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Microplastic-associated bacterial assemblages in the intertidal zone of the Yangtze Estuary

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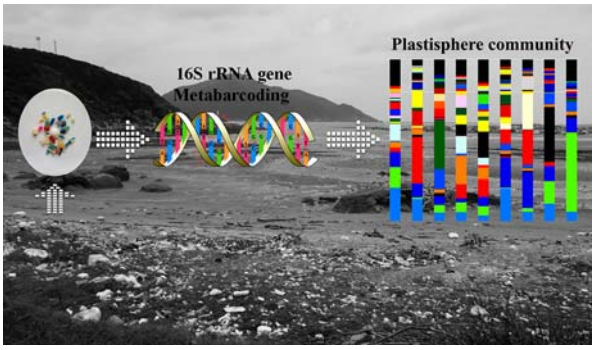
HIGHLIGHTS

- The information of plastic-associated microbial community in the intertidal zone is scanty.
- Microbial communities attached plastics discriminates among three intertidal ecosystems around the Yangtze estuary, China.
- Keystone species were Alphaproteobacteria, Gammaproteobacteria, Flavobacteriia, Acidobacteria and Cyanobacteria.
- Putatively pathogenic species acts as hitchhikers on microplastic particles.

GRAPHICAL ABSTRACT



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ABSTRACT

Plastic trash is common in oceans. Terrestrial and marine ecosystem interactions occur in the intertidal zone where accumulation of plastic frequently occurs. However, knowledge of the plastic-associated microbial community (the plastisphere) in the intertidal zone is scanty. We used high-throughput sequencing to profile the bacterial communities attached to microplastic samples from intertidal locations around the Yangtze estuary in China. The structure and composition of plastisphere communities varied significantly among the locations. We found the taxonomic composition on microplastic samples was related to their sedimentary and aquatic origins. Correlation network analysis was used to identify keystone bacterial genera (e.g. Rhodobacterales, Sphingomonadales and Rhizobiales), which represented important microbial associations within the plastisphere community. Other species (i.e. potential pathogens) were considered as hitchhikers in the plastic attached microbial communities. Metabolic pathway analysis suggested adaptations of these bacterial assemblages to the plastic surface-colonization lifestyle. These adaptations included reduced "cell motility" and greater "xenobiotics biodegradation and metabolism." The findings illustrate the diverse microbial assemblages that occur on microplastic and increase our understanding of plastisphere ecology.

1. Introduction

Worldwide plastics production was 322 million tons in 2015 (Plastics Europe, 2016). Improper plastic disposal has led to an accumulation of plastic marine debris in the oceans of the world (Cózar et al., 2014). Plastic litter has been ranked as a new addition to the list of global threats, including climate change, ocean acid and ozone depletion

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(Amaral-Zettler et al., 2015). Plastic marine debris fragments into smaller particles (microplastic, <5 mm) (Thompson et al., 2004). Deleterious effects of plastic debris on marine organisms, such as the physical and chemical effects of entanglement and ingestion by a range of marine organisms from zooplankton to fish, seabirds, sea turtles and cetaceans, have been documented (Gall and Thompson, 2015). Microplastic (3.0 or 9.6 μm) ingested by mussels translocated from the gut to the circulatory system (Browne et al., 2008). Microplastic inside cells can cause significant impacts at the tissue and cellular level, and interfere with energy reallocation, reproductive success, and offspring performance (Moos et al., 2012; Sussarellu et al., 2016).

Microplastic can be colonized by various marine organisms, that may affect the fate and ecological impact of plastic pollution (Kiessling et al., 2015). Due to its light weight and persistence, plastic debris transported by ocean currents and winds may transfer organisms to non-native habitats, posing a threat to biodiversity and coastal environments (Barnes, 2002; Gregory, 2009). Plastic debris lasts much longer than most natural floating substrates such as macroalgae, feathers, or wood, and represents a novel type of pelagic substrate for microbial colonization and transportation (Zettler et al., 2013). Zettler et al. (2013) characterized epiplastic microbial communities (termed the 'plastisphere') from the North Atlantic ocean using 16S metabarcoding. They found that the taxonomic composition of microbial communities on plastic particles was distinct from the surrounding seawater. These plastic inhabitants could be capable of degrading plastic polymers (Zettler et al., 2013; Reisser et al., 2014), altering the buoyancy of polymers (Ye and Andrady, 1991; Lobelle and Cunliffe, 2011), and affecting the toxicity of plastics (Pham et al., 2012; Zettler et al., 2013). The oceanic interactions between microorganisms and plastics are of significant ecological concern. Microbial assemblages associated with plastics differ in relation to the polymers in the plastic substrate, the geographical location, and the sampling date (Oberbeckmann et al., 2014; Amaral-Zettler et al., 2015; De Tender et al., 2015). The metagenomic approach was used for determining the genes expressed in the plastisphere from the North Pacific Subtropical Gyre. The results demonstrated that the microbial populations on microplastics have lifestyles, metabolic pathways, and biogeochemical activities that differ from those of microbes in the surrounding seawater (Bryant et al., 2016; Debroas et al., 2017).

Due to the ecological importance of the plastisphere, additional study of the plastic-colonizing community in different environments may provide information useful for mitigation of plastic debris. Serving as the hydrographic link between anthropogenic activities in the upland and the adjacent marine environments makes the intertidal zone become a hot spot for plastic debris accumulation, which has been documented by previous studies (Aguilera et al., 2016; Browne et al., 2010). Previous studies of the ocean plastisphere focused on floating plastic litter on the water surface. Most plastic debris accumulates in the sediment, particularly in the coastal zones (Harrison et al., 2014). However, knowledge of the microbial community attached to plastic marine debris in the sediment environment is limited (De Tender et al., 2015). We used next-generation amplicon sequencing to study the bacterial community on microplastics collected in the intertidal zone of the Yangtze estuary, China. Our aims were to study the: 1) plastic-associated microbial communities in the intertidal zone; 2) patterns in plastic-associated community structure related to geographic locations and 3) key bacterial groups and their corresponding functionality in plastic attached communities.

2. Materials and methods

2.1. Sample collection

Microplastic samples were collected at three stations in April 2016: Xiangshan Bay (29°30′29.9″N, 121°27′27.3″E), Chongming Island (31°36′57.9″N 121°23′30.0″E) and Lvsi Port (32°04′52.4″N 121°36′10.1″E) (Fig. S1). Chongming Island is located in the Yangtze estuary and is strongly influenced by the freshwater from the Yangtze River. During low tide, a total of thirty four microplastics stranded on the surface of the muddy intertidal areas were collected with sterile forceps. Microplastics were placed into sterile 15 ml plastic tubes, immediately stored on ice, and transported to the laboratory where the samples were stored at −20 °C until analysis.

2.2. Spectroscopic analysis

The polymeric matrix of plastic pieces was studied using micro-FTIR spectroscopy (BRUKER LUMOS). Two replicate FTIR spectra were acquired on different spots of each PMD piece. All spectra were recorded with the average of 32 scans, at 4 cm^{-1} resolution. Spectra processing and analysis were done according to a recent plastic identification protocol (Zhao et al., 2017). Each measured FTIR spectra was compared with spectra in a commercial library (BioRad-KnowItAll Informatics System, Thermo Fisher Scientific Inc.).

2.3. DNA extraction and sequencing

DNA in plastic samples was extracted using MoBio Powersoil DNA extraction kits (MoBio Laboratories, Carlsbad, CA) following manufacturer instructions. The V3-V4 variable region of the 16S rRNA genes was amplified using the primer sets of 319F (5′-ACTCCTACGG GAGG CAGCAG-3′) and 806R (5′-GGACTACHV GGGTWTCTAAT-3′). PCR amplification was conducted as follows: initial denaturation at 98 °C for 30 s; 35 cycles of 98 °C, 10 s; 54 °C, 30 s; and 72 °C, 45 s, and a final extension step at 72 °C for 10 min. The 25 μl PCR master mixes contained 50 ng of template DNA, 12.5 μl of the Premix Ex Taq™ hot start version, and 0.1 mM of each primer. PCR products were purified using beads. The resulting amplicons were pyrosequenced using Illumina MiSeq v3 technology (2 × 350 bp, paired-end) by LC SCIENCES, Hangzhou, China. DNA samples (3 DNA samples × 3 sampling sites) were successfully assessed from 9 (4.61 ± 0.27 mm) of the 34 microplastic particles collected from the 3 locations, and used for further high-throughput sequencing. The raw sequence reads were deposited into the NCBI sequencing read archive under Accession No SRP125152.

2.4. Sequencing data treatment

Raw sequences were processed using MOTHUR v.1.33.3 software (Schloss et al., 2009). Briefly, barcode and adapter primer sequences were clipped off the reads. Paired reads were assembled. The sequences met the following criteria: (1) the sequence matches the 806R primer and one of the used barcode sequences; (2) no ambiguous bases were found within the sequence; (3) the sequence had a length of ≥200 bp; (4) the sequence had an average quality score ≥25; (5) homopolymers in the sequence were <8 bp. Sequences containing an N (undetermined nucleotides) ratio >5% of the sequence and generating low-quality value (Q) reads (the base number of Q < 10 >20% in the entire read) were removed from the data set.

2.5. Data analysis

CD-HIT was used for clustering raw sequences into operational taxonomic units (OTUs) at a 97% similarity level. The OTU statistics and graphical output were conducted in R program version 3.3.1. The longest sequence in each category was designated as the representative OTU sequence which was annotated by RDP, Greengenes and the NCBI 16S Microbial database. Alpha Diversity was calculated in QIIME v1.3.0. PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states), encompasses data from the United States Department of Energy Joint Genomic Institute's Integrated Microbial Genomes (IMG) database (Markowitz et al., 2012). PICRUSt was utilized to normalize the 16S copy number for each OTU and predict the

functionality represented by KEGG Orthology (Kanehisa and Goto, 2000; Kanehisa et al., 2014). Associations between the microbial communities were examined by using network analysis and Pearson rank correlations. Only correlations with an R-value >0.9 were considered to be a valid interaction event. We used NetworkX (Hagberg et al., 2008) to explore and visualize the network. To describe the topology of the resulting networks, degree (DC), closeness (CC) and betweenness centrality (BC) were calculated.

3. Results

3.1. Polymer types of plastic samples

The chemical composition of nine plastic samples was composed of commonly used plastic types. Nine plastic particles were confirmed as polyethylene (PE, n = 4), polypropylene (PP, n = 3), and polystyrene (PS, n = 2) (Fig. 1 and S2).

3.2. Bacterial richness and diversity across plastic polymers and sampling sites

Diverse bacterial communities residing on plastic debris were found, with observed OTUs of 498, 661, 778 in Lvsi port, Chongming island, and Xiangshan bay, as well as 588, 920, 876 different OTUs of PS, PP and PE type. The values estimated by the Good's coverage for Lvsi port, Chongming Island, Xiangshan Bay were 99.2%, 99.2%, 98.7%, respectively. Chongming Island had a higher Chao1 richness and Shannon-Wiener index than Lvsi port (*t*-test, $P = 0.0317 < 0.05$) and Xiangshan Bay (*t*-test, $P = 0.03216 < 0.05$) (Table 1). No differences of bacterial richness and diversity were found among the three plastic types.

The nonmetric multidimensional scaling (nMDS) analysis, based on bacterial community OTUs, discriminates Lvsi port, Chongming island and Xiangshan bay (Fig. 2). The stress value is 0.069. Furthermore, Chongming island is plotted alone and far from Lvsi port and Xiangshan bay, indicating a different microbial assemblage OTU. The ANOSIM analysis also indicated significant variances in community composition among all sample sites ($R = 0.9588$, $P = 0.003 < 0.01$).

Table 1
Bacterial richness (Chao1 index) and diversity (Shannon-Wiener) across the sampling sites and plastic types.

Site	Replicate	Polymer type	Chao1	Shannon-Wiener
Lvsi Port	1	PE	242.54	3.45
	2	PE	223.71	3.75
	3	PP	318.13	3.16
Chongming Island	1	PS	468.39	3.92
	2	PP	428.25	4.12
	3	PS	394.88	4.26
Xiangshan Bay	1	PE	351.09	3.496
	2	PP	304.89	3.51
	3	PE	316.00	3.84

Microbial assemblages on plastic particles were dominated by Proteobacteria, Cyanobacteria, Bacteroidetes and Actinobacteria across all sampling sites (Fig. S3). Acidobacteria, Gemmatimonadetes and Chloroflexi significantly discriminated samples from Chongming island from samples of Lvsi port and Xiangshan bay. On the level of family, bacterial community also varied among the three geographical areas (Fig. 3). The five most common families in Chongming island were Erythrobacteraceae (10.6%), Sphingomonadaceae (9.5%), Comamonadaceae (8.6%), norank_c_Cyanobacteria (8.1%), and Blastocatellaceae_ Subgroup_4_ (7.9%). In the Lvsi port the most common families were Rhodobacteraceae (16.4%), Erythrobacteraceae (13.9%), Moraxellaceae (10.1%), FamilyI_o_SubsectionIII (9.6%), Planococcaceae (8.6%). The most frequent families of Xiangshan bay included norank_c_Cyanobacteria (22.4%), Saprospiraceae (12.5%), Pseudoalteromonadaceae (10.5%), Flavobacteriaceae (8.5%), Erythrobacteraceae (8.0%). The Cyanobacterium genus *Phormidium* was common in Lvsi port, Chongming island and Xiangshan bay, accounting for 2.1%, 1.2% and 0.8% respectively. Additionally, *Leptolyngbya* accounted for 1% of the total sequences from the Chongming island. Genus *Vibrio* was detected in Xiangshan bay, occupying 0.4% of total sequences. Genus *Pseudomonas* existed on all plastics, with less than <0.01% of the abundance in each sampling site.

3.3. KEGG pathways associated with the plastics

Among the main KEGG orthologs (KOs) found in the plastic-attached communities, some metabolic pathways were underrepresented. These

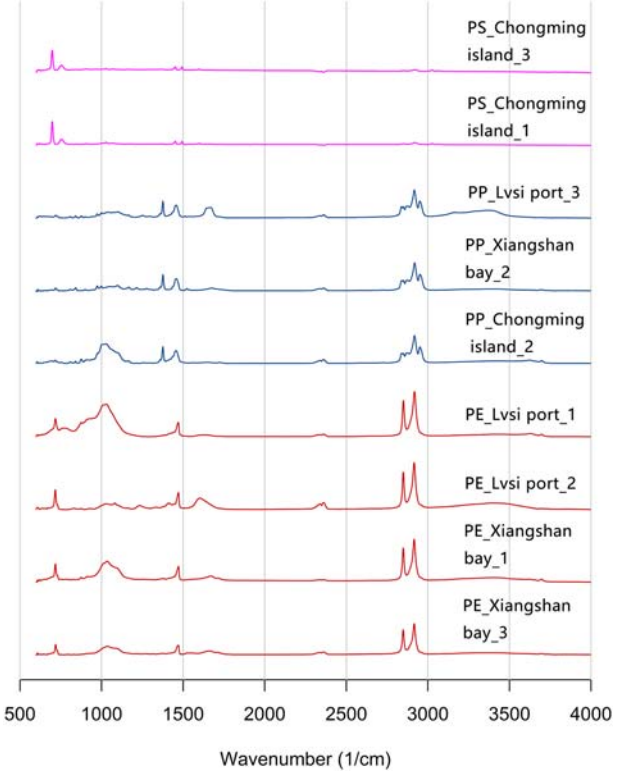

Fig. 1. FTIR spectra of microplastics collected from three locations sequenced in the current study (polyethylene, PE; polypropylene, PP; polystyrene, PS).

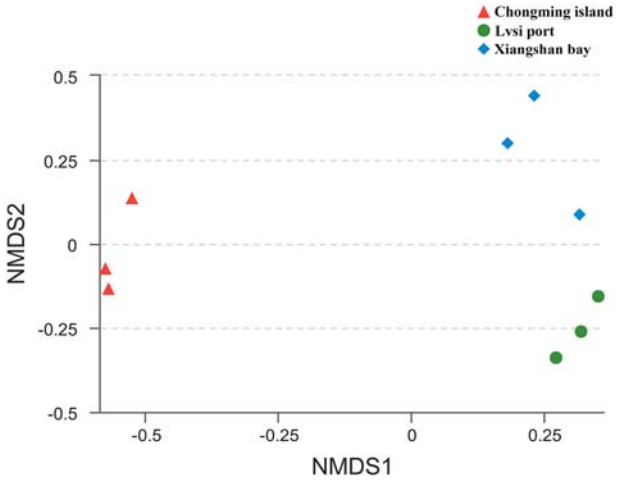

Fig. 2. Nonmetric multidimensional scaling (nMDS) analysis of bacterial communities colonizing microplastics across three sampling stations (stress: 0.069). Bray-Curtis dissimilarity of plastisphere communities was based on the OTUs level across samples.

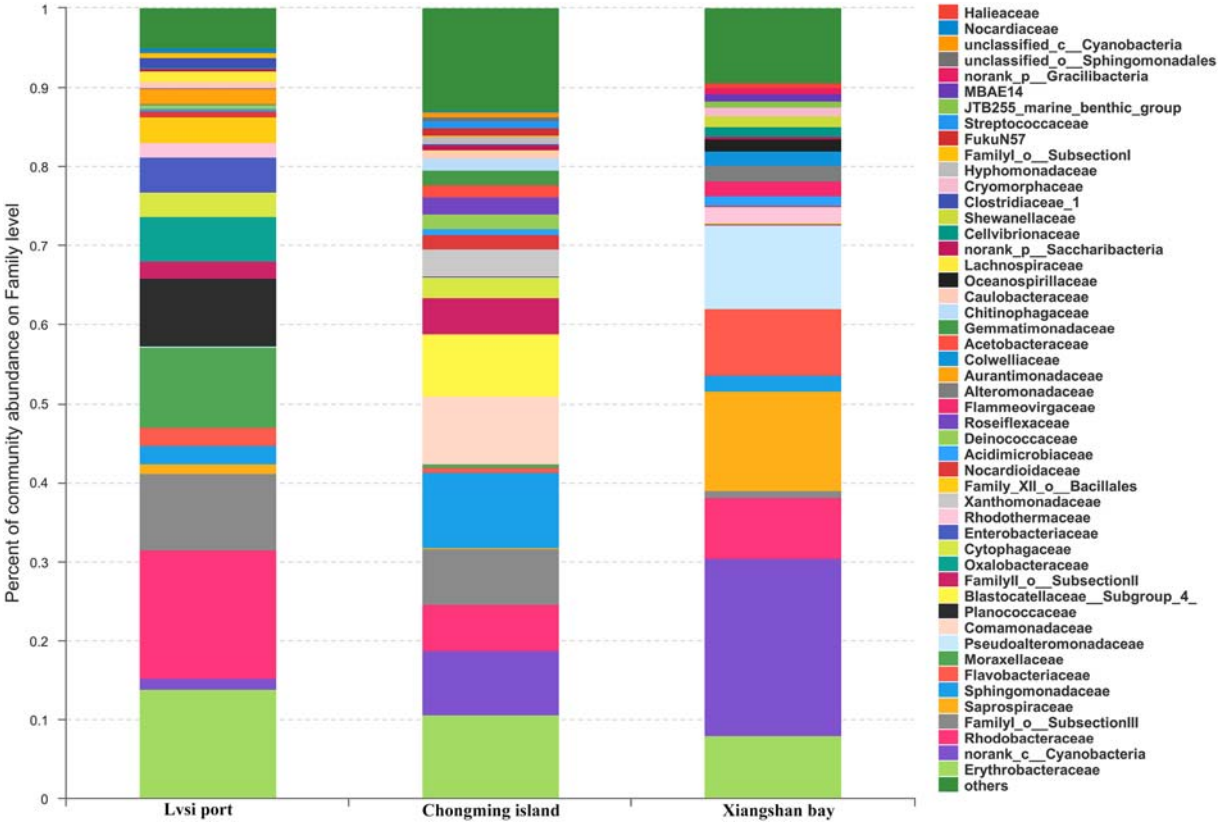

Fig. 3. Abundance of bacterial families at three locations. Clades with abundances >0.1% in samples are shown.

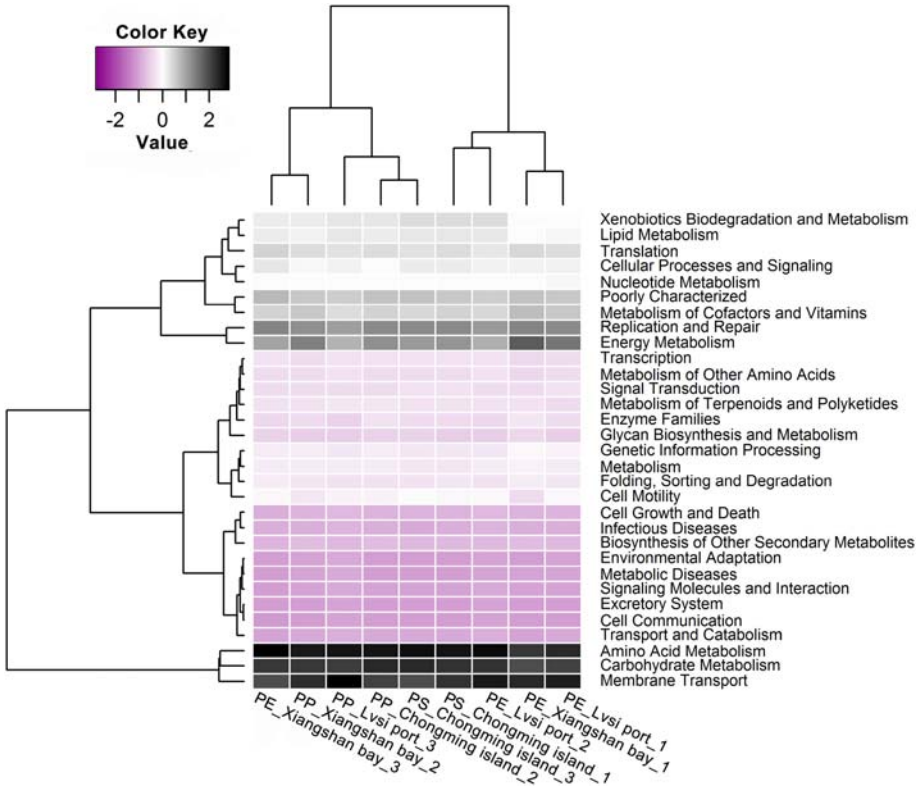

Fig. 4. Abundance of KEGG pathways present at the surface of each plastic particle. Positive values and black colors suggest the enrichments, (PE: polyethylene, PP: polypropylene, PS: polystyrene).

included "Environment Adaptation", "Signaling Molecules and Interaction" and "Cell Motility". Other KO abundances associated to the plastisphere communities seem enriched including "Amino Acid Metabolism", "Carbohydrate Metabolism", "Membrane Transport", "Energy Metabolism" and "Xenobiotics Biodegradation and Metabolism" (Fig. 4).

3.4. Network analyses of the plastisphere community

The plastisphere network was constructed with the first 100 dominant OTUs, comprising about 69.2% of the total number of taxonomic units. The resulting bacterial network consists of two disconnected sub-networks. One sub-network was fully connected consisting of 91 OTUs as nodes (Fig. 5). The other sub-network of 9 OTUs was removed from the analysis. The mean number of neighbors within the main cluster was 11.0 and 3 OTUs belonging to Alphaproteobacteria (OTU292), Cyanobacteria (OTU250), Acidobacteria (OTU349) presented 36 neighbors, respectively. Degree (DC), closeness (CC) and betweenness (BC) centrality were computed to evaluate the taxa importance within the network. Based on high scores of these topological properties (arbitrarily determined as DC > 0.2, CC > 0.4 and BC > 0.02), 10 OTUs were selected, representing putative keystone genera within this molecular network (Figs. 5 and S5). These taxa mainly belonged to the Proteobacteria (Table S1). Taxonomic units belonging to Cyanobacteria, Bacteroidetes, and Acidobacteria were characterized as keystone genera.

4. Discussion

Microbial colonization of plastic marine debris is well known (Carpenter et al., 1972), but detailed investigations of the plastisphere community are uncommon. This is especially true for studies using culture-independent methods and these studies have mostly been performed on plastics floating on surface waters (Amaral-Zettler et al., 2015; Bryant et al., 2016; De Tender et al., 2015; Didier et al., 2017; Oberbeckmann et al., 2014; Zettler et al., 2013). The present study compared plastisphere communities at three locations with intertidal environments and found different bacterial communities among the locations (Fig. 2). This result is consistent with previous plastisphere studies from the surface of the seawater (Amaral-Zettler et al., 2015; Oberbeckmann et al., 2014; Oberbeckmann et al., 2016). Proteobacteria and Bacteroidetes attached to microplastics were common in the present study (Fig. S3). Relative microbial abundance has been used to indicate the different biofilm formation stages (De Tender et al., 2015). Proteobacteria appear to be early biofilm colonizers of non-natural substrates in marine environments, followed by secondary colonizers such as Bacteroidetes (Lee et al., 2008). The relatively higher abundance of Bacteroidetes in Xiangshan bay suggests a biofilm formation stage differing from the other two sites and may be a contributor to the variation of plastisphere communities across the sample sites. The significantly higher bacterial richness and diversity in Chongming island (in the Yangtze estuary, Fig. S1) may be explained by the complex environment there. The influx of freshwater from the Yangtze river might influence

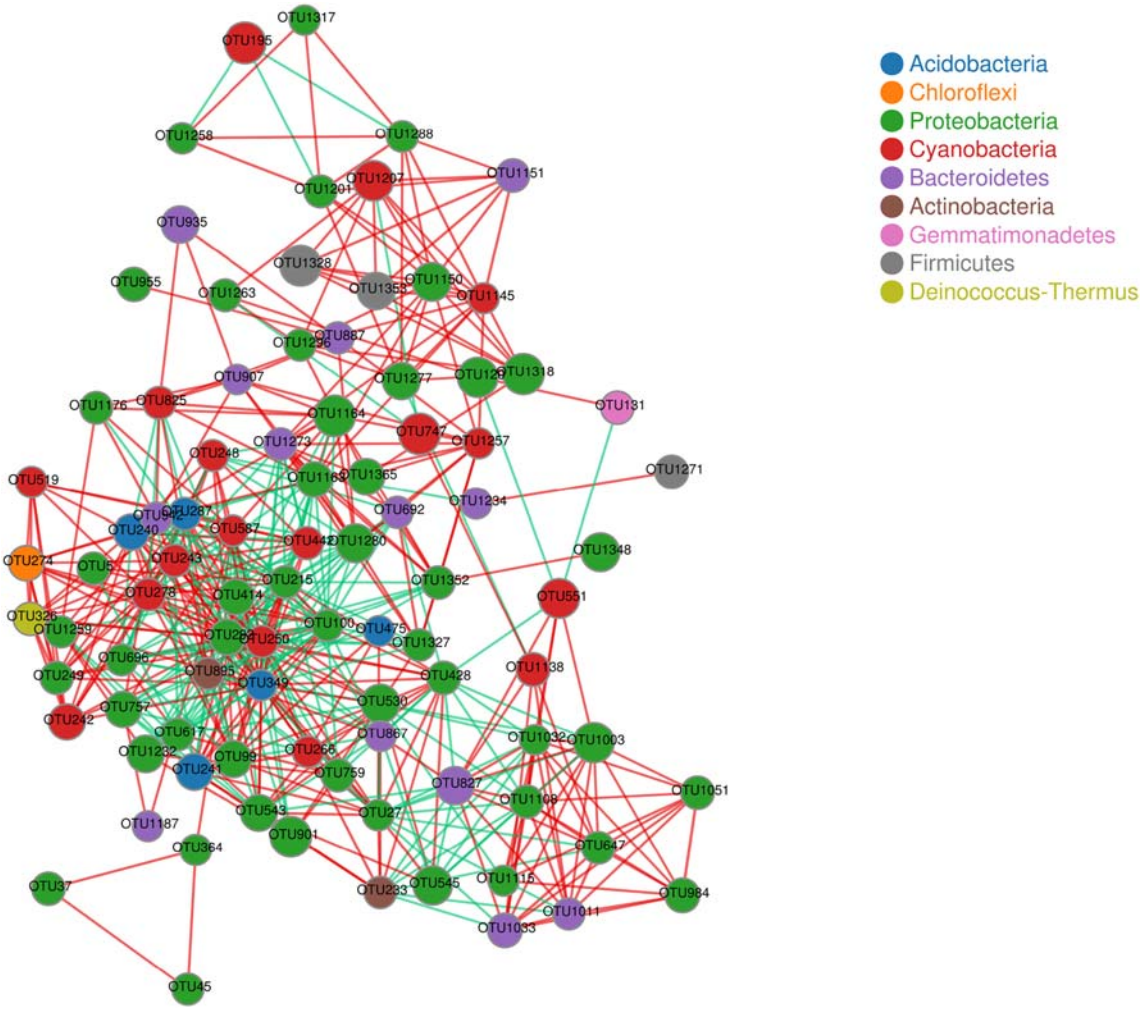

Fig. 5. Network diagram of the 100 most abundant OTUs colonized on the plastic surfaces. Table S1 shows the full taxonomy for all the OTUs within the network.

the microbial assemblages colonizing the microplastics. Additionally, estuaries act as a transition zone between terrestrial and marine environments, and complex matter exchange in this area may also contribute to the characteristic plastic community profile on Chongming island. Environment-related physicochemical properties such as salinity, temperature, turbidity, which can be correlated with the diversity of plastisphere communities (De Tender et al., 2015), were lacking in the present study and should be employed in future studies on the richness and diversity of bacterial structures on plastics. In addition, microbial communities on other types of substrates such as macroalgae and feathers should be studied and compared with communities on plastic particles. Contrary to plastisphere communities in seawater (Bryant et al., 2016; Zettler et al., 2013), a relatively high abundance of Actinobacteria was observed at all sampling sites (Fig. S3). This might have occurred because microplastics in the intertidal environments are impacted by factors from both terrestrial and aquatic environments (Elifantz et al., 2013; Servin et al., 2008). The plastisphere on Chongming island included higher abundances of sediment microbes such as Acidobacteria, Gemmatimonadetes and Chloroflexi. Strong freshwater runoff from the Yangtze River, high rates of particle deposition, and sediment resuspension in Chongming island might have resulted in stronger exchange between the water and sediment bacterial communities (Feng et al., 2009). Although the taxonomic profiles of the seawater and sediment samples are lacking in the current study, bacterial families detected on microplastics are similar to those in natural microbial communities of the surrounding environments, suggesting the origin of plastic colonizers (De Tender et al., 2015; Feng et al., 2009; Hu et al., 2014). Lack of significant discrepancies in microbial composition among the PS ($n = 2$), PE ($n = 4$) and PP ($n = 3$) types was inconsistent with a previous study by Zettler et al. (2013), who reported distinctions of bacterial profiles in the PP and PE samples. The results of nMDS analysis based on taxonomic community OTU also did not discriminate the three polymer types (Fig. S4).

By correlation network analysis, a core bacterial group that had important microbial associations within the plastisphere community was identified. The Alphaproteobacteria were the most abundant class in this network as well as that in the microbial communities attached to plastics from the North Pacific and North Atlantic Gyres (Bryant et al., 2016; Didier et al., 2017). Six of ten putative keystone OTUs from this associated group belonged to the Alphaproteobacteria (Table S1). The Order Rhodobacterales, which are dominant and ubiquitous primary surface colonizers in temperate coastal waters of the world (Dang et al., 2008), had 11 OTUs in this network and two of these are keystone species. Rhodobacterales can produce quorum-sensing signals, involved in various microbial processes such as biofilm formation and development (Gram et al., 2002; Martens et al., 2007). Thus, these bacteria may shape the subsequent biofilm community structure and development via intercellular communication and interaction. Additionally, Roseobacter clade members, alternating between planktonic and sessile lifestyles (Didier et al., 2017; Orr et al., 2004), are the initial fastest utilizers of various carbon resources in coastal environments. For example, *Rhodococcus ruber* degrades PET in biofilms (Orr et al., 2004). Bacteroidetes phylum (13 OTUs in the network), are frequently found on organic matter particles (DeLong et al., 1993) and play an important role in the marine carbon cycle. Whole genome analysis of *Gramella forsetii* revealed that Bacteroidetes representatives could adapt to degrading polymeric carbon sources (Bauer et al., 2006). One keystone OTU (OTU1273) in our network clustered with *Gramella*. In addition, the Cyanobacterium genus *Phormidium* (3 OTUs in the network), including putative hydrocarbon degrading taxa, were detected in the microbial network which is consistent with previous reports (Bryant et al., 2016; Zettler et al., 2013). The results of comparing selected KEGG genes from each plastic indicated an underrepresentation of metabolic pathways and genes (i.e. “Cell Motility”), which is consistent with the attached lifestyle. Some metabolic pathways, such as, “Xenobiotics Biodegradation and Metabolism”, are enriched in the plastic-associated communities (Fig. 4). These KOs findings are consistent with earlier research (Bryant et al., 2016; Didier et al., 2017). The Cyanobacteria (= phototroph bacteria, 17 OTUs) also play an important role within the network. The occurrence of oxygen producing bacteria like Cyanobacteria on plastic surface is especially important during oxidation of the polymers (Eich et al., 2015).

Consistent with previous reports (Didier et al., 2017; Keswani et al., 2016; Zettler et al., 2013), some bacterial taxa found on our microplastic samples were associated with human and animal pathogens. Although not statistically significant, *Pseudomonas* spp. (<0.01% of the abundance) containing several fish pathogens (i.e. *Pseudomonas anguilliseptica*) in marine and brackish waters worldwide were also detected on the plastic surface (Lalucat et al., 2006). With a small proportion of taxonomic abundance (0.4%), the potential pathogens in *Vibrio* were only found at the plastic surfaces from Xiangshan bay. Similarly, *Leptolyngbya*, members of the coral black band disease group, had a relative higher abundance (1.6%) on microplastic from Chongming island (Myers et al., 2007). In contrast to earlier research, the abundances of potentially harmful microorganisms detected in the present study were rare. Zettler et al. (2013) found *Vibrio* spp. dominated the plastisphere community on one of the PP samples from the North Atlantic, constituting nearly 24% of the bacterial OTUs. The family Vibrionaceae was present on 13% of microplastic samples collected from the North Sea (Kirstein et al., 2016). These putative toxic species were only detected on a few of the plastic samples in the current and previous studies, suggesting that they are not representative. The present pattern of potentially pathogenic species shows that they are microbial hitchhikers and opportunistic microbes colonizing plastics marine debris (Didier et al., 2017; Keswani et al., 2016). Microplastic debris is ubiquitous in intertidal environments and could be ingested by various organisms that inhabit these areas (Browne et al., 2010; Cauwenberghe and Janssen, 2014; Mizraji et al., 2017). These potentially toxic bacteria on stranded microplastic can enter the food chain, possibly survive in the animal digestive system and be transferred to different trophic levels. This could lead to undesirable impacts on the intertidal ecosystems, commercial farming and human health (Cluzard et al., 2015; Kirstein et al., 2016; Mincer et al., 2016). Although were unable to identify potential pathogens on the species level based on 16s rRNA gene data, the possibility that microbes on our plastic surface such as *Vibrios*, *Pseudomonas*, and *Leptolyngbyas* could be pathogens, cannot be excluded.

5. Conclusion

Our data demonstrated that microbial communities colonized the surface of microplastic particles in the intertidal environments around Yangtze estuary. These communities were clustered by geographical locations rather than by polymer types. Microplastic particles harbored bacterial assemblages originating from both aquatic and sedimentary areas. Consistent with previous studies (Bryant et al., 2016; Didier et al., 2017), specific metabolic pathways were enriched within the plastic-colonizing bacterial communities. Network analysis predicted a core bacterial group on the plastic surface. Despite the limited abundance, several genera clades, including possible pathogens, were detected in the samples. An understanding of plastisphere ecology may expedite environmental policy changes and guide effort to reduce or eliminate plastic marine debris. Future research should study the factors (e.g. physical and chemical plastic properties, microbial populations on other non-plastic substrates and environmental parameters) that shape plastisphere communities. The relatively inefficient approach of extracting DNA from micro-sized plastic particles produced a small dataset in present study. This could be improved using more advanced protocols (Debeljak et al., 2017).

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2017.12.105.

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