areas. In addition, PD has a higher potential for pathogenicity based on the abundance of pathogenic species.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b03659.

Studies on microbial communities attached to marine PD in the world, studies on degradation of PD by marine bacterial communities, physical and chemical parameters of the surface water in the Haihe estuarine areas, photos of PD samples, difference analysis of biomass on PD after the six-week exposure, difference analysis of the average growth rate of biofilm on PD at different in situ sites, difference analysis of alpha diversity, PCA profile of pairwise community, heat map of marker genera on PD at different in situ sites, relative abundance of top 10 bacterial genera on PD at different in situ sites, Chao 1 index of different PD types in three time periods, major genera of bacterial communities (top 10) on different PD types in three time periods, heat map of marker genera on different PD types, correlation between the relative abundance of conditional pathogen *Vibrio* on different substrates (SW, SD, and PD) and environmental factors, and heat map of metabolic function of bacteria genera on SW, SD, and PD samples after the six-week exposure (PDF)

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