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# Microplastics from mulching film is a distinct habitat for bacteria in farmland soil

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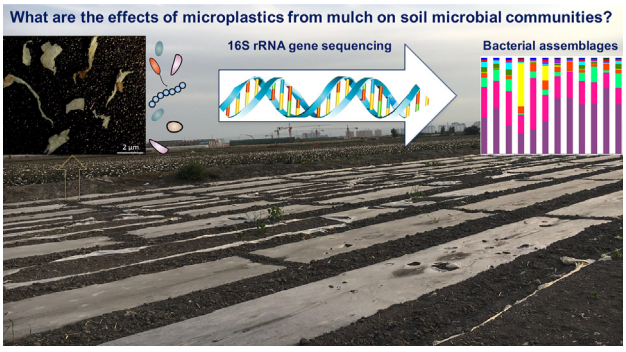
## HIGHLIGHTS

- The effects of microplastics on soil microbial communities are poorly described.
- Microbial community on microplastics differed structurally from those on other residues and in soil.
- Keystone species were Acidobacteria, Chloroflexi, Gemmatimonadetes and Bacteroidetes.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Microplastics, as an emerging pollutant of global importance, have been well documented in aquatic ecosystems. However, little is known about the effects of microplastics on agroecosystems, particularly for soil microbial communities. Herein, microplastics collected from cotton fields in Xinjiang, China, were analysed with a scanning electron microscope (SEM) and high-throughput sequencing to investigate the attached bacterial communities. Microplastic surfaces, especially pits and flakes, were colonized by various microorganisms, suggesting active hydrolysis of plastic debris. The bacterial communities colonizing microplastics were significantly different in structure from those in the surrounding soil, plant litter and macroplastics. In addition, statistical analysis of differentially abundant OTUs showed that microplastics serve as a "special microbial accumulator" in farmland soil, enriching some taxa that degrade polyethylene, such as Actinobacteria, Bacteroidetes and Proteobacteria. Co-occurrence network analysis revealed that the biotic interactions between microorganisms on microplastics are as complex as those in soil, and Acidobacteria, Chloroflexi, Gemmatimonadetes, and Bacteroidetes are considered keystone species in bacterial communities. Collectively, the findings imply that microplastics acted as a distinct habitat for bacteria in farmland soil, which increases our understanding of microplastic pollution.



## 1. Introduction

Microplastics, defined as plastic debris <5 mm in diameter (Duis and Coors, 2016), are ubiquitous throughout various environments, including marine (Auta et al., 2017; Law et al., 2014; Thompson et al., 2004), freshwater (McCormick et al., 2014; Redondo-Hasselerharm et al.,

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2018), terrestrial ecosystems (Ng et al., 2018), and even atmospheric fallout (Gasperi et al., 2018), and have been identified as emerging pollutants of global importance. It is widely acknowledged that microplastics have a severe negative impact on aquatic ecosystems and even human health due to leaching of toxic chemicals, ingestion by consumers, and accumulation of organic pollutants (Sharma and Chatterjee, 2017; Syberg et al., 2015; Wright et al., 2013). Currently, most research still focuses on microplastics in aquatic ecosystems, while knowledge of microplastics in terrestrial ecosystems is limited. In fact, terrestrial ecosystems have been demonstrated to be a major sink for microplastics (Mahon et al., 2017; Browne et al., 2011; Nizzetto et al., 2016b). According to statistics on global plastic production, use, and fate between 1950 and 2015, the quantity of global plastic waste is estimated to be 6.3 billion tones, 79% of which is in terrestrial ecosystems (Geyer et al., 2017).

Agroecosystems are likely to be the most microplastic-contaminated terrestrial system other than landfills and urban spaces; microplastics in agroecosystems are mainly derived from municipal solid waste (sludge application), sewage irrigation and plastic mulching film (Nizzetto et al., 2016a). Considering the increasingly stringent management of sewage and sludge used on farmland, plastic mulching film could become the main source of microplastics in farm soil. Because of its function in improving crop production, increasing water use efficiency by regulating the soil microclimate, and blocking pests (Ekebafe et al., 2011), the usage of plastic mulching film is tremendous worldwide and has continued to increase every year for the last 30 years (Brodhagen et al., 2017). China uses the largest quantity of plastic mulching film, covering 19.8 million hectares of agricultural land (Liu et al., 2014). The removal of plastic film residues from farm soil is extremely difficult owing to their small size and thorough incorporation into the soil (Brodhagen et al., 2017; Steinmetz et al., 2016). Therefore, agricultural land, especially land where plastic mulching film has been intensively used in the long term, is likely to have high microplastic concentrations. Recent studies have made progress in evaluating the effects of microplastics in agroecosystems on soil physicochemical properties (Liu et al., 2017), animals (Browne et al., 2013; Rillig et al., 2017), and plant growth (Qi et al., 2018). However, less attention has been paid to exploring the effects of anthropogenic substrates on the soil microbiome, which is essential for soil biogeochemical cycling and crop production (Fierer, 2017; Philippot et al., 2013).

A large number of studies have shown that microplastics in aquatic ecosystems are a vector that can be colonized by various algae and microorganisms (McCormick et al., 2014; Harrison et al., 2011). Microplastics, as exogenous particles with a hydrophobic surface, are highly likely to provide new substrates for heterotrophic microbial activities (Arias-Andres et al., 2018), making their surface microbial communities significantly different from those of the surrounding environment and other organic residues (McCormick et al., 2014; Dussud et al., 2018; Jiang et al., 2018). In marine and freshwater ecosystems, microplastics constitute a unique microbial habitat known as the "plastisphere" (Zettler et al., 2013). Therefore, it is meaningful to explore whether and how the presence of microplastics affects soil microorganisms to gain further insight into the ecological consequences of plastic mulching residue as a pollutant of agricultural land.

In this study, we performed SEM and next-generation amplicon sequencing to examine the microbial communities associated with microplastics and other samples (i.e., macroplastics, plant litter and surrounding soil). All samples were collected from cotton fields in Xinjiang Province where plastic film mulching technology has been applied for >30 years (Wang et al., 2016). The objective of this study is to improve our understanding of the consequences of plastic mulching residue on farmland by investigating the following: 1) the polymer type and surface morphology of soil microplastics; 2) the diversity and composition of microbial communities adhered to microplastics and adjacent habitats; 3) the microplastic-associated microbial groups in agricultural land; and 4) the co-occurrence patterns of microbial assemblages attached to microplastics. This study presents comprehensive information on microbial assemblages colonizing microplastics in agroecosystems.

## 2. Materials and methods

### 2.1. Study site

Samples were collected from cotton fields (44°21′48″ N, 85°58′47″ E, 427 m a.s.l.) in Shihezi City, Xinjiang Uygur Autonomous Region, Northwest China (Fig. S1), which is one of the most important cotton cultivation areas and has used intensive plastic mulching practices for >30 years (Wang et al., 2016). This region has a northern temperate continental climate with an average annual temperature of 7.8 °C and mean annual precipitation of 225 mm. The soil has the following physicochemical properties: pH 8.20, moisture content 8.45%, total carbon (TC) 41.07 g $kg^{-1}$, and total nitrogen (TN) 1.90 g $kg^{-1}$.

### 2.2. Microplastic collection and quantification

Three sampling plots (10 m × 10 m) were established randomly in an area covering approximately $2.5 \times 10^3$ $m^2$, with a distance of approximately 14 m between adjacent plots. From each plot, five soil cores with a depth of 0–30 cm and a diameter of 5 cm were randomly collected using an auger and mixed thoroughly to generate a soil sample representing the plot. Each sample was divided into two parts and sealed in sterilized sampling bags on ice for transport to the laboratory, where all samples were stored at 4 °C. In order to collect samples for microbial community analysis, one part of each sample was lightly ground on ice, and individual macroplastics and pieces of plant litter were directly picked up using sterilized forceps. Then, the ground soil was sieved through 5 mm meshes, and microplastic samples were separated from sieved soil in the same way as macroplastics and plant litter. All collected plastic residues and plant litter were rinsed gently with sterile water to remove large soil particles adhering to the surface and them placed in a sterile 2 mL centrifuge tube. The remaining soil was defined as the surrounding soil, and samples were collected. Except for some microplastics stored at 4 °C for spectral analysis and electron microscopy observation, all samples were preserved at −80 °C for DNA extraction within a week.

A protocol designed for the quantification of soil samples to measure microplastic concentrations was adapted for this study. Considering that the smallest size of microplastics that can be identified under a stereoscope is 450 μm, while fine clay adds to the difficulty of identification, the 0.45–5 mm soil fractions were used for microplastic quantification. The other part of each sample was air-dried and ground to pass through stacked 5 mm and 450 μm sieves, and suspected microplastic debris was separated from the soil by flotation using NaI solution. Specifically, 200 mL saturated NaI solution and 20 g of the soil sample were added to a 250 mL glass beaker successively. The beaker was magnetically stirred for 10 min and then filled with NaI solution to the rim. After the suspension had settled for 20 min, approximately 100 mL of the supernatant was decanted into a 1 L glass beaker by consistently adding NaI solution into the 250 mL beaker with a washing bottle. The supernatant in the 1 L glass beaker was subsequently poured into a vacuum filtration unit equipped with a 0.45 mm filter. The glass beaker was thoroughly rinsed three times with distilled water, which was poured into the vacuum filtration unit. The filters were dried at 65 °C overnight using a drying cabinet. Microplastics on the filters were then filtered and counted under a stereoscope (OPTEC, TP510). Concentration was calculated by dividing the number of particles by the weight of the soil (no. particles/kg). The control samples were treated the same as the environmental samples to measure procedural contamination (N = 3).

### 2.3. Spectroscopic analysis

Microplastics collected were identified using micro-Fourier transform infrared spectroscopy (μ-FTIR). Samples were randomly selected and pretreated according to a recent plastic identification protocol (Li et al., 2016). All spectra were recorded with an average of 128 scans at 4 $cm^{-1}$ resolution and compared with spectra in a commercial library (BioRad-KnowItAll Informatics System, Thermo Fisher Scientific Inc.).

### 2.4. Scanning electron microscope (SEM) imaging

Microplastic samples for SEM were rinsed gently with sterile water, dehydrated with a graded ethanol series, and dried in a Tousimis Samdri-795 critical-point dryer. Subsequently, samples were sputter coated with 5 nm of platinum and visualized on a Merlin Compact Field Emission Scanning Electron Microscope (ZEISS) at an accelerating voltage of 15 kV.

### 2.5. DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from frozen samples using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Three selected residues (i.e., microplastics, macroplastics and plant litter) were thawed on ice and rinsed gently with sterile water before DNA extraction. The extracted DNA was evaluated by 1% agarose gel electrophoresis, and the quality of the extracts was checked with a spectrophotometer (NanoDrop, ND2000, Thermo Scientific, Wilmington, DE, USA). The universal 16S rRNA gene primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGA CTACHVGGGTWTCTAAT-3′) were chosen for the amplification and subsequent high-throughput sequencing of the PCR products. The PCRs were performed using the following protocol: 3 min of denaturation at 95 °C, 27 cycles of 30 s at 95 °C, 30 s for annealing at 55 °C, and 45 s for elongation at 72 °C, and a final extension at 72 °C for 10 min. PCRs were performed in triplicate 20 μL mixtures, each containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase and 10 ng of template DNA. The resulting PCR products were extracted from a 2% agarose gel and further purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol. Purified amplicons were pooled in an equimolar mixture and subjected to paired-end sequencing (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to the standard protocols provided by Majorbio Bio-Pharm Technology Co. Ltd. The raw bacterial sequences in this study were deposited in the Sequence Read Archive (SRA) of the NCBI database and are available under accession number SRP187007.

### 2.6. Bioinformatics analysis

Raw sequences yielded from Illumina sequencing were processed using QIIME 1.9.1 (Caporaso et al., 2010). Paired-end reads were joined with fastq-join, demultiplexed and quality filtered with default parameters (Aronesty, 2013). Briefly, sequences with a quality score < 20 or with any truncated reads shorter than 50 bp were removed. Operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using UPARSE 7.1, and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analysed by the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using a confidence threshold of 80%.

### 2.7. Statistical analysis

All statistical analyses were carried out in the program R version 3.5.1. To minimize the impact of read count variation in each library, the sequences were normalized based on the minimum number of sequences per sample. The α-diversity of bacterial communities was evaluated based on the Shannon diversity index, and significant differences were calculated using one-way analysis of variance with Tukey's honest significant difference test. The β-diversity of bacterial communities was visualized using non-metric multidimensional scaling (NMDS) ordination based on weighted UniFrac distances and significance assessed with permutation tests using the function "Adonis" in the vegan package (Dixon, 2003). Statistical analyses of differentially abundant OTUs were performed using the "edgeR" library by fitting a negative binomial generalized linear model to the OTUs and visualized using the ggplot2 package (Dimont et al., 2015). Co-occurrence network analysis of bacterial communities was conducted using the "igraph", "psych", "WGCNA", and "multtest" packages (Langfelder and Horvath, 2012). In the network analysis, taxa with a relative abundance of <1% were excluded, and correlational relationships (the Spearman's correlation coefficient was >0.7 or < −0.7; the *P*-value was <0.01) were established.

## 3. Results and discussion

### 3.1. Polymer type and surface morphology of microplastics

The polymer type of soil microplastics was examined using micro-Fourier transform infrared spectroscopy (μ-FTIR). A majority of microplastics collected were positively confirmed as polyethylene (Fig. 1A). This was expected, since this polymer is the main component of mulching film (Koutny et al., 2006), which is widely and intensively used in agricultural production systems (Wang et al., 2016).

Then, other microplastics were imaged with a scanning electron microscope (SEM) to explore their surface morphology. Remained in the soil for a long period of time, the surface of microplastics from mulching film residues turned from the smooth texture of unweathered plastic mulch to a rough texture full of pits, flakes, grooves and attached particles (Fig. 1B; C), which might be caused by the weathering process according to research on plastic debris in a marine environment (Ter Halle et al., 2016; Veerasingam et al., 2016). Weathering is a key process in governing the fate of plastic materials in nature, as it leads to the loss of the physical integrity of the material by abiotic and biotic influencing factors and related degradation of the material. In the weathering process of plastic mulching films, photooxidation degradation is the major abiotic degradation occurring on plastic surfaces (Gijsman and Diepens, 2009). The weathered surfaces display a modified topography with increased surface roughness and altered chemical properties, which provides fairly suitable conditions for the colonization of various heterotrophic microorganisms, in terms of habitat and substrate (Feldman, 2002; Webb et al., 2013). Considering the high specific surface area of microplastics and the heavy colonization of its surface by numerous bacterial microorganisms, mostly living in large pits and flakes (Fig. 1C), it might be inferred that the microplastic surface is a special microbial microhabitat completely different from the adjacent environments, with the potential to recruit distinct microplastic-associated microorganisms, such as plastic-degrading bacteria and pathogens (Reisser et al., 2014; Viršek et al., 2017). Therefore, further analyses were carried out to investigate the microbial communities colonized on microplastic surfaces and to compare them with those on macroplastics, litter and soils to explore their potential adverse ecological impact in the context of intensive plastic mulching applied to farm soil.

### 3.2. Characteristics of bacterial community structure

All soil samples collected after harvest from cotton fields where plastic mulching has been practised for a long time. Because fine plant litter left in the field is often colonized quickly by microorganisms (Aneja et al., 2006), soil and 3 types of residue, i.e., plant litter (size approximately 5 mm), macroplastics (defined as plastic mulching film residue

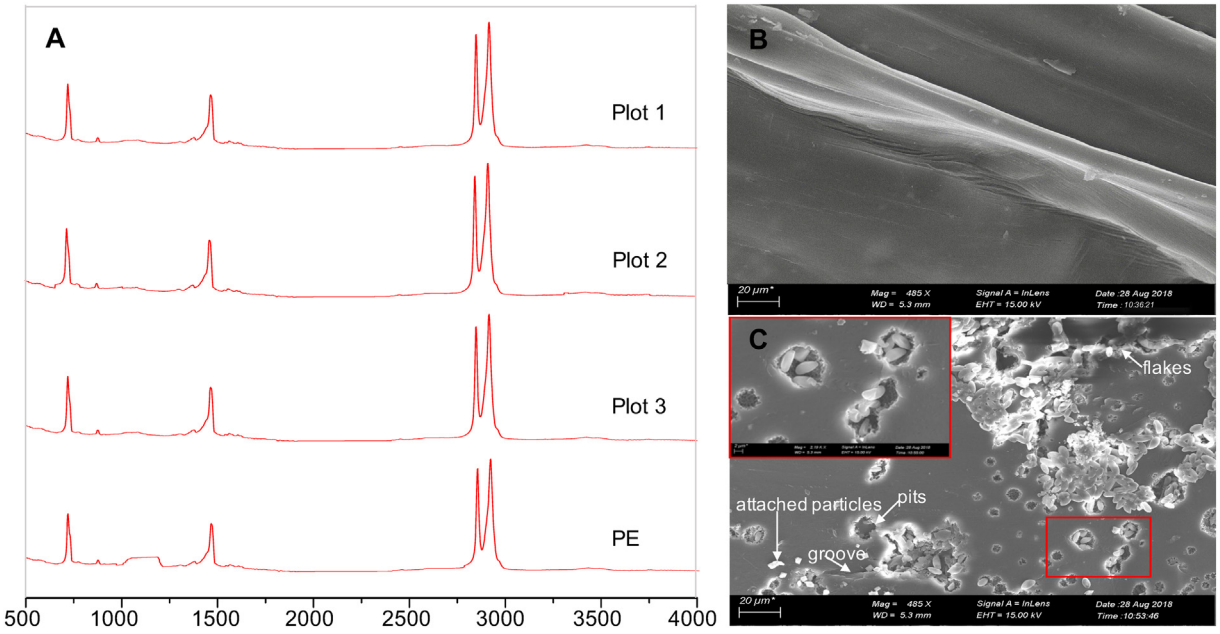


**Fig. 1.** Identification of soil microplastics. (A) FTIR spectra of microplastics collected from three pots (polyethylene). There are 4 characteristic peaks in the infrared spectrum: the asymmetric stretching vibration peak of $-CH_2-$ at 2919 $cm^{-1}$; the symmetrical stretching vibration peak of $-CH_2-$ at 2851 $cm^{-1}$; the bending vibration peak of $-CH_2-$ at 1467 $cm^{-1}$ and $-(CH_2)n-$ ($n \geq 4$) in-plane rocking vibration peaks at 725 $cm^{-1}$. (B) SEM image showing the surface morphology of unweathered polyethylene plastics. (C) SEM image showing examples of various degradation forms and numerous microbes on microplastic surfaces.

measuring >5 mm), microplastics (size <5 mm) as well as soil, were selected from soil samples to understand the impact of the substrate on microbial assembly from adjacent ambient environments. The amplicon 16S rRNA gene V3-V4 region for 12 samples was amplified using PCR and sequenced using the Illumina MiSeq platform. A total of 554,408 high-quality sequences were recovered with a median read count per sample of 46,201 (range: 30,764–67,484; Dataset S2). The high-quality reads were clustered using a genetic distance of 97%, and low-abundance OTUs (<5 total counts) were removed, resulting in 2571 OTUs (Dataset S3). Good's coverage estimators (in all cases above 98.5%) and the rarefaction curves showed clear asymptotes (Fig. S2), which together indicated a near-complete sampling of the bacterial community in this study.

Bacterial community characteristics were represented by the diversity and relative abundances of species, which indicate the community complexity and composition of samples (Schneider, 1994). At 30,000 sequence counts, an average of 70, 73, 226 and 70 different OTUs of microplastics, macroplastics, plant litter and soil particles were illustrated with a Venn diagram (Fig. S3), indicating variance in the number of unique species among sample types. Alpha diversity (within-sample diversity) of bacterial communities varied among the three selected residues and soil (Fig. 2A). The Shannon diversity index was highest in soil, followed by microplastics, plant litter and macroplastics; however, significant differences existed only between macroplastics and surrounding soil (Dataset S4). When we applied non-metric multidimensional scaling (NMDS) of weighted UniFrac distances to further investigate the patterns of separation between bacterial communities, it illustrated strong clustering of bacterial communities according to different sample types (Fig. 2B). The two types of plastic residue and the surrounding soil were clearly distinguished from plant litter along the first principal coordinate, while the separation between two plastic residues and surrounding soil was seen along the second principal coordinate. Consistent with the results of NMDS analysis, ANOSIM analysis also revealed significant differences in the structure of bacterial communities among different samples ($R = 0.417$, $P = 0.002$).

Furthermore, the relative abundance of bacterial OTUs at the phylum level was also variable among the three residues and surrounding soil (Fig. 2C). The most dominant bacterial phyla across all samples were Actinobacteria and Proteobacteria, accounting for 26.79–57.52% and 18.78–33.68% of the pyrosequencing reads, respectively; the predominance of these phyla is well documented in many studies of soil microbiology (Delgado-Baquerizo et al., 2018; Schloss et al., 2016). The proportion of Actinobacteria and Bacteroidetes on macro- and microplastics was significantly higher than the proportion in the surrounding soil, whereas the proportions Proteobacteria, Gemmatimonadetes and Acidobacteria showed the opposite pattern. For plant litter, the proportions of Cyanobacteria, an important phototrophic bacterium (Roper and Ladha, 1995), and of Bacteroidetes were significantly higher than in the surrounding soil, whereas the proportions of Actinobacteria, Chloroflexi and Acidobacteria were significantly lower (Dataset S6). The results indicated that macro- and microplastics, as anthropogenic substrates, are likely to select distinct microbial assemblages profoundly different from those of natural substrates and surrounding soil, which proved in previous research in aquatic environments (McCormick et al., 2014; Bryant et al., 2016; Jiang et al., 2018; Zettler et al., 2013).

### 3.3. Enrichment effects of microplastics on bacterial community

It is known from the above results that the bacterial communities colonizing plant litter, macroplastics and microplastics were significantly different. Hence, we further identified OTUs correlated with the differences between three selected residues to explore the enrichment or exclusion of different bacterial taxa by microplastics. We performed differential OTU abundance analysis by fitting a generalized linear model with a negative binomial distribution to normalized values for each of the 2571 OTUs retrieved from samples and examining for differential abundance using a likelihood ratio test (Dataset S7). Taking the OTU counts from the surrounding soil as a control and an adjusted *P*-value cutoff of 0.05, there were different numbers of OTUs with significant variation among the three residues (Fig. 3). The plant litter enriched and depleted almost equal proportions of OTUs (407 vs 404), while the macroplastics showed a weak exclusion effect, enriching for 100 OTUs and depleting for 137 OTUs. In comparison, the microplastics were the most similar to surrounding soil as shown by the "tail" in the

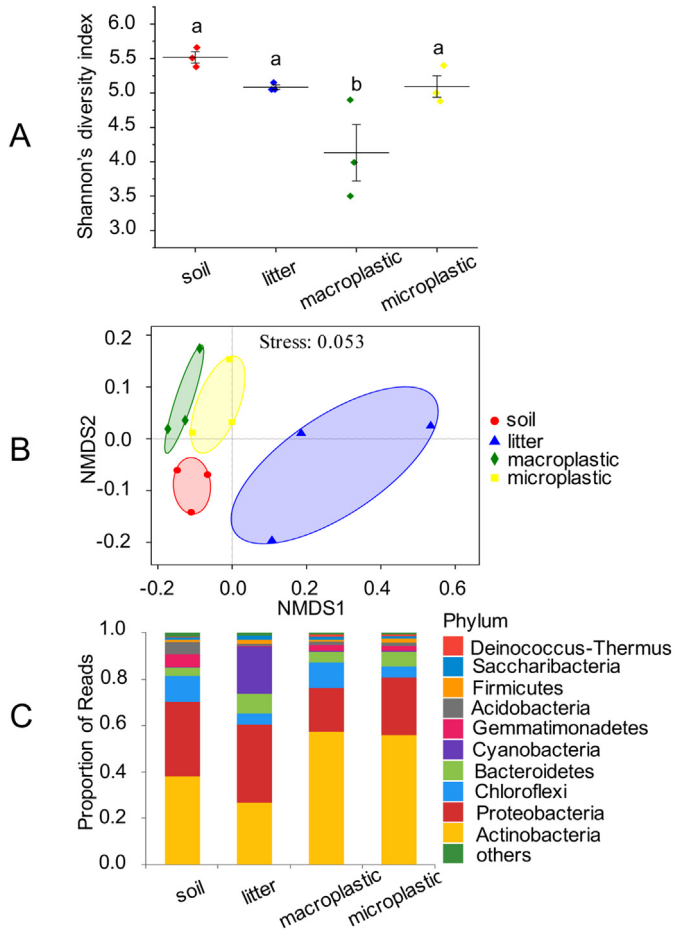


**Fig. 2.** Bacterial communities are separable by different organic residues and surrounding soil. (A) Within-sample diversity (α-diversity) of microbial communities based on the Shannon index. The Wilcoxon rank sum test was used to assess the significance between two samples. Different letters indicate statistical significance among different samples at $P < 0.05$. (B) Non-metric multidimensional scaling (NMDS) ordination of 16S sequencing data (Weighted UniFrac) comparing assemblages of bacteria retrieved from different samples. The circles represent group membership, assuming the 95% confidence limit as the cutoff distance. (C) Stacked bar chart of phylum abundances in different organic residues and surrounding soil.

MA plot, but an enrichment effect was indicated by the statistically significant ratio of highly enriched OTUs to depleted OTUs (80 vs 16). Moreover, the enrichment effect in the microplastics was also implied by the enrichment of 40 OTUs and the depletion of 11 OTUs compared with the macroplastics (Fig. S4; Dataset S8). Combined with SEM imaging (Fig. 1B), these results might indicate that microplastics provide a distinct microbial habitat in the soil environment and serve as an "accumulator" by providing special spaces and substrates, as in many previous studies of plastic debris in aquatic environments (McCormick et al., 2014; Arias-Andres et al., 2018; Bryant et al., 2016; De Tender et al., 2015; Jiang et al., 2018; Zettler et al., 2013).

Then, we further dissected the community shifts by arranging OTUs based on their taxonomy and illustrating their enrichment or exclusion in the three residues using a set of Manhattan plots (Fig. 4). The plant litter significantly enriched most of the Bacteroidetes, Proteobacteria, Saccharibacteria, and depleted most of the Acidobacteria, Actinobacteria, Chloroflexi, and Tectomicrobia. The macroplastics enriched Bacteroidetes and Saccharibacteria but depleted many bacterial phyla, including Acidobacteria, Actinobacteria, Chloroflexi, Gemmatimonadetes, Proteobacteria and Tectomicrobia. Strikingly, the microplastics had different enrichment effects on a wide range of bacterial phyla, including Actinobacteria, Bacteroidetes, Proteobacteria, Cyanobacteria and Deinococcus-Thermus.

The phyla Bacteroidetes are widely distributed in the environment, and many of them have the ability to degrade cellulose (Naas et al., 2014), crude oil (Viñas et al., 2005) and other organic polymer compounds (Bauer et al., 2010); thus, it is reasonable to enrich in the three residues. However, some members of Bacteroidetes are pathogenic bacteria, such as *Bacteroides nodosus*, which can cause hoof rot disease in domestic ruminants (Stewart et al., 2010). Due to the durability of microplastics, the concentration of pathogenic bacteria in soil will increase with the continuous input of plastic debris, which may threaten the health of agroecosystems. The phylum Actinobacteria was significantly enriched in microplastics but decreased in plant litter and macroplastics. A large number of studies have shown that some species of Actinobacteria can biodegrade polyethylene (PE) through the synthesis of hydrolytic enzymes, especially in terrestrial environments (Kleeberg et al., 1998; Amouric et al., 2011; Gilan and Sivan, 2013; Santo et al., 2013; Wei et al., 2014; Singh and Sedhuraman, 2015; Abraham et al., 2016; Muhonja et al., 2018). In addition, the differences in community shifts between macro- and microplastics implied that the size of plastic debris is also an important factor affecting colonization by biodegrading microorganisms. Thus, it is further established that after being buried in the soil for a long period of time, weathered plastic debris became a special microbial accumulator dominated by a core microbiome that related to plastic decomposition.

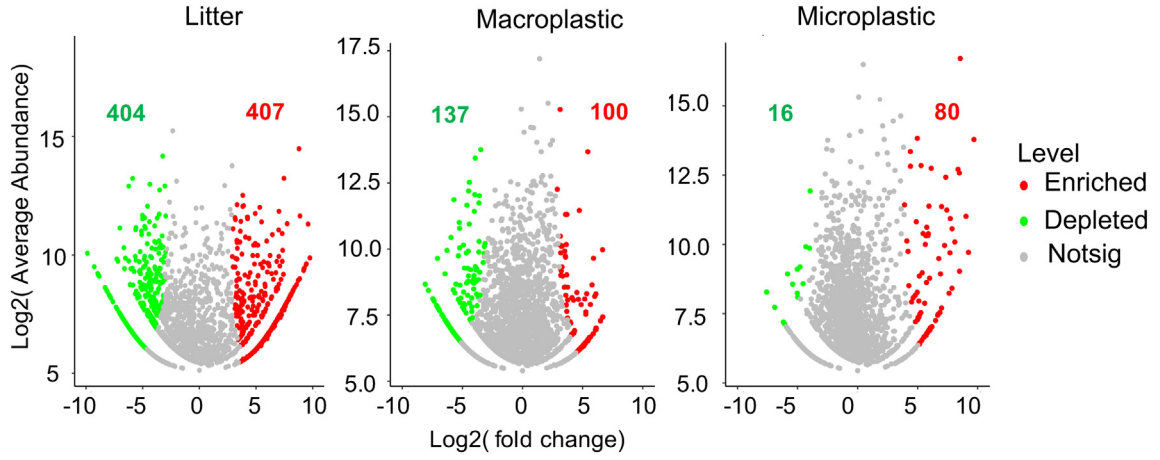


**Fig. 3.** Enrichment and depletion of the 2571 OTUs for litter, macroplastics and microplastic compared with surrounding soil as determined by differential abundance analysis. Each point represents an individual OTU. Red or green circles represent OTUs depleted or enriched compared with soil, respectively, whereas grey circles represent OTUs that are not significantly enriched or depleted in a sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

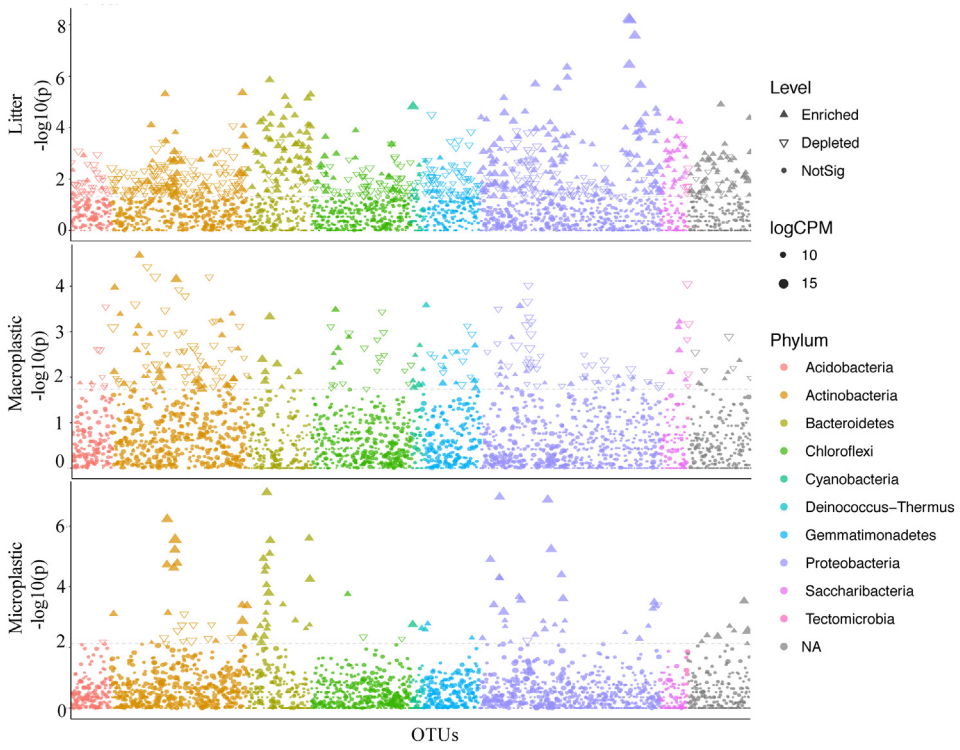


**Fig. 4.** Manhattan plots showing OTUs with significant differences in relative abundance in three selected residues with respect to soil. Each triangle or circle represents an individual OTU. Upward solid triangles or downward hollow triangles represent OTUs enriched or depleted, respectively, whereas circles represent OTUs that are not significantly enriched or depleted. The dashed line corresponds to the false discovery rate-corrected threshold *P*-value for significance ($\alpha = 0.05$). The colour of each dot represents the taxonomic affiliation of the OTU (phylum level), and the size corresponds to its relative abundance in the samples.

### *3.4. Co-occurrence network analysis*

Individuals of microbial communities coexist in natural ecosystems based on their nutritional preferences and functional distinctiveness (Blagodatskaya and Kuzyakov, 2008; Mau et al., 2015). Thus, to better understand the microbial communities colonized on microplastics, four bacterial co-occurrence networks were constructed for different samples to insight into potential biotic interactions among various microbes and identify the keystone species of bacterial communities (Barberán et al., 2012; Berry and Widder, 2014).

Three selected residues formed their own distinctive bacterial networks compared with surrounding soil, among which the microplastic network differed profoundly from that of macroplastics and plant litter (Fig. 5). In addition, multiple network topology parameters (i.e., nodes, edges, average degree and modularity), which normally represent the size and complexity of bacterial networks (Berry and Widder, 2014; Newman, 2006), further illustrated markedly different among three selected residues and soil (Dataset S9). The plant litter and macroplastic networks incorporated 234 nodes, 312 edges, and 194 nodes, 288 edges, respectively, and were obviously smaller than the soil network (350 nodes and 716 edges). The microplastic network (332 nodes and 701 edges) was approximately the same size as the soil network despite microplastic bacterial assemblages having lower α-diversity than surrounding soil (Fig. 2A). Therefore, it may be inferred that the bacteria

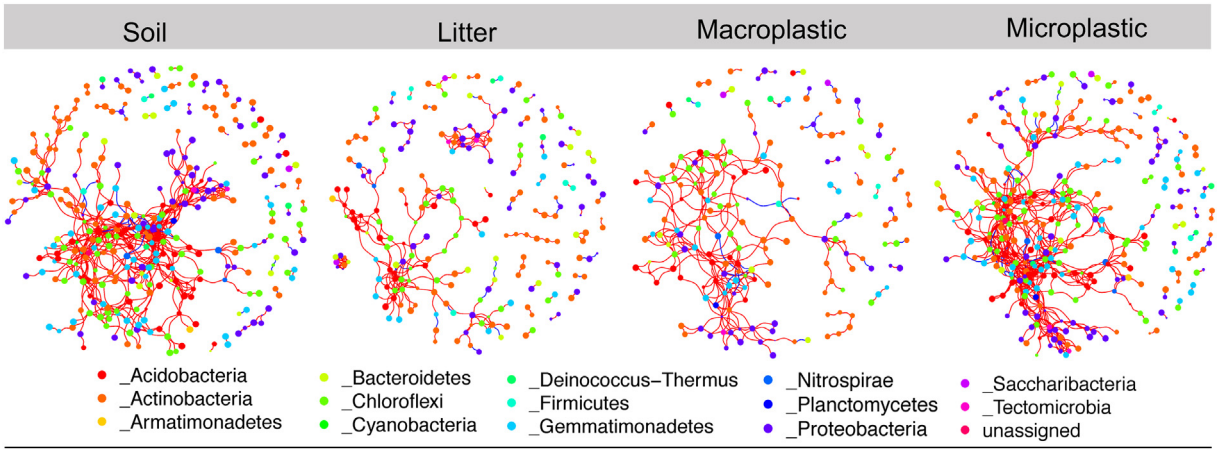


**Fig. 5.** Co-occurrence networks of the bacterial community based on correlation analysis. Each node represents an individual OTU coloured by taxonomy at the phylum level, and the size of each node is proportional to the relative abundance. The connection stands for a strong (Spearman's $\rho > 0.7$) and significant (*P*-value < 0.01) correlation. Red edges represent positive correlation, and blue edges represent negative correlation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

colonized on the surface of microplastics form closer connections than the initial state in the soil. Furthermore, the differences were also reflected in the average degree and modularity, in which the plant litter and macroplastic networks had a lower average degree and higher modularity than those of soil, while the microplastic network was very similar to that of the soil. Collectively, the above results indicated that the bacterial network of the microplastics was larger and more complex than those of the plant litter and macroplastics, which could be explained through two plausible interpretations. First, microbiota living on the surfaces of microplastics, macroplastics and plant litter are recruited from the surrounding soil and interact closely with soil microbes. Considering that the particle size of microplastics was much smaller than that of plant litter or large plastic residues and thus increased the specific surface area in contact with the surrounding soil (Brodhagen et al., 2017; Steinmetz et al., 2016), it is highly likely that microplastics support more complex interactions between microbial groups than would occur on macroplastics or plant litter. A second possible explanation is that microplastics also remain in the soil for much longer than macroplastics or plant litter; therefore, they may form a more thriving core microbiome involved in metabolizing their own substrate (Arias-Andres et al., 2018; Jiang et al., 2018), an explanation that is also supported by the results of the differential OTU abundance analysis (Figs. 3; 4). In addition, biological co-occurrence networks are mainly affected by environmental conditions, especially the availability of food and other resources (Barberán et al., 2012; Fosterab et al., 2012; Henzi et al., 2009), and microplastics that have larger specific surface areas (Fig. 1B) and release larger amounts of additive small molecular compounds may contribute to niche sharing between active taxa in bacterial communities (Arias-Andres et al., 2018).

The degree (defined as the number of connections per node) distribution of various taxa involved in the networks differed significantly among the four samples (Fig. 5 and Fig. S5). Nodes with a high degree are often referred to as keystone species, which play a vital role in maintaining the stability of ecosystem structure and function (Berry and Widder, 2014; Shi et al., 2016). According to this criterion, members of the Acidobacteria phylum were inferred to be keystone species in all networks. Previous studies have shown that Acidobacteria is an important bacterial taxon in soil, participating in soil material circulation and environmental construction (Eichorst et al., 2011; Jones et al., 2009; Navarrete et al., 2013). Moreover, the significant effects of soil pH on Acidobacteria in different soil types and spatial scales have been well documented (Lauber et al., 2009; Rousk et al., 2010), and the soil in cotton fields where our samples were collected was high in pH (Dataset S1). Thus, it is implied that Acidobacteria, as a mediator of environmental pH, play a key role in the formation and stability of the microbial community in the alkaline habitat. On the other hand, Acidobacteria had relatively low abundances (0.15–5.28%), while Actinobacteria, despite high abundance (26.79–57.52%), were not keystone species, which might hint that rare taxa play an important role in maintaining microbial networks (Jousset et al., 2017). For the microplastics, members of Chloroflexi, Gemmatimonadetes, and Bacteroidetes were also characterized as keystone species in their bacterial network, which is consistent with the results from microplastics collected from aquatic ecosystems (Jiang et al., 2018). Finally, more attention must be paid to the ecological impact that microplastics, as a special accumulator of soil bacteria, exert on plants and soil organisms.

## 4. Conclusion

Our results demonstrated that the microplastics derived from mulching film are a distinct habitat for microbial communities. Microplastics remained in the soil for long periods of time, and their surfaces, especially pits and flakes, were heavily colonized by numerous microorganisms. We found that the bacterial communities colonizing microplastics were significantly different in structure from those in the surrounding soil, plant litter and macroplastics. Unexpectedly, microplastics in soil may be a "special microbial accumulator", enriching the bacterial groups involved in their own biodegradation. Moreover, the interactions between microorganisms on microplastics were more complex than those on plant litter and macroplastics, and the keystone species were Acidobacteria, Chloroflexi, Gemmatimonadetes and Bacteroidetes. However, as with the majority of studies, the design of the current study is subject to limitations. The interpretations of these data are limited to three replicate plots in a small area of cotton field. It is well known that soil microorganisms are significantly affected by a variety of abiotic and biotic factors, including soil types; plant varieties; and human-controlled variables such as duration of mulching, cultivation methods and fertilization systems (Bissett et al., 2013; Fierer, 2017; Tripathi et al., 2018). Therefore, whether our findings in this study can be extended to other soil ecosystems remains to be rigorously examined. Future research will need to implement a comprehensive sampling scheme involving more regions and crop systems to better elucidate the impact of microplastics on microorganisms, which will be meaningful for assessing the ecological consequences of plastic mulching residual pollution on agricultural land.

## Declaration of Competing Interest

This work has no actual or potential conflict or interest including any financial personal or other relationships with other people or organizations.

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## Appendix A. Supplementary data

The following files are available free of charge. Fig. S1. The location of the sampling site in Shihezi, Xinjiang, China. Fig. S2. Rarefaction curves comparing the number of sequences with the number of observed OTUs for bacterial communities in each sample. Fig. S3. Enrichment and depletion of the 2,571 OTUs for microplastics compared with macroplastics. Fig. S4. The degree distribution of various taxa involved in the networks.

*Dataset*

The following files are available free of charge. Dataset S1. Soil physicochemical properties and microplastic contents. Dataset S2. Table displaying sequencing effort for each sample. Dataset S3. Table showing operational taxonomic units with taxonomy information recovered from samples. Dataset S4. One-way ANOVA of bacterial diversity. Dataset S5. ANOSIM results using weighted UniFrac as a distance metric for samples.

Dataset S6. Comparison of sample differences in abundance of phyla. Data are means ± SD in parentheses. Different letters indicate significance levels (Tukey's HSD, $P < 0.05$). Dataset S7. OTUs that are significantly differentially abundant in each sample compared to soil. Dataset S7. OTUs that are significantly differentially abundant in microplastics compared to macroplastics. Dataset S8. Multiple topological parameters of networks. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.06.108.

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