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Impacts of addition of natural zeolite or a nitrification inhibitor on antibiotic resistance genes during sludge composting

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ABSTRACT

Composting is commonly used for the treatment and resource utilization of sewage sludge, and natural zeolite and nitrification inhibitors can be used for nitrogen conservation during sludge composting, while their impacts on ARGs control are still unclear. Therefore, three lab-scale composting reactors, A (the control), B (natural zeolite addition) and C (nitrification inhibitor addition of 3,4-dimethylpyrazole phosphate, DMPP), were established. The impacts of natural zeolite and DMPP on the levels of ARGs were investigated, as were the roles that heavy metals, mobile genetic elements (MGEs) and the bacterial community play in ARGs evolution. The results showed that total ARGs copies were enriched 2.04 and 1.95 times in reactors A and C, respectively, but were reduced by 1.5% in reactor B due to the reduction of conjugation and co-selection of heavy metals caused by natural zeolite. Although some ARGs (bla_{CTX-M}, bla_{TEM}, *ermB*, *ereA* and *tetW*) were reduced by 0.3–2 logs, others (*ermF*, *sulI*, *sulII*, *tetG*, *tetX*, *mefA* and *aac(6′)-Ib-cr*) increased by 0.3–1.3 logs after sludge composting. Although the contributors for the ARGs profiles in different stages were quite different, the results of a partial redundancy analysis, Mantel test and Procrustes analysis showed that the bacterial community was the main contributor to the changes in ARGs compared to MGEs and heavy metals. Network analysis determined the potential host bacteria for various ARGs and further confirmed our results.

1. Introduction

Alexander Fleming once warned: “the ignorant may someday misuse his life-saving discovery—penicillin—and select for resistant bacteria” (Price et al., 2015). His warning has come to pass with the increasing worldwide emergence and spread of antibiotic resistance genes (ARGs) that pose increasingly serious risks to human health, especially when ARGs are acquired by human pathogenic bacteria through horizontal gene transfer (HGT). *Acinetobacter baumannii* strain AYE, which caused an epidemic in France, has a resistance island in which 45 resistance genes from various environments and microbes are clustered (Fournier et al., 2006). Wastewater treatment plants (WWTPs) are considered to be hotspots for ARGs investigation, and sewage sludge contributes far more (ca. 1000 times) to the release of ARGs into the environment compared with WWTPs effluent (Munir et al., 2011). Furthermore, antibiotic resistance factors from WWTPs have been demonstrated to be associated with clinical pathogens (Szczepanowski et al., 2009; Kumaraswamy et al., 2014), and HGT in sewage sludge might occur more frequently due to its high bacterial density and diversity of bacterial communities (Su et al., 2015). In China, 6.25 million tons of dry solids were produced in 2013, and total sludge production grew by 13% annually from 2007 to 2013 (Yang et al., 2015). Thus, sewage sludge treatment could be a key

Abbreviations: ARGs, antibiotic resistance genes; ARB, antibiotic resistance bacteria; DMPP, 3,4-Dimethylpyrazole phosphate; MGEs, mobile genetic elements; HGT, Horizontal gene transfer; WWTPs, Wastewater treatment plants; MRGs, Heavy metal resistance genes; qPCR, Quantitative PCR; DW, Dry weight.

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point for implementing strategies to reduce the risks of ARGs coming from WWTPs (Burch et al., 2013).

Interest in sludge composting as an effective resource utilization strategy, along with subsequent land applications, continues to grow because of constraints and environmental concerns around the alternative landfill and incineration strategies. Composting is an effective and widely used method for sludge treatment in which sewage sludge is transformed into mature products for soil amendments and fertilizer (Wei et al., 2000). However, sludge composting has been demonstrated to enrich ARGs; thus, the direct application of sewage sludge compost onto fields may lead to the increased spread of ARGs in the soil (Su et al., 2015). This suggests that operational parameters of sludge composting must be further optimized to reduce ARG accumulation in sludge compost. The effects of various sludge digestion conditions on the removal of ARGs, including thermal hydrolysis pretreatment, thermophilic and mesophilic anaerobic digestion conditions, have been investigated in detail (Ma et al., 2011; Zhang et al., 2015c). While natural zeolite is usually added for nitrogen conservation, to reduce NH_3 emissions and to reduce atmospheric pollution, and a nitrification inhibitor addition has also be demonstrated to be a promising way for nitrogen conservation during sludge composting (Zhang et al., 2015a). The addition of natural zeolite and a nitrification inhibitor during sludge composting might offer an added benefit for the removal of ARGs, although this has not been experimentally determined. Furthermore, there is still no information about the effects of natural zeolite or nitrification inhibitors on the evolution of the bacterial community during sludge composting.

Heavy metals, which are found in high levels in sewage sludge (Cheng et al., 2014), are considered to be co-selection factors for antibiotic resistance (Baker-Austin et al., 2006) and have been shown to select for antibiotic resistance among pathogenic and commensal bacteria from various environments (Becerra-Castro et al., 2015; Yazdankhah et al., 2014; Amachawadi et al., 2015). When a large number of ARGs and their host bacteria come into contact with heavy metals, heavy metal-driven selection of antibiotic resistant bacteria (ARB) can occur (Becerra-Castro et al., 2015). However, the effects of heavy metals on the evolution of ARGs during sludge composting are still not clear. There are several key unanswered questions that require further study in the context of sludge composting: (1) the effects of natural zeolite and a nitrification inhibitor on the levels of ARGs; (2) the degree to which different variables, including heavy metals, MGEs and the bacterial community, affect the enrichment of ARGs; and (3) how to best control ARGs during sludge composting for resource utilization. Therefore, in this study three identical lab-scale reactors (A, B and C) were established to investigate the effects of the addition of natural zeolite and one of the most effective nitrification inhibitors, 3,4-dimethylpyrazole phosphate (DMPP), on the evolution of twelve frequently detected ARGs. These ARGs include three tetracycline resistance genes (*tetG*, *tetW* and *tetX*), two sulfonamide resistance genes (*sulI* and *sulII*), one fluoroquinolone resistance gene (*aac(6′)-Ib-cr*), two β-lactam resistance genes (bla_{CTX-M} and bla_{TEM}) and four macrolide resistance genes (*ereA*, *ermB*, *ermF* and *mefA*). Additionally, to determine the mechanisms underlying the selection of ARGs associated with HGT and heavy metals, four MGEs were also quantified, including the class 1 integrase gene (*intI1*), the conjugative transposon Tn*916*-Tn*1545* family (Tn*916/1545*), one insertion sequence common region I gene (IS*CR1*) and one type of conjugative plasmid (IncQ *oriV*). Five heavy metal resistance genes (MRGs), *tcrB*, *copA*, *cueO*, *cusA* and *pcoA*, were used to determine heavy metal resistance. Moreover, heavy metals and bioavailable heavy metals were quantified, and changes in the composition of the bacterial community were investigated using 16S rRNA gene high-throughput sequencing.

2. Materials and methods

2.1. Composting experimental setup

Sewage sludge composting was carried out in three identical lab-scale reactors labeled A, B and C. Reactor A was treated as the control, and reactors B and C were added with natural zeolite (1% wet weight) and DMPP (1% total nitrogen), respectively, as previously described (Zhang et al., 2015a). Raw materials for composting, including dewatered sewage sludge collected from Qinghe wastewater treatment plants in Beijing, China, and spent mushrooms were thoroughly mixed at a ratio of 1:3 (*v*/*v*), and then approximately 45 kg of the mixture was added to each reactor. The composting experiment was conducted for 183 days. Detailed information about the composting experimental setup was described in supporting information.

2.2. Sampling and heavy metal analysis

Samples were collected on days 1, 3, 13, 21, 73, and 183, ranging over the four composting stages (Fig. S1): the mesophilic stage (days 1 and 3), the thermophilic stage (day 13), the cooling stage (day 21), and the maturation stage (days 73 and 183). Samples were taken at five positions randomly at a depth of 10–15 cm for each reactor, and the five sub-samples were then mixed well, resulting in a representative sample. All the samples were stored at −80 °C for further analysis.

After being freeze-dried and ground to sieve through 100-μm mesh, 0.1 g of each sample was digested in a 8-mL mixture of aqua regia and hydrofluoric acid (3:1, *v*/*v*) using the microwave assisted digestion method 3051A by a microwave digestion system (Mars 5, CEM Corp, Matthews, NC, USA) (USEPA, 2007). Then, perchloric acid (0.5 mL) was added, and the sample was evaporated at 150 °C until white fumes appeared. The residue was dissolved in deionized water for total heavy metals analysis. The bioavailable fractions of heavy metals were determined by the method reported by Huerta-Diaz and Morse (1990) and Diop et al. (2015). Briefly, approximately 0.4 g of each sludge composting sample was leached using 20 mL of 1 M HCl for 24 h with continuous agitation at room temperature, and the solution was filtered through a 0.45-μm membrane. The measurements for total and bioavailable heavy metals were run in duplicate for each sample. All the heavy metal concentrations were determined using inductively coupled plasma-mass spectrometry (ICP-MS) or optical emission spectrometry (ICP-OES) according to the concentrations of different heavy metals.

2.3. DNA extraction

Total genomic DNA was extracted in triplicate from 0.2 g of freeze-dried samples using a FastDNA Spin Kit for soil (MP Biomedical, France) according to the manufacturer's instructions. The triplicate DNA extracts were then merged together for further analysis. Extracted genomic DNA was detected and quantified using 1% agarose gel electrophoresis and a NanoDrop 2000 (Thermo Scientific, USA), respectively, and then stored at −20 °C until use.

2.4. Quantitative PCR (qPCR)

Twelve frequently detected ARGs, four MGEs and five MRGs were quantified by qPCR. The plasmids containing these specific genes, used as standards in a 10-fold dilution for making qPCR standard curve, were manufactured by Zhejiang Tianke Biotechnology Company (Zhejiang, China). The 25-μL PCR reaction mixtures contained 12.5 μL of SYBR Green qPCR Super-Mix-UDG with Rox (Invitrogen, USA), 0.5 μL each of 10 μM forward and reverse

primers, 10.5 μL of DNA-free water, and 1.0 μL of standard plasmid or DNA extract. The thermocycling steps for qPCR amplification were as follows: (1) 50 °C, 2 min; (2) 95 °C, 5 min; (3) 95 °C, 20 s; (4) annealing temperature, 30 s; (5) 72 °C, 31 s; (6) plate read, repeat steps (3) through (5) 39 more times; (7) melt-curve analysis: 60 °C–95 °C, 0.2 °C read. The reaction was conducted using an ABI Real-time PCR system 7500 (ABI, USA). Primer specificity was confirmed by melting curves and gel electrophoresis. Each gene was quantified in triplicate for each sample using a standard curve and a negative control. The primers, annealing temperature used in qPCR and the corresponding amplification efficiencies were summarized in Tables S1 and S2.

2.5. High-throughput sequencing and bioinformatics analysis

PCR primers 515F/806R targeting the bacteria and archaea 16S V4 region were selected for the microbial community structure analysis (Caporaso et al., 2010). The reverse primer contains a 6-bp error-correcting barcode unique to each sample. The barcode was permuted for each sample and permitted the identification of individual samples within a mixture in a single Illumina MiSeq sequencing run. DNA was amplified in triplicate for each sample following a protocol described previously (Caporaso et al., 2010). PCR amplicons were further purified using a DNA purification kit (BioFlux, Japan), and the concentrations were determined by spectrometry using a QuantiFluor™-ST (Promega, USA). Amplicons from different samples were then mixed to achieve equal mass concentrations in the final mixture, which were sent out to Majorbio Co., Ltd. in Beijing for small-fragment library construction and pair-end sequencing using the Illumina MiSeq sequencing system (Illumina, USA).

Sequencing reads were assigned to each sample according to the unique 6-bp barcode for each sample. Pairs of reads from the original DNA fragments were merged using FLASH (Magoč and Salzberg, 2011) and then were filtered using QIIME quality filters. PCR chimeras were filtered out using UCHIME (Edgar et al., 2011). After the above filters were applied, the minimum number of selected sequences in the 16 samples was 26,710. To fairly compare the 16 samples at the same sequencing depth, the sequence number was normalized by extracting the first 26,710 sequences from each sample for further analysis. The normalized sequences were uploaded to MG-RAST (http://metagenomics.anl.gov/linkin.cgi?project=13356). The taxonomic classification of the sequences in each sample was carried out individually using RDP Classifier, and the sequences of different taxonomy levels were assigned at the bootstrap cutoff of 50%, as suggested by the RDP (Wang et al., 2007). In addition, diversity and richness indices were calculated using the relevant RDP pipeline modules as previously described (Zhang et al., 2015b).

2.6. Statistical analysis

The generation of plots for the evolution of ARGs, MGEs, MRGs and heavy metals was performed with OriginPro 9.0 (OriginLab, USA), and Excel 2013 (Microsoft, USA) was used to determine the averages and fold change values of ARGs. The gene copies indicated the absolute copy numbers present per unit of dry weight (DW), while the normalized copy number by 16S rRNA was regarded as the abundance. The Spearman correlation was performed using SPSS 21.0 (IBM, USA), and a p value <0.05 was considered statistically significant. Principal component analysis (PCA), redundancy analysis (RDA), partial RDA and Procrustes analysis were conducted using Canoco 5.0 (Microcomputer Power, USA). To clarify the correlation between the evolution of ARGs and the bacterial community, a Mantel test was conducted using PAleontological STatistics software (PAST 3.07). To determine which ARGs were primarily responsible for an observed difference between different treatments, SIMPER (Similarity Percentage) was also conducted by PAST. The heat maps illustrating the evolution of the quantified genes and the top ten genera in each sample were built by Heml 1.0 (Deng et al., 2014). Network analysis based on the Spearman analysis between ARGs and the bacterial community (based on OTU), MRGs and MGEs was determined using the Gephi platform (Bastian et al., 2009).

3. Results and discussion

3.1. Effects of natural zeolite and DMPP addition on ARGs evolution

The ARGs copy numbers and abundance of total ARGs changed dramatically during sludge composting, and the addition of natural zeolite and DMPP certainly affected the evolution of total ARGs (Fig. 1). However, the evolution tendency of biomass after natural zeolite and DMPP addition revealed by 16S rRNA did not differ drastically upon natural zeolite or DMPP addition (Fig. S2). The gene copies of total ARGs were significantly and positively correlated with 16S rRNA gene copies for the control ($p < 0.0001$) and natural zeolite addition ($p = 0.019$) groups but not for the DMPP addition ($p = 0.072$) group. Additionally, the peak gene copies of ARGs for the DMPP addition group were delayed until D21 compared with the control and natural zeolite addition groups, which peaked at D13 (Fig. 1A). In terms of absolute gene copies, sludge composting showed some enrichment in ARGs in the control (2.04 times) and DMPP addition (1.95 times) groups, while the natural zeolite addition group showed a slight reduction in total ARGs (1.5%). However, in terms of gene abundance, total ARGs were reduced by 12.4%, 16.2% and 1.9% for reactors A, B and C, respectively, after sludge composting. The lowest reduction of the ARGs abundance in reactor C indicated that nitrification inhibitor may also select the ARGs. The selective pressure caused by nitrification inhibitor was observed through the inhibitory of microbial activity reflected by the temperature (Fig. S1), while the highest reduction in reactor B indicated that the selective pressure was reduced, and this needed further clarification. Furthermore, these results indicated that, without additives, ARG abundance increased during sludge composting, which corroborated the report previously (Su et al., 2015) who showed that the abundance of total ARGs increased in the control group at the maturation stage as shown in Fig. 1B. According to SIMPER analysis based on the Bray–Curtis measure of distance, *ermB*, *sulI* and *sulII* contributed the most to the differences between treatments, and their cumulative contribution ranged from 62.1% to 71.4%. The dissimilarity between reactors A and B (51.4) was higher than that of reactors A and C (49.8).

The change in the prevalence of each ARG was quite different during sludge composting. The *erm*B gene was predominant at the beginning of composting, and *sulI*, *sulII* and *tetX* became dominant later, as shown in Figs. 1 and 2. Detailed information on the gene copies of each gene is shown in Table S3. Sludge composting reduced the abundance of some ARGs, including $bla_{\text{CTX-M}}$, bla_{TEM}, *ereA*, *ermB* and *tetW*, in all the three reactors, while enriching others, including *aac(6′)-Ib-cr*, *ermF*, *sulI*, *sulII*, *tetG* and *tetX*. The levels of reduction and enrichment in the different reactors are shown in Table S4, which reflected the fact that *ereA* was reduced the most (2.3 logs) by natural zeolite addition while *tetX* was enriched the most in the control (1.25 logs). The increase of *tetX* may be not a bad thing, because it can degrade tetracyclines and reduce the further selective pressure from tetracyclines. The reduction of ARGs with the addition of natural zeolite (0.5–2.3 logs) was generally greater than that in the control group (0.003–0.9 logs) and the DMPP addition group (0.5–1.5 logs), and

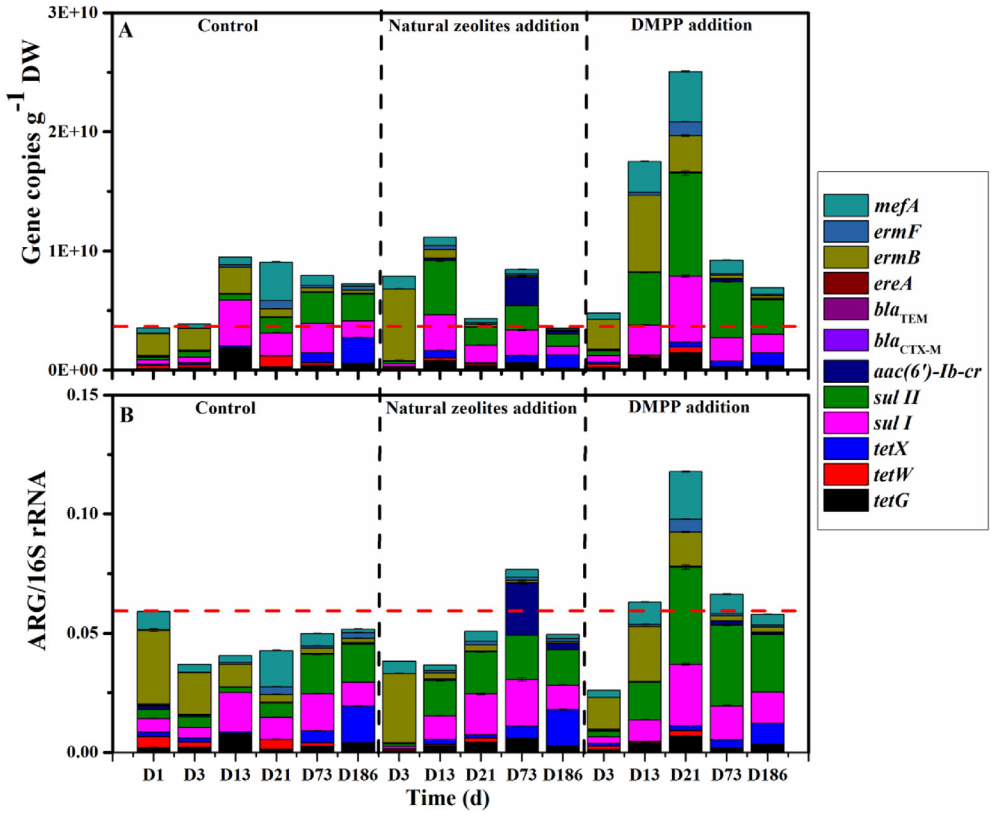

Fig. 1. Changes in the absolute gene copy numbers (A) and abundance (B) of the ARGs in different treatments during sludge composting.

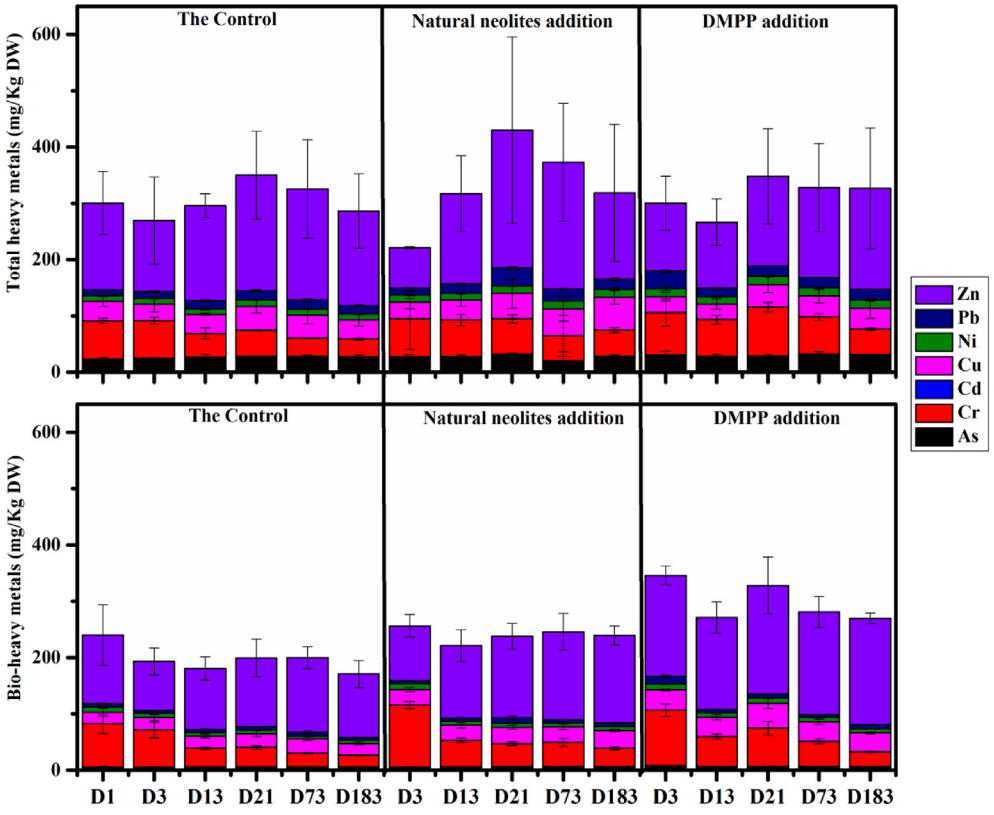

Fig. 2. Changes of total heavy metals (up) and bioavailable heavy metals (down) in different treatments of the control (A), natural zeolites (B) and DMPP (C) addition, respectively.

the enrichment of ARGs was always lower for reactor B (0.3–0.9 logs) except for the *aac(6′)-Ib-cr* gene, which increased by 0.3 logs. ARGs abundance showed similar trends, as shown in Table S5. According to the risk ranking in the antibiotic resistome published previously (Martínez et al., 2015), genes encoding β-lactamases, including $bla_{\text{CTX-M}}$ and bla_{TEM}, belong to the highest risk category (RESCon 1). Our data indicated that sludge composting could effectively reduce the abundance of these high-risk ARGs. However, this was in contrast to a previous study (Su et al., 2015), and the gene enrichment was generally much lower in our study. A detailed comparison of gene enrichment is shown in Table S6. The discrepancy was likely due to the different raw materials used (sawdust and rice straw vs spent mushroom), which led to the significantly different bacterial community structures reported in these two studies. These differences highlighted the importance of optimization of operational parameters for the control of ARGs in sludge composting.

3.2. Role of heavy metals and MRGs in the selection of ARGs

While considering the co-selection of heavy metals, both total heavy metal concentrations and their speciation should be analyzed because it is the bioavailable fractions of heavy metals that impose selective pressure on microbes (Seiler and Berendonk, 2012). Moreover, MRGs could reflect the real response of bacteria to the selective pressures of heavy metals, and copper has been widely demonstrated to co-select for antibiotic resistance (Stepanauskas et al., 2005; Ji et al., 2012), and the evolution of copper resistance genes has been investigated in various environments (Amachawadi et al., 2013, 2015; Jia et al., 2013; Xiong et al., 2015c). Thus, the copper resistance genes quantified in this study were used to represent MRGs.

Fig. 2 shows the changes in heavy metals and bio-heavy metals during sludge composting for the three reactors, and the MRGs changed significantly as shown in Fig. 3. The absolute gene copies of MRGs are shown in Table S3, and different MRGs showed different abundances over the course of sludge composting. The *cusA* and *copA* genes were predominant in the control group, while *cusA* was the predominant gene in sludge composting with either natural zeolite or DMPP addition. The dominance of *cusA* indicated that the selective pressure imposed by copper was high because *cusA* dominates at higher copper levels compared with *copA*, as demonstrated previously (Besaury et al., 2014; Outten et al., 2001). According to the network analysis based on Spearman correlations, significantly positive correlations ($p < 0.05$) were found between ARGs and heavy metals along with MRGs, as shown in Fig. S3. This was particularly true for *sulII*, which had a significantly positive correlation with 13 heavy metals factors, indicating that *sulII* may be co-selected by more heavy metal relevant factors. Moreover, ARGs generally co-occurred with plasmid-encoded MRGs than with chromosome-encoded MRGs. For instance, *tcrB* was generally thought to exist on a conjugative plasmid, and the co-occurrence of *tcrB* with *ermB* has been demonstrated (Amachawadi et al., 2013). In this study, *tcrB* was significantly and positively correlated with 5 ARGs, including *sul*II, bla_{TEM}, $bla_{\text{CTX-M}}$, *ereA* and *ermB*. Additionally, *pcoA*, which was present only on plasmids and encoded a multi-copper oxidase protein (Besaury et al., 2014), was significantly and positively correlated with 5 ARGs, including *sulII*, bla_{TEM}, $bla_{\text{CTX-M}}$, *ereA* and *tetG*, and the same for *cueO* (*sulII*, $bla_{\text{CTX-M}}$, bla_{TEM} and *ereA*). In summary, network analysis elucidated that co-selection of heavy metals and the co-occurrence of MRGs and ARGs generally existed and that heavy metal relevant factors should be emphasized when considering the control of ARGs during sludge composting.

Natural zeolite has been widely considered to be able to inactivate the heavy metals, and this was confirmed by the lowest abundance of copper resistance genes at the end (Fig. 3). This further elucidated that the selective pressure from copper was the lowest, which led to the removal of ARGs due to the less co-selection from heavy metals in comparison with the control and DMPP addition groups.

3.3. Role of MGEs on the evolution of ARGs

The role of HGT in the proliferation of ARGs has been widely elucidated, and generally, conjugation, as opposed to transduction and natural transformation, was considered the main mechanism for HGT of ARGs in various environments (Huddleston, 2014). Thus the relevant genes concerning conjugative transfer elements were quantified to probe the following hypothesis: ARGs may have been integrated into integrons (*intI1* and IS*CR1*), then integrons were further integrated into transposons or plasmids, and conjugative transposons (Tn*916/1545*) or plasmids (IncQ *oriV*) could transfer between microbes through cell-to-cell contact. The genes *intI*1 and

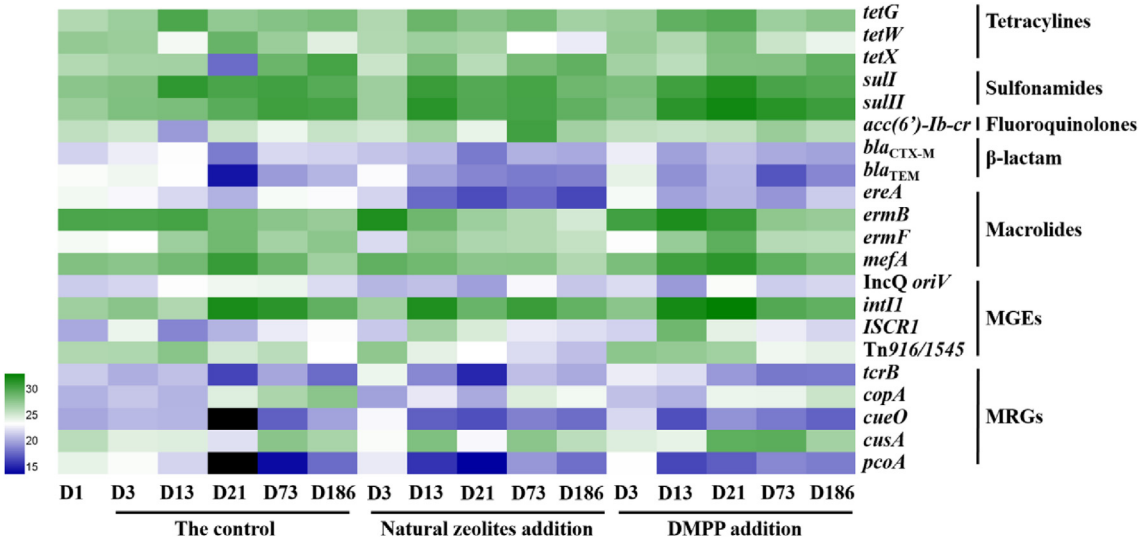

Fig. 3. Heatmap of changes in ARGs, MGEs and MRGs during sludge composting in different reactors. Values plotted are the natural log2 transformed of the copies of each ARG.

IS*CR1* were used as a marker for the capture of ARGs, and Tn*916/1545* and IncQ *oriV* represented for the common conjugative elements of transposons and plasmids, respectively.

As shown in Fig. 3, *intI1* was the predominant MGE throughout sludge composting, and both gene copies and abundance of *intI1* increased in the final stages of composting. The significantly positive correlation ($p < 0.001$) between MGEs and total ARGs indicated that HGT occurred frequently during sludge composting and that it had a significant impact on the changes in ARGs. MGEs had a significantly positive correlation ($p < 0.05$) with almost all of the increased ARGs, including *tetG*, *sulI*, *sulII*, *ermF* and *mefA*, except *tetX* ($p = 0.35$), which suggested that HGT aided in the enrichment of these genes during sludge composting. The correlation between *intI1*, which has often been considered a marker for the proliferation of ARGs (Gillings et al., 2014), and each ARG showed a similar pattern as seen with MGEs. In this study, *intI1* appeared to be a proper marker for ARG proliferation during sludge composting. However, it is interesting to note that the changes in conjugative-relevant MGEs (Tn*916/1545* and IncQ *oriV*) increased in the thermophilic stage but decreased during the maturation stage, which corresponded to the increased ARGs. This may be due to the need for cell-to-cell contact and higher microbial activity for conjugation and thus highlighted the importance of maturation for sludge composting.

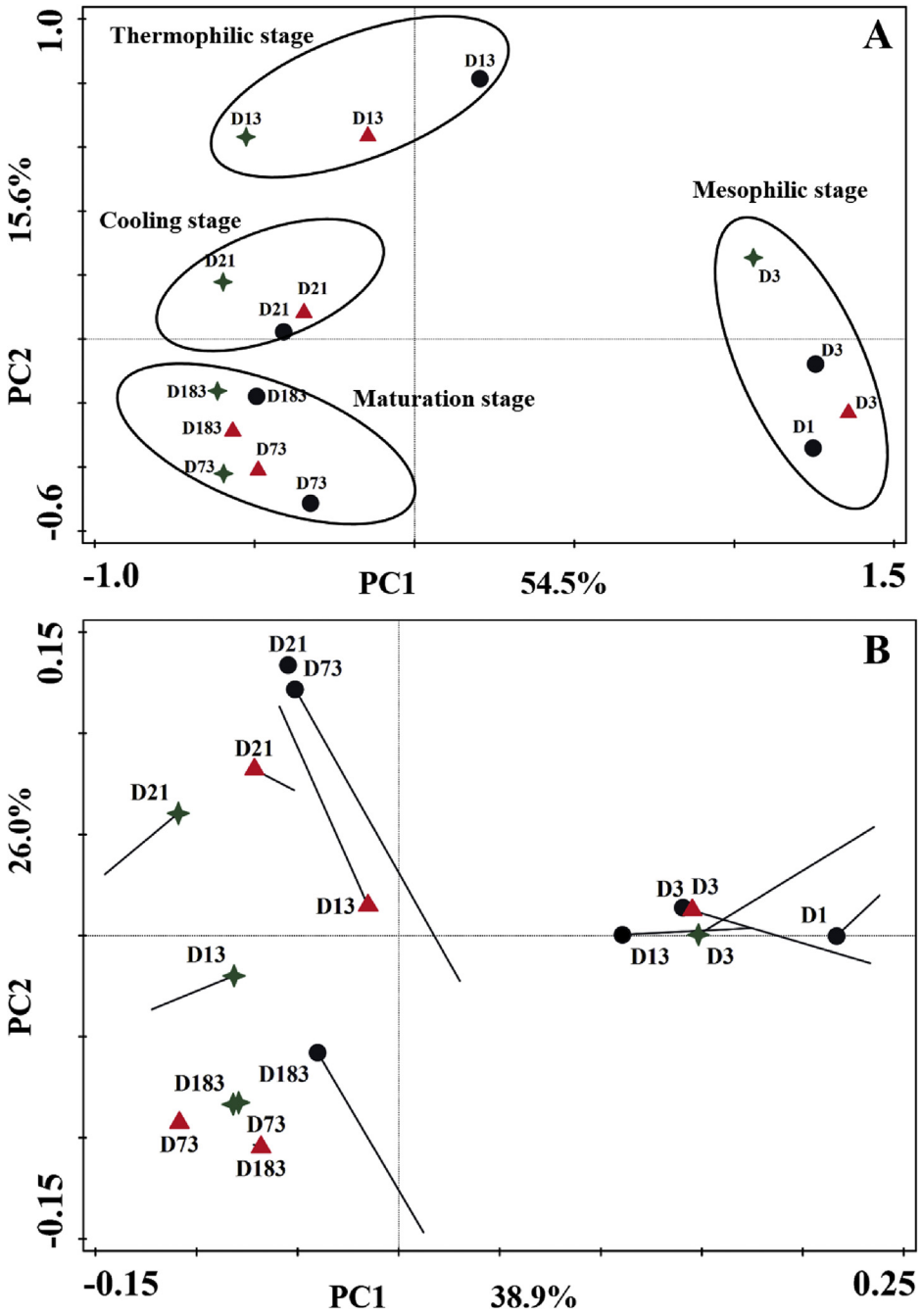

Fig. 4. Principal component analysis (PCA) showing the changes of the bacterial community for sludge composting in the three reactors (A) and Procrustes analysis showing the significant correlation between ARG profiles and the bacterial community (B). Black circles, green stars and red triangles represent samples from reactors A (the control), B (natural zeolite addition) and C (DMPP addition), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Besides, natural zeolite has sporous structure, and it could enlarge the space between the microbes or reduce rate of the microbe contact, while microbe contact is the first step for conjugation. Thus, it was assumed that the effects of natural zeolite on ARGs removal may be also associated with the reduction of HGT through conjugation compared to the control and DMPP addition groups, and this was further confirmed by the lowest abundance of Tn*916/1545* and IncQ *oriV* for natural zeolite addition at the end of sludge composting.

3.4. Changes in the bacterial community

According to the rarefaction curve, community richness and diversity analysis (Fig. S4 and Table S7), the diversity of the bacterial community for the three reactors decreased during the mesophilic and thermophilic stages and then increased at the cooling and maturation stages, which was typical for composting (Ryckeboer et al., 2003). Previous studies have demonstrated that pile temperature played an important role in shaping the microbial community (Zhang et al., 2015b). Principal component analysis (PCA) showed that the bacterial community structure changed significantly over the course of sludge composting. The bacterial community developed as shown in Fig. 4A during the four stages of composting. This profile was further confirmed by UPGMA clustering analysis (Fig. S5). The addition of natural zeolite and DMPP exerted a limited influence on the changes in the bacterial community and did not delay or accelerate the evolution of the bacterial community.

The composition of the bacterial community changed significantly at the phylum and genus levels, as shown in Figs. 5 and S6. The phyla *Actinobacteria*, *Firmicutes*, *Proteobacteria* and *Bacteroidetes* were the four predominant phyla, ranging from 71.4% (D73 of A) to 96.3% (D13 of C) of the sequences in each sample during sludge composting. The abundance of *Actinobacteria* decreased at the mesophilic and thermophilic stages in the control and DMPP addition groups, then increased significantly at the cooling and maturation stages. However, after natural zeolite addition, it increased during the entire sludge composting process. The final abundance of *Actinobacteria* was 28.4%, 41.1% and 38.1% for the control, natural zeolite and DMPP addition groups, respectively. *Actinomycetales* was the predominant order and the main contributor to the evolution of *Actinobacteria* during sludge composting. The abundance of the *Actinobacteria* phylum has long been considered a marker for compost maturity (Wang et al., 2013); thus, the increased abundance of this phylum after natural zeolite and DMPP addition indicated the higher maturity of the resulting compost. The main genera were *Saccharomonospora*, *Actinomadura*, *Nonomuraea* and *Corynebacterium*. The evolution of these genera differed significantly, as shown in Fig. 5. The *Corynebacterium* genus, which contained potential human pathogens, was the dominant *Actinobacteria* genus at the mesophilic stage but became undetectable by the final stages of composting. *Saccharomonospora* was the main thermophilic *Actinobacteria*, while *Actinomadura* was predominant at the cooling and maturation stages, and

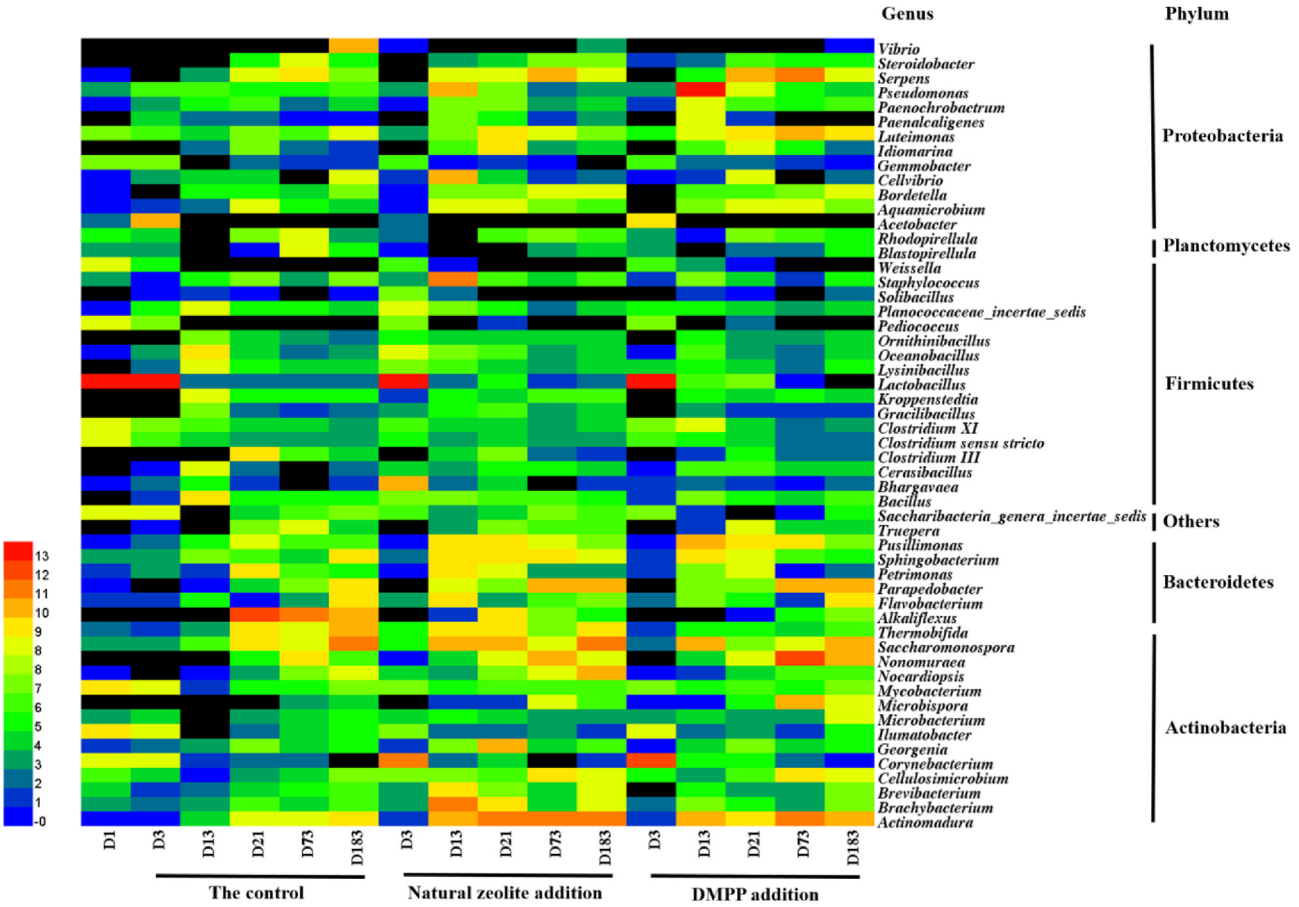

Fig. 5. Heatmap of the top ten genera for each sample. The top 10 abundant genera in each sample were selected, and a total of 54 genera for 16 samples were chosen. The color intensity shows the log2-transformed number of reads as the color key indicates at the bottom left. Black indicates that no reads were detected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Nonomuraea was mainly found at the maturation stage. *Firmicutes* was the most abundant at the mesophilic and thermophilic stages (ranging from 56.0% to 92.5% for each sample) but decreased significantly at the cooling and maturation stages (ranging from 8.5% to 16.3%), and the *Bacilli* class was the main contributor to the changes in *Firmicutes* under the different treatments. The main genus, *Lactobacillus*, belonging to the order of *Lactobacillales*, was dominant at the mesophilic stage, accounting for 64.2%, 31.5% and 58.1% of the control, natural zeolite and DMPP addition groups, respectively, but decreased to nearly undetectable levels at the final stage of composting. The order of *Bacillales* was the predominant order belonging to *Firmicutes* during the cooling and maturation stages.

The *Proteobacteria* phylum decreased at the mesophilic or thermophilic stages but increased at the cooling and maturation stages and was enriched 2.6, 1.5 and 2.6 times after sludge composting in reactors A (26.3% at the end), B (15.6% at the end) and C (26.9% at the end), respectively. *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were the dominant classes in the phylum. The *Alphaproteobacteria* class was dominant at the mesophilic stage, and the order *Rhizobiales* was predominant. As sludge composting progressed, the *Gammaproteobacteria* class became dominant in the *Proteobacteria* phylum. The orders *Xanthomonadales* and *Pseudomonadales* were used as markers for the *Gammaproteobacteria* class. The *Serpens* genus was predominant at the maturation stage, while *Pseudomonas* and *Cellvibrio* were the main *Gammaproteobacteria* at the thermophilic stage. The order *Burkholderiales*, belonging to *Betaproteobacteria*, was predominant throughout the sludge composting, and *Pusillimonas* was the predominant genus. The phylum *Bacteroidetes* also increased significantly after sludge composting and was enriched 16.1, 8.3 and 10.5 times with a final abundance of 23.8%, 12.2% and 15.5% for reactors A, B and C, respectively. *Sphingobacteriia*, *Bacteroidia* and *Flavobacteriia* were the predominant classes in this phylum. The genera *Sphingobacterium* and *Parapedobacter* were the predominant *Sphingobacteriia*, while *Alkaliflexus* was the dominant genus belonging to *Bacteroidia* at the cooling and maturation stages, especially for the control group. *Flavobacteriaceae* was the predominant family belonging to the *Flavobacteriia* class.

3.5. The role of the bacterial community in the changes in ARGs compared with the role of heavy metals and MGEs

Procrustes analysis was conducted by rotating the ordination of changes in the bacterial community to match the profiles of ARGs based on PCA analysis, and the results (Fig. 4B) indicated that most of the ARGs and bacterial communities clustered according to different composting stages. The explanatory variables accounted for 49.8%, and the correlation between the first two axes was significantly positive (R = 0.94 and 0.74, respectively). A Mantel test confirmed that ARG profiles were significantly correlated with bacterial community based on Bray–Curtis distance (R = 0.7833,

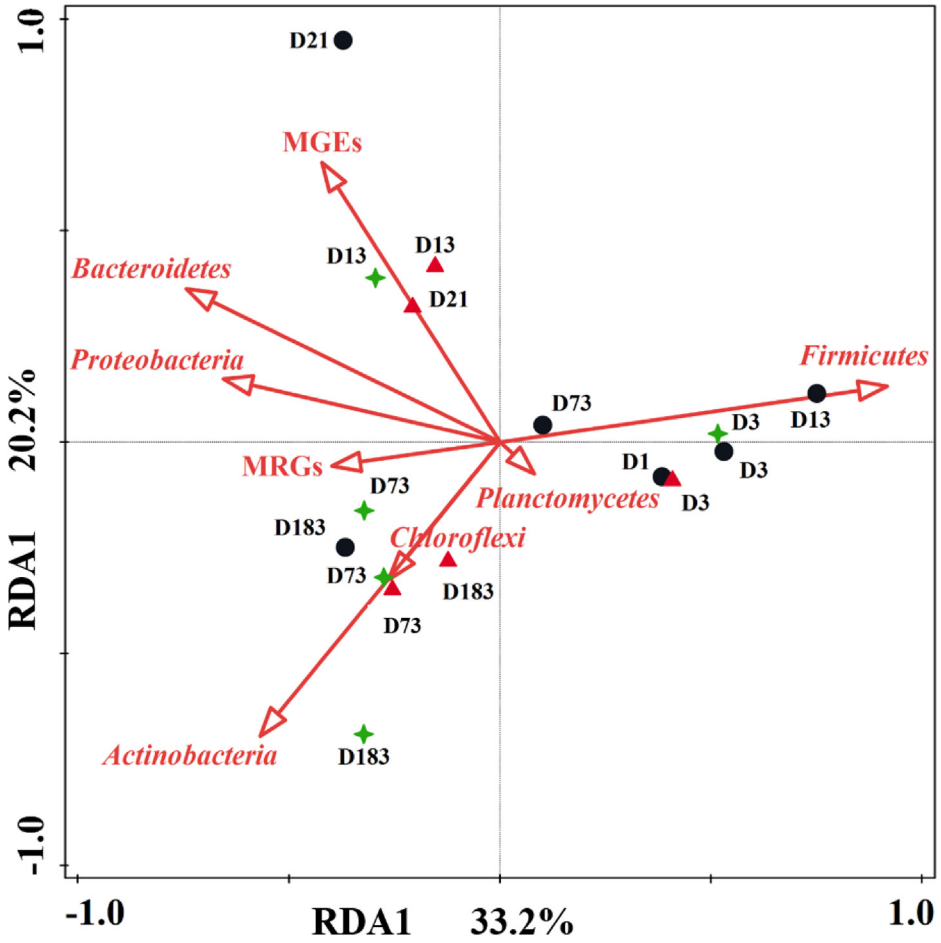

Fig. 6. RDA analysis results showing the relationship between environmental factors including bacterial community, MRGs and MGEs and ARGs profiles in sludge composting of the control (A), natural zeolite (B) and DMPP (C) addition, respectively. Black circle, green star and red triangle represented samples from reactor A (the control), B (natural zeolite addition) and C (DMPP addition), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$p = 0.0003$, permutation N = 9999). Together, changes in microbial community structure as sludge composting progressed contributed significantly to the profiles of ARGs.

RDA analysis was conducted to investigate the relationships between environmental factors, including bacterial community (based on the abundance of phylum), MGEs and MRGs and ARGs (Fig. 6), and the results showed that the explanatory variables accounted for 76.2% of the total variation. The contributors for the ARGs profiles in different stages were quite different. *Firmicutes* mainly contributed to the ARG profiles in the mesophilic stage, while *Actinobacteria* contributed at the maturation stage, and MGEs contributed at the thermophilic and cooling stages. These were corresponding to the different characteristics of the stages of sludge composting. Phylum *Firmicutes* dominated at the beginning, while at thermophilic and cooling stage, nutrition was released and microbial activity increased to the maximum, and these availed the HGT, thus, MGEs dominated the evolution of ARGs. Phylum *Actinobacteria* was typical for the sludge composting at maturation phase, and it contributed most to the development of the ARGs profiles at this stage.

To determine the key contributor to the explanatory variation as a whole and separate the influences of bacterial community, MGEs and MRGs, partial RDA was conducted by designating the explanatory variables and covariates. It was concluded that the bacterial community contributed the most (68.2%) to the ARG profiles, followed by MGEs (12.6%), and MRGs (10.9%). Indeed, the evolution of the bacterial community was the main driver for the changes in ARGs rather than HGT induced by MGEs or co-selection/co-occurrence determined by MRGs. The dominant contribution of the bacterial community to ARG profiles has been elucidated in various environments, including soils (Forsberg et al., 2014), rivers (Czekalski et al., 2014), WWTPs (Yang et al., 2014) and others (Xiong et al., 2014, 2015a, 2015b; Udikovic-Kolic et al., 2014). Nevertheless, the less frequent HGT incidents should not be overlooked, as even one HGT event into a human pathogen has the potential for great harm (Berendonk et al., 2015).

A previous study confirmed that network analysis could be used to provide new insights into ARGs and their possible hosts in complex environmental scenarios (Li et al., 2015). As shown in Fig. 7, various ARGs quantified in this study were significantly ($p < 0.05$) and positively correlated with various species. The network was clearly divided into two groups (Fig. 7): the ARGs that increased during sludge composting clustered together, and the host bacteria identified through the Spearman correlation analysis

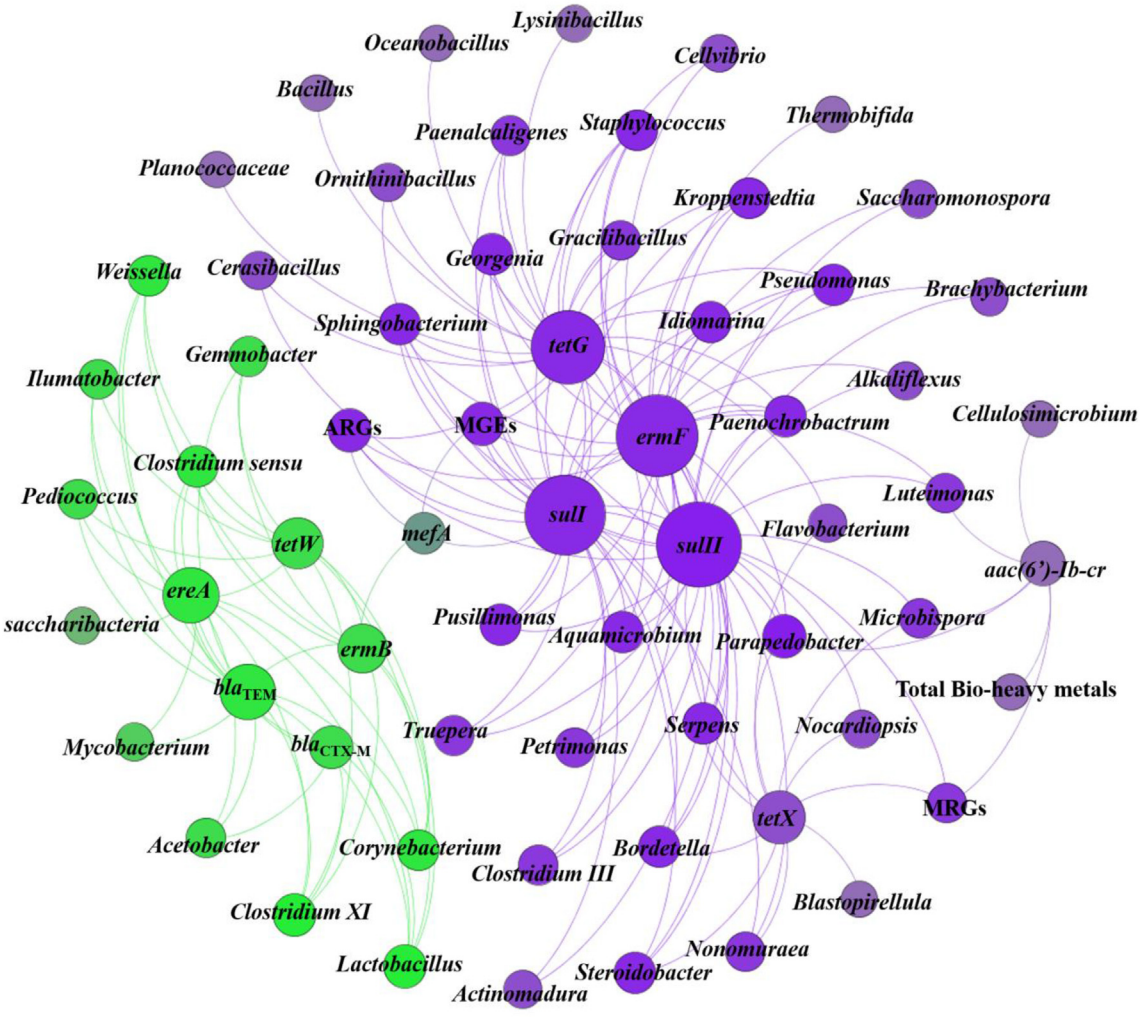

Fig. 7. Network analysis showing the co-occurrence of ARGs and their potential host bacteria. The nodes are colored according to whether the ARGs increased (purple) or decreased (green) during sludge composting, and a connection represents a significant positive correlation ($p < 0.05$). The bigger the size of each node, the greater the number of connections. The darker purple, the more the ARG is connected to others for the increased ARGs, while for the greener, the more the ARG is connected to others for the decreased ARGs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

clustered more (37) than the ARGs that decreased (11). The three most abundant ARGs, including *sulI*, *sulII* and *ermF*, had the most diversity in terms of host bacteria, with 24, 22 and 24 potential hosts, respectively. The abundance of the host bacteria of these genes generally increased as sludge composting progressed. For instance, the genera *Pseudomonas*, *Flavobacterium*, and *Nocardiopsis* all increased as sludge composting progressed (Fig. 5). The significantly positive correlation between different ARGs and the same host bacteria indicated by network analysis demonstrated the co-occurrence of different ARGs. The ARGs that decreased and increased were only linked by the gene *mefA*. As for the ARGs that decreased during sludge composting, their host bacteria also showed the same tendency. Thus, network analysis further elucidated the primary effects of bacterial community on the evolution of ARGs by determining their potential host bacteria.

The high pile temperature retention (>5 days) was enough to destroy pathogens through the guidelines for in-vessel composting (USEPA, 2000). However, no significant reduction in the abundance of ARGs was detected after the thermophilic stage. One reasonable explanation could be that environmental bacteria, and not only pathogens, harbored diverse and abundant ARGs. In other words, some non-pathogenic microbes may have also carried certain specific ARGs, a mechanism that has been reported (Forsberg et al., 2014). The potential host bacteria reflected by network analysis may be not ARB yet, and the significantly positive correlation with ARGs may be due to its functional connection with real ARBs. However, these potential host bacteria may have the biggest chance of becoming the ARBs through HGT due to their functional connection. This would make sense in case that the function connection was found between pathogens and non-pathogens.

4. Conclusions

Natural zeolite and DMPP have been demonstrated to be able to conserve nitrogen during sludge composting, and the effects of natural zeolite and DMPP addition on the changes in ARG abundance were investigated in this study. Conclusions and proposals derived from present work are as follows:

- Natural zeolite could be used to reduce some environmental risks of ARGs in sludge compost, and this may be due to the sporous structure and the ability of reduce the selective pressure from heavy metals. The sporous structure reduced the rate of microbe contact, and then HGT through conjugation was reduced. The passivation of heavy metals retarded the co-selection of heavy metals to ARGs.
- DMPP addition even increased the abundance of ARGs compared to the control, and this may be associated with the selective pressure as reflected by the evolution of the lowest pile temperature at thermophilic stage.
- The contributors for the ARGs profiles in different stages were quite different during sludge composting. *Firmicutes* mainly contributed to the ARG profiles in the mesophilic stage, while *Actinobacteria* contributed at the maturation stage, and MGEs contributed at the thermophilic and cooling stages. These indicated that more work should be done at the thermophilic and cooling stages to control the environmental risks of ARGs caused by HGT during sludge composting.
- Natural zeolite was little more than physical changes for sludge composting, and DMPP addition inhibited the microbial activity rather than changed the evolution of bacterial community structure. Their effects on the changes in the bacterial community were limited.
- Heavy metals, MGEs and bacterial community had varying impacts on the changes in ARGs, and the bacterial community was the main driver for the changes in ARGs rather than MGEs and selective pressures imposed by heavy metals. The key to controlling ARG proliferation during sludge composting is the regulation of the bacterial community. However, MGEs may brought more environmental risks than bacterial community concerning the evolution of ARGs.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.01.010.

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