Abstract

Primitive electronic waste (e-waste) recycling activity released lots of organic pollutants and heavy metals to the environment. As the crucial moderator in geochemical cycling processes and pollutants remediation, microbes might be affected by these contaminants. In the present study, we collected soil samples heavily contaminated by e-waste recycling from China and Pakistan. The results revealed that polycyclic aromatic hydrocarbons (PAHs), polybrominated diphenyl ethers (PBDEs) and heavy metals (e.g. Cu, Zn, Pb) affected microbial community composition and diversity both in whole and core community level significantly. Besides, the ecological network, which revealed the co-occurrence, competition and antagonism influence, was constructed and its response to soil properties and e-waste was investigated. There were three main modules constructed by core OTUs and two modules were sensitive to nutrition and pollutants. This is the first study that investigated the effects of e-waste pollutants on soil microbes over a large scale and evaluated the role of pollutants on microbial network, which gives us a deeper understanding on the ecological influence of crude e-waste recycling activity on soil microbes.

Introduction

The crude recycling technology of electronic waste (e-waste) released persistent organic pollutants and heavy metals with extreme high amount to the environment, such as PBDEs, polychlorinated biphenyls (PCBs), Cu, Pb and Zn[1-4]. Due to the increasing risk of human health and the potential threat to the ecosystem, the e-waste pollution has been paid considerable attention[5-7].

Microbes, crucial to the functioning of almost all ecosystems, are sensitive to the environmental change due to the rapid growth and active metabolism[8]. Numbers of studies reported that the environmental pollution (organic pollutants and heavy metal) could inhibit the enzyme activities, damage the microbial metabolic ability, weaken the resistance of soil microbial community to subsequent disturbance and decrease the microbial communities' diversity[9-16]. Besides, these pollutants alter the microbial structures via significantly increasing the abundance of some species possessing remarkable adaptability or biodegradability and reducing the other species' abundance[12,17]. Thus, the microbial community in e-waste sites could be the possible indicators of soil environmental quality[18] and reflect the ecological risk to the environment. Recently, the influence patterns of microbes by organic pollutants and heavy metals in e-waste recycling sites draw considerable attentions. Several laboratory experiments were performed to investigate the ecotoxicological effects of e-waste pollution on microbes. It was reported PBDEs and Cu had combination toxic effects on urease, catalase, saccharase[19]. PBDEs and Pb decreased the microbial biomass and suppressed microbial basal respiration[20]. It also discovered that

decabromodiphenyl ether and tetrabromobisphenol A had antagonistic and synergistic toxic effects on microbes during the different periods[21]. However, these results hardly represented the actual situation in *situ*. To overcome this shortage, Liu et.al conducted a comprehensive survey of the microbial communities in e-waste contaminated soils in 2014[22]. The results exhibited that environmental variables explained approximately 70% of the observed variation in microbial communities, where moisture content, decabromodiphenyl ether, Cu were identified as the most important factors[22]. Nevertheless，all the samples in Liu's study were derived from the Town of Guiyu, South China, which was limited to give a wide perspective covering a large scale.

The ecological network can discover the co-occurrence, competition and antagonism among different microbial populations within microbial communities. One approach developed by Zhou et.al was successfully used to characterize ecological networks in microbial communities based on high-throughput metagenomic sequencing data, which is originated from novel random matrix theory [23]. In this network, co-occurrence such as commensalism or a mutualistic relationship is represented by positive correlations, whereas negative correlations may suggest the presence of competition and antagonism[24]. In addition, the mantel tests could detect the relationship between microbial network interactions and soil properties[23]. Therefore, constructing and analyzing the microbial network can make a deep understanding on microbial communities in e-waste recycling sites, especially the inner relationship between different species, and identify the influence of organic pollutants and heavy metals. However, no studies were performed to disclose the

interactions among microbes in e-waste recycling soils in *situ* so far.

Here, we collected samples from the e-waste contaminated soils in China and Pakistan, two major e-waste recycling countries across the world. The 454 high-throughput sequencing targeting the 16S rRNA was utilized to present the composition and diversity of microbial communities. The roles of general soil properties and pollutants in microbial communities constructing were surveyed and the factors influencing the microbial network were examined. Our results exhibited a depth view of microbial communities in e-waste contaminated soils on a wide scale and disclosed the patterns that the microbial communities were shaped by e-waste pollution consisting of organic pollutants and heavy metals. These findings provide important insights into ecological effect caused by e-waste recycling activity.

Materials and methods

Sample collection

Soil samples were collected from five sites (Shijiao and Longtang towns of Qinyuan city, Guiyu town of Shantou city, Fengjiang and Xinqiao towns of Taizhou city) in China and three sites (Karachi, Multan, Lahore) in Pakistan, where the ewaste-recycling activity was intense (Figure S1). Samples were named as following: C1 (Shijiao), C2 (Longtang), C3 (Guiyu), C4 (Fengjiang), C5 (Xinqiao), P1 (Karachi), P2 (Multan) and P3 (Lahore). In June 2012, soil samples were derived in triplicate at a depth of 0-10 cm in each site and transported into the lab with ice pack.

Soil properties analyses

Total organic carbon (TOC) and Total nitrogen (TN) were determined as

previously descriped[25] with modifications. Briefly, 2 g of freeze-dried soil were blended and treated with 25 mL of 1 M HCl twice, followed by ultrapure water washing with a final pH of 6-7. After drying and homogenizing, TOC and TN were analyzed using an elemental analyzer. The soil dissolved organic carbon (DOC) was extracted in a 100 mL centrifuge bottle with 10 g of soil and 50 mL of ultrapure water. After shaking for 30 min and centrifuging at 5000rpm for 10 min, the supernatants were filtered through 0.45 μL polycarbonate filter membrane[26]. Then DOC was measured using TOC-VCPH analyzer. Soil pH was measured in a suspension with 1:5 (w/v) soil/0.01 M $CaCl_2$ solution using pH meter [27].

PAHs, PCBs, PBDEs and DechloranePlus (DPs) were extracted by dichloromethane (DCM) in a Soxhlet apparatus for 48h spiking with relevant recovery standards (Table S1). During the extraction, the activated copper was employed to remove sulfur. After solvent exchange to hexane, the extracts were concentrated to ~0.5 mL. The PCBs, PBDEs, DPs extracts were purified on a multilayer column containing (from bottom to top) neutral alumina (3% deactivated), neutral silica gel (3% deactivated), 50% (w/w) sulphuric acid-silica gel and anhydrous Na_2SO_4, via elution with 20 mL hexane/DCM. PAHs extracts were clean up using the same multilayer column without sulphuric acid-silica gel. Then the internal standards were added into the corresponding extracts followed the evaporated process using N_2 to concentrate the extracts to approximately 50 μL. A total of 16 PAHs (using DB-5MS column, 30 m, 0.25 mm, 0.25 μm), 17 PCBs (using CP-Sil 8 CB column, 0.25 mm, 0.25 μm), 8 PBDEs (using DB5-MS column, 30 m, 0.25 mm, 0.25 μm and

CP-Sil 13 CB column) and 2 DPs (using DB5-MS column, 30 m, 0.25 mm, 0.25 μm) congeners (Table S2) were detected via Agilent 7890 GC-MS as previously described[28-30].

Cu, Pb, Zn in the soils were determined using the flame-atomic absorption spectrometer (AAS; novAA 400, Analytik Jena AG) after homogenization and strong acid digestion (4:1 concentrated HNO_3 and $HClO_4$ (v/v)) for 32 h. The detail extraction protocol was described as previously[31].

DNA extraction, 16S rRNA amplification and sequencing

DNA was extracted triplicate from 0.5 g of soil using a PowerSoil DNA Isolation Kit according to the manufacturer. The combined DNA solutions were stored at -20 ℃ for further analysis after measuring the concentration/quality via a NanoDrop 2000 Spectrophotometer.

The universal primer (341F: 5'-CCTACGGGNGGCWGCAG-3', 802R: 5'-TACNVGGGTATCTAATCC-3') target V3~V4 hypervariable region was used to amplify the 16S rRNA gene. PCR mixtures (50 μL) contained: 50-100 ng (1 μL) of DNA, 25 μL of Taq premix buffer (TaKaRa), 100 nM of each primer (1 μL) and 22 μL of H_2O. The 16S rRNA gene were amplified in triplicate as the following process: 94 ℃ for 5 min; 28 cycles at 94 ℃ for 30 s, 55 ℃ for 30 s, 72 ℃ for 90 s; and a final extension at 72 ℃ for 5 min. The products were tested by agarose gel (1.5%) and purified using the MicroElute Cycle-Pure Kit (Omega Bio-Tek) following the manufacturer. Then, the products from the same sample were combined and quantified as previously. The DNA was sent to company for sequencing based on 454

platform.

Processing of pyrosequencing data

The data were analyzed using Mothur[32] and QIIME[33]. Briefly, reads with low quality (quality score<25, length<250, ambiguous base>0) were removed. Operational taxonomic units (OTUs) with 97% similarity were picked out and the representative sequence set was chosen. Then the chimeric sequences were identified and discarded[34]. The phylotypes information was identified according to the Greengenes 13.5 database using "assign_taxonomy.py".

The relative abundance of each taxon was estimated by comparing the number of sequences classified as the specific taxon versus total sequences of individual sample. To analyze the microbial diversity at the same level, 5270 sequences were randomly selected as subset per sample. The core OTUs were picked up, which existed in all 5 samples. The α-diversity indexes (Chao1, Shannon and ACE) and β-diversity (weighted UniFrac: the phylogenetic distance between samples[35]) were measured using QIIME script.

Network construction

occurrence ecological network constructing was performed using the online MENA (Molecular Ecological Network Analyses) pipeline (http://ieg2.ou.edu/MENA)[36]. The OTUs existed over 5 samples were analyzed in the network construction. The relationship between OTUs was tested via Pearson correlation and the threshold was determined by random matrix theory (RMT) based approach. Then, the network was visualized via Cytoscape. The relationships between the network and soil properties

were measured by Mantel test [37].

Statistical analysis

The statistical analysis was performed using SPSS and R packages. The relationship between soil properties was determined by Spearman's correlation analysis (two tailed). The influence of soil properties on bacterial α-diversity was detected using the Spearman's analysis (two tailed). To pick out the major soil properties affected the microbial communities (whole microbial community and core OTUs), "Bioenv" function in "vegan" package was used with default parameters. Then, redundancy analysis (RDA) was performed to explain the relationship between microbial communities and selected soil properties using "vegan" package. The Mantel test was used to find the influence of soil properties on microbial communities and β-diversity with two-tailed test and 999 times permutations.

Results:

Microbial community profiles

After filtering the low quality reads (score<25), total number of 84403 reads were generated from 454 sequencing. The 68610 sequences in 8 samples ranging from 5270 to 11397 were further analyzed in downstream investigation.

On phylum level, 45 phyla assigning to bacteria were identified. More than 97.6% sequences were classified to the phyla with abundance larger than 0.1% (Figure 1). Only less than 2.41% of total sequences were classified as rare phyla (<0.1%). Six predominant phyla (>5%), *Proteobacteria*, *Actinobacteria*, *Firmicutes*, *Chloroflexi*, *Acidobacteria*, *Planctomycetes*, accounted for 84.7% (average) of the total sequences.

Based on 97% similarity, 19407 OTUs were identified. The 183 core OTUs, existing in more than 5 samples, were selected and presented in Figure S2. The OTU with highest abundance (ID: 1051517) was classified as *Bacillus*.

Bacterial diversity was calculated based on OTUs level. Chao1, Shannon, Ace indexes ranging from 3420 to 11752, 7.55 to 11.68, 3656 to 12724 were used to characterize the alpha diversity (Table S3). Beta diversity (weighted Unifrac in this study), which contains the phylogenetic information, is presented in table S4 and Figure S3. It was find that the microbial communities of P3's was more similar with that of these samples from China, rather than other sites of P1 and P2 from Pakistan.

Effects of soil properties on the microbial composition and diversity

The soil properties are summarized in Table 1. Both general soil properties and contamination levels were in a wide range. For instance, pH ranged from 4.75 to 12.55; PAHs and Cu concentration ranged from 307 ng/kg to 2940 ng/kg and 151 mg/kg to 3003 mg/kg, respectively.

To identify the influence of soil properties on the microbial communities, "bioenv" was applied and two subsets were picked based on the whole community level and core OTUs level: (1) pH, TN, TOC, PAHs, Cu, Zn (whole community level); (2) pH, TN, DOC, PAHs, Cu, Pb (core OTUs level). Variables of both two subsets were significantly related to the compositions on community and core OTU levels, respectively (mantel test, $p<0.05$). The relationship between microbial composition and subset of variables was detected using redundancy analysis (RDA). On the whole community level, the six variables explained 93.56% of the microbial community

compositions variation (Figure 2A), and the contributions of general soil properties and pollutants to community altering were 21.6% and 15.8%. The co-effects explained 56.2% of communities' variation. Besides, Zn was the most important factor explaining 5.5%, which was followed by PAHs (4.8%) and pH (4.8%). On core OTUs level, general soil properties explained 51.6% and pollutants explained 50.5% of core OTUs variation. Notably, the subset variables could only explain 89% of the variation (Figure 2B), which indicated the antagonistic affection existed between soil properties and pollutants. Here, PAHs (30.7%), Cu (28.7%) and Pb (22.4%) were the most relevant factors explaining the core OTUs variations. The contributions of all the individual variables are shown in Table S5.

PBDEs were significantly negatively related to alpha diversity: Chao1 (spearman, r=-0.81, p=0.015), and Ace (spearman, r=-0.81, p<0.05). No obvious relationship between alpha diversity and other pollutants was detected. In addition, soil properties and pollutants did not affect the Unifrac distance (beta-diversity) (mantel test, p<0.05).

Network structure and its association with soil properties

The topological properties, commonly used to describe the complex patterns of bacterial inner-relationships, are listed in Table 2. The empirical network indexes, average clustering coefficient (avgCC), average geodesic distance (GD) and modularity, were much higher than the random network indexes, which indicated the network possessed typical small-world and modular characteristics (Table 2). Ten phyla constructed three dominant modules, and *Acidobacteria*, *Actinobacteria*,

Proteobacteria were the three main dominant phyla (Figure 3). Total of 15 modules were detected, but the three dominant modules possessed 118 notes which made up 75.64% of the total network sizes. The OTUs topological roles were identified and shown as a Z-P plot in Figure 4. Five module hubs assigning to *Acidobacteria*, *Proteobacteria*, *Nitrospirae*, play an important role in bacterial interaction. Their special taxonomic information is presented in Table 3. No network hubs and connector OTUs were observed in the present study.

The relationship between bacterial networks interactions and soil properties were tested using Mantel test. The square of the correlation between the signal intensity of individual OTU and soil properties was calculated to determine the role of soil variable in network modules[23,38]. The relationship between soil properties and modules is presented in Table 4. Module 1 was sensitive to pollutants, its inner interaction was significantly affected by PAHs, PCBs and Cu ($p<0.05$). Module 3 was closely related with TOC, TN and DPs. The interactions of OTUs in module 2 were independent of soil properties.

Discussion

In this study, 45 phyla of bacteria were identified, which was remarkably various in comparison with the previous study in the e-waste recycling site of Qingyuan which detected 29 phyla bacteria using Miseq platform[22]. However, most of the novel bacteria were classified to the rare phyla with small abundance. The dominant phyla were similar in most of the e-waste contaminated soil samples. Among the core OTUs, which represented the main microbe in e-waste soils (Figure S2), *Bacillus* was the

most abundant OTU indicating its highly adaptive capacity to the contaminants. *Bacillus* could produce highly dormant endospores in response to the environmental stress[39]. Besides, its ability of resisting heavy metal and degrading organic pollutants has been reported [40,41].

It is interesting that the divergence of microbe community in samples with close distance is more significant than that in samples with remote distance (Figure S3, table S4). Previous researches showed spatial distance was the most powerful driver of microbial communities constructing [42-44]. In our study, it seems that the environmental conditions might play a more important role in constructing microbial communities, which was supported by some studies[45] and consisted with the Baas-Becking tenet: "Everything is everywhere, but the environment selects". We infer that both distance and environmental conditions influence the constructing of microbial community.

To detect the main soil variables affecting microbial community's construction, "Bioenv" was performed and several important soil properties were picked out. Based on the influence of soil properties on microbial community in different level, different patterns were formed. On the whole community level, the co-effects of general soil properties (e.g. pH and nutrition content) and pollutants explained the large part (Figure 2A) of microbial communities construction. The relationship between general soil properties and pollutants was presented in many previous studies. pH and organic matter could alter heavy metal mobility[46,47]; PAHs, PCBs and PBDEs were tightly related to organic carbon[48,49]. Thus, it was foreseeable that the co-effects being

detected. Here, Zn was the most important individual factor. Zn is a necessary micronutrient for cell metabolism, but the extremely concentration was harmful to the microbe. Zn could alter the microbial communities, decrease the microbial diversity and inhibit the enzyme activity [50-52].

Though general soil properties and pollutants both affected the whole and core microbial communities, the co-effect did not exist in core OTUs, not like the whole communities. We inferred that the more adaptive ability of core OTUs than the whole communities might be the reason. However, the mechanism was unclear and there were short of studies for us to make an accuracy speculation. A further study about the effects of general soil properties and pollutants on core microbial communities are needed. PAHs and Cu were detected as the most important factors influencing the core microbial communities. On the whole community level, PAHs was also important. The PAHs effects on microbial communities[53] have been observed previously, microbial communities structure and diversity could be altered by PAHs addition[54]. In the e-waste contaminated soils, the role of PAHs was dependent on its concentration, it was revealed that PAHs did not simply reduce the diversity of bacterial community and slight contamination could increase the diversity in e-waste contaminated soil[55]. Cu, as the second powerful factor here, commonly represented one of the most important toxic substances in e-waste contaminated soils[56] and shifted the microbial communities. Similar result was also revealed in Liu's research. PBDEs, a group of typical contaminants, were frequently detected in soils around e-waste recycling sites[57] and could produce reactive oxygen species to selectively inhibit the

growth of specific lineages[12]. In our work, the negative relationship between PBDEs and alpha diversity supported this conclusion.

We examined the topological roles of core OTUs to explore cooperative way of the microbial community in e-waste soils, and three main patterns were identified: (1) sensitive to pollutants; (2) sensitive to nutrition; (3) independent of soil properties (Table 4). Module 1, sensitive to pollutants, was significantly related to PAHs, PCBs, Cu. The increasing of the three pollutants reduces the connections between the OTUs clustered in module 1. It indicated that high concentration of pollutants could break the cooperation of microbes, and the damage was sorted as: Cu>PAHs>PCBs (Table 4). Besides, TOC was also negative to OTUs connection. However, the mechanism was quite different from pollutants though the same result was detected. Carbon source benefiting to microbes had been discovered previously[58]. We speculate that microbes need to cooperate much more tightly to resist stress[59] and high TOC ease the nutrition stress, which lead to weak connection. Compared with module 1, module 3 was more sensitive to nutrition content. Both TOC and TN affected microbes connection. The interesting thing is DPs influenced the network. Although DPs has been used for nearly 50 years, its environmental behavior and toxicological data are limited[60,61]. Recently, bioaccumulation of DPs in fish and humans has been reported[60], but the role in microbial communities is still unclear. According to our knowledge, it is the first time that the relationship between DPs and microbial communities was discovered. As for module 2, no soil variables altered the interaction strength of microbes indicating its excellent adaptability to e-waste contaminated soils.

The module hubs and connectors of network might be the key species that play a crucial role in microbial communities[62]. No connectors were detected in the present study, which may be attributed to the fact that heavy contamination in e-waste contaminated soils breaks the connection among different modules. Five module hubs were identified through *Z-P* plot (Figure 4, Table 3). Iii1-15 affiliating with *Acidobacteria* and *Piscirickettsiaceae* are uncultured and no information about their function was reported to date. *Bradyrhizobiaceae* and *Nitrospira* were related to nitrogen metabolism[63,64], which indicated that the metabolism of nitrogen play a crucial role in interaction of microbe in e-waste contaminated soils. *Kaistobacter* belonging to Sphingomonadaceae, exhibited power ability to degrade pollutants[65,66], which suggested the capability of organic pollutants utilization was important in microbial communities.

In this study, we took an in-depth look at the microbial community profiles in e-waste contaminated soils and the impact of soil properties on microbes. It was found that general soil properties (TOC, TN, pH), organic pollutants (PAHs, PCBs, PBDEs) and heavy metals (Cu, Zn, Pb) affected microbial communities in different dimensions such as microbial abundance, diversity and inner associations. This study help us understand the   ecological risk assessment of e-waste pollution on natural microbial communities with an overall viewpoint on a large scale.

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