Fate and behavior of antibiotic resistance genes and their relations with microbial profiles during vermicomposting of excess activated sludge

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抗生物質耐性遺伝子の挙動と微生物との関連性の解明

March 2019

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Abstract

Excess activated sludge is a reservoir of various pollutants and pathogens contained in wastewater. As a newly emerging environmental pollutant, antibiotic resistance genes (ARGs) have been highly concerned in recent years due to their likely adversary effects to humans and ecosystems, and their existence in excess activated sludge has been reported. Incineration, landfilling, anaerobic digestion and composting (including vermicomposting) are the major methods used for treatment and disposal of excess activated sludge. Of these methods, anaerobic digestion and composting are considered as more eco-friendly, economical and sustainable. According to previous studies, anaerobic digestion and conventional composting are less effective in attenuating ARGs existed in excess activated sludge, and the removal efficiency for ARGs is greatly affected by such factors as temperatures and the sludge properties. Compared to conventional composting, vermicomposting is more effective in alleviating the effect of toxic chemicals in organic waste due to the joint actions of earthworms and microorganisms. However, concerning the fate and behavior of various ARGs in excess activated sludge during its treatment by vermicomposting, little is known. Stabilization of organic waste through vermicomposting is driven by abundant and diverse microorganisms; however, the presence of earthworms can directly and/or indirectly alter the activities, abundances and communities of microorganisms through various actions like burrowing, gut digestion, mucus stimulation and cast discharge. Therefore, it is reasonable to infer that the involvement of earthworms may affect the dynamics of ARGs in excess activated sludge through their likely roles in regulating the microbial profiles involved in the treatment system (activity, density and structure). The objective of this study was to investigate the fate and behavior of ARGs and their association with microbial profiles in the vermicomposting treatment of domestic excess activated sludge. For this, the focus of this

study was placed on clarification of the effects of earthworm density, temperature and earthworm's gut digestion.

In regard of the effects of earthworm density, the changes of *qnr*A and *qnr*S, the two well-detected quinolone resistance genes in excess activated sludge, were investigated through vermicomposting experiment for three different earthworm densities (427, 854, 1281 earthworms/kg dry sludge); and the relationship of the quinolone resistance genes with the microbial profiles during vermicomposting treatment of excess activated sludge was discussed through redundancy analysis that used the environmental factors (including pH, electrical conductivity, dissolved organic carbon, ammonium and nitrate) and the integrase gene that transmits resistance genes as variables. The results showed that vermicomposting could significantly reduce *qnr*A and *qnr*S genes and integrase gene *intl*1. The extent of reduction at the final stage of vermicomposting was more obvious for the treatment introduced with the higher density of earthworms. Principal component analysis revealed that the behavior of the resistance genes was correlated more closely with the microbial activity and abundance than diversity.

The effect of vermicomposting temperature was investigated through experiments performed for three different temperatures (15, 20 and 25°C). The abundances of three tetracycline resistance genes (*tet*G, *tet*M and *tet*X), one sulfonamide resistance gene (*sul*1), two quinolone resistance genes (*qnr*A and *qnr*S) and the integrase gene (*intl*1) were determined by quantitative PCR with specific primers. In order to clarify the underlying mechanisms regarding the responses of the targeted genes to vermicomposting at different temperatures, the composition of bacterial community in the vermicomposing systems was analyzed through high-throughput sequencing. Vermicomposting at 25°C did not show significant reduction of the resistance genes in comparison to the vermicomposting at either 15°C or 20°C, which was not in coincidence with the results of stabilization during vermicomposting. Vermicomposting demonstrated higher removal efficiency for *qnr*A, *qnr*S and *tet*M, and an obvious selectivity in eliminating the three different types of ARGs. The

significantly increased activity of nitrification and the resulting increased formation of nitrate nitrogen at 25°C was probably a reason that contributed to the rebound of *qnr* and *tetG* genes at the end of vermicomposting.

For the effects of earthworm gut digestion, three different resistance genes, *qnr*, *sul* and *tet,* in viable bacterial cells were quantified by fluorescence quantitative PCR after PMA pretreatment. The bacterial community structure was determined by high-throughput sequencing. The abundant and diverse cell-free resistance genes and *intl1* gene in the domestic excess activated sludge probably implied that transformation is a potential mechanism in the proliferation of the ARGs. Earthworm gut digestion performed the selective effects on the potential host bacteria carrying these resistance genes. The reduction of cell-free resistance genes contributed greatly to the reduction of the total resistance genes. High-throughput sequencing further confirmed that earthworm gut digestion significantly sharped the ratio of viable to total bacterial community in the excess activated sludge. Pearson correlation analysis of the relations between the ARGs and the bacterial community revealed that the potential host bacteria carrying the targeted genes were affiliated to 11 phyla including *Acidobacteria*, *Euryarchaeota*, *Ignavibacteriae*, *Fibrobacteres*, *Deinococcus-Thermus*, *Gemmatimonadetes*, *Spirochaetes*, *Euryarchaeota*, *Firmicutes*, *Proteobacteria* and *Chloroflexi*.

In conclusion, the process of vermicomposting can significantly attenuate the ARGs (*tet*, *sul* and *qnr*) contained in domestic excess activated sludge through sharping microbial profiles. The gut digestion of earthworms is an important step that had remarkable effect in the fate and behavior of the resistance genes, especially for cell-free resistance genes existed in the sludge. Further research needs to clarfy the effects of different functional regions of earthworm gut.

Acknowledgement

Time flies. Looking back to life and study for 3 years in Gifu University, I would like to thank many people.

Firstly, I am very grateful to Prof. Li Fusheng and Prof. Fu Xiaoyong for providing me with a valuable opportunity to study in Japan, and giving me the chance to experience the culture and life of a foreign country. Also, I appreciate my supervisor, Prof. Li Fusheng for giving me a great liberty to independently conduct scientific research project, which will be very helpful to my future work. Besides, I am indebted to Prof. Li for his great guidance to complete my doctoral thesis and the writing of English.

I would like to thank Prof. Ichiro Tamagawa from Basin Hydrology Dynamics Laboratory for his valuable suggestion and comments in my mid-term presentation. I also thank Associate Prof. Toshiro Yamada for sharing many interesting Japanese culture and giving meaningful suggestions in my academic presentations.

I also want to thank Associate Prof. Kui Huang from Lanzhou Jiaotong University for providing me with many conveniences when I came to Japan. Special thanks are given to Assistant Prof. Yasushi Ishiguro for giving me many help in sampling and gene analysis.

I would like to thank Mr. Li Shuailei for his lots of assistance in vermicomposting experiment. I also appreciate Dr. Sartaj Ahmad Bhat for guiding me submit paper and improving my writing skill. Additionally, special thanks are given to all members from Water Quality Research Laboratory for bringing me many unforgettable and valuable experiences.

Finally, from the heart, I would like to appreciate my parents and my wife Li Yaqin for always understanding, supporting and encouraging me to keep forward.

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Chapter 1 Introduction

1.1 Background

The discharge of large amounts of sewage sludge into the environment by treatment plants is a main cause of environmental pollution. As a treatment center of sewage, the level of domestic sewage treatment plant can reflect the development level of a city. Sewage treatment plant is designed to not only treat sewage, but also make the final byproduct (excess sludge) treated and disposed properly. However, the reality is that large amounts of excess sludge have not been properly treated before entering the natural environments in many developing countries, where the sludge is directly returned to the open landfill (Gupta *et al*., 2015; Zhang *et al*., 2016), hence damaging the ecological environment and posing a serious threat to human health.

Excess sludge has been confirmed to be a hotspot of various pollutants, including pathogens and micro-pollutants (Rizzo *et al*., 2013). In recent years, antibiotic resistance genes (ARGs) as a new type of environmental pollutant have received much attention around the world. Their potential threat to humans is far greater than that of antibiotics themselves (Zhang *et al*., 2018). Excess sludge contains abundant ARGs such as tetracycline resistance genes, macrolide resistance genes, sulfonamide resistance genes, β-lactam resistance genes and fluoroquinolones resistance genes (Ezzariai *et al*., 2018). Numerous studies have revealed that resistance genes can be readily transmitted to other microbial genetic information by means of horizontal transfer elements (HGTs) including integrons, transposons and plasmids. Resistance genes contamination (germs from food and animals) has caused about 1 in 5 resistant infections (CDC, 2013). Therefore, it is necessary to take adequate measures to control ARGs contained in excess sludge to minimize their adverse effects on ecological environment and human health.

The widely used methods for biological treatment of excess sludge include anaerobic digestion and composting. Anaerobic digestion not only effectively reduces sludge volume, but also generates methane gas. Recent studies have revealed that anaerobic digestion had excellent reduction effects on different types of ARGs and mobile gene elements contained in sludge, but some ARGs rebound or changed slightly (Diehl *et al*., 2010). In addition, environmental factors such as temperature, can significantly affect the abundance of ARGs during anaerobic digestion of excess sludge (Diehl *et al*., 2010). For composting, Su *et al*. (2015) confirmed its effectiveness on the removal of ARGs, but others suggested the increase of ARGs concentration after composting. Bacterial communities, mobile gene elements are considered to be critical factors involved in the removal or proliferation of ARGs. Besides, composting stages and environmental factors (pH, moisture, ammonium, and water-soluble carbon contents) have important effects on the dynamics of ARGs. It can be seen that anaerobic digestion and composting did not show the expected good results regarding the attenuation of ARGs in excess sludge. Many environmental stress (anaerobic status and temperature), operating conditions, and feed properties would greatly influence the occurrence and dissemination of ARGs.

Vermicomposting has been widely applied for the treatment of organic solid wastes due to its high efficiency, low investment and easy operation. Organic solid wastes can be well stabilized and transformed into final products-vermicompost with abundant nutrients by the joint action of earthworms and microorganisms. Although vermi-stabilization of organic waste depends mainly on the action of microorganisms, the participation of earthworms indirectly alters microbial activities, abundances and communities by various actions like burrowing, gut digestion, mucus stimulation and cast discharge (Huang and Xia, 2018). Lv *et al*. (2018) explored the changes in the microbial community in the process of vermicomposting of mixture of excess sludge and cow dung by means of high throughput sequencing, and found that the inoculation of earthworms could promote the growth of some microbes such as Flavobacteria, Acidobacteria, and Planctomycetes. Huang *et al*. (2018)

observed that Chao1 and Shannon indices were significantly increased after vermicomposting of pelletized excess sludge, and dominant bacteria in the final vermicompost presented remarkable changes. It can be speculated that earthworm activity would correspondingly affect ARGs in excess sludge through altering microbial profiles. In addition, microbial endogenous respiration could release nucleic acid, hence leaving large amounts of cell-free DNA in excess sludge. Previous studies have confirmed that cell-free DNA not only maintains the structural stability of biofilm, but also plays an important role in the transmission of genetic information (Nagler *et a*l., 2018). Furthermore, vermicomposting could promote the degradation of oxytetracycline in the chicken manure/waste paper mixtures, which was probably related to specific microbes and enzymes in the gut of earthworms (Ravindran and Mnkeni, 2017). The results suggested that earthworm's gut digestion may decline the selective pressure of formation of antibiotic resistance. Collectively, it is reasonable to speculate that vermicomposting would affect the fate and behavior of resistance genes in the domestic sludge, and the degree of its impact would also vary with the changes of earthworm density and environmental parameter such as temperature.

1.2 Objectives of the study

The objective of this study was to investigate the fate and behavior of antibiotic resistance genes and their association with microbial profiles in the vermicomposting treatment of domestic excess sludge. Research idea on the effects of vermicomposting on ARGs is shown in **Figure 1.1**: (1) the evaluation based on the results of experiment under different earthworm densities; (2) the evaluation based on the results of experiment under different temperatures; (3) the evaluation based on the results of experiment for clarifying the role of earthworm gut digestion.

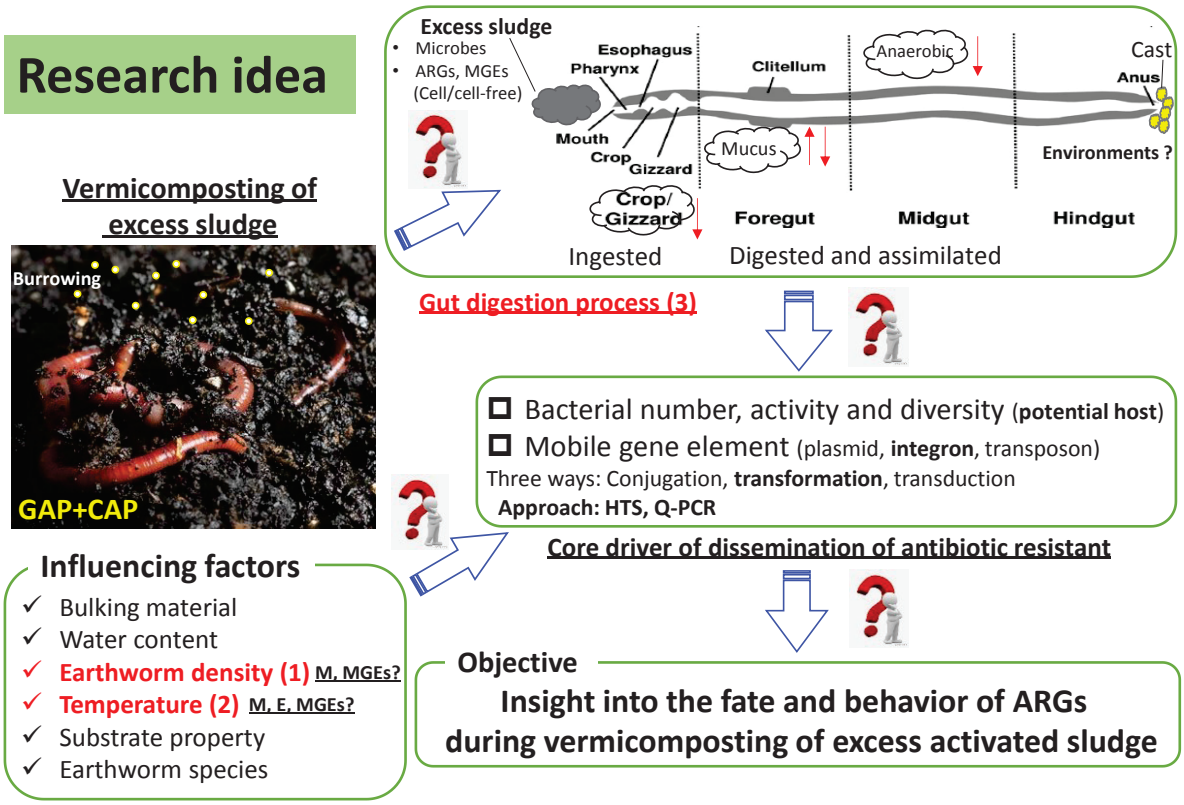

Figure 1.1 Main study contents and concerned points in this dissertation

Note: GAP-gut associated process, CAP-cast associated process, M-microorganisms, E-earthworm, MGEs-mobile gene elements, HTS-high throughput sequencing, Q-PCR-quantitative polymerase chain reaction.

1.3 Structure of the dissertation

Chapter 1 introduces the research background, objectives, and the paper framework; Chapter 2 is literature review about characteristics of domestic excess sludge, new micropollutant-ARGs and integrase gene, main biological treatment methods of excess sludge, and state-of-art on vermicomposting technology; In Chapter 3, the effect of earthworm density on ARGs during vermicomposting of excess sludge. Chapter 4 investigates how temperature affects ARGs during vermicomposting of excess sludge through high throughput

sequencing; Chapter 5 clarifies how earthworm gut digesion as the core function of vermicomposting affects ARGs in excess sludge; Chapter 6 conculdes for this dissertation based on Chapter 3 to Chapter 5.

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Chapter 2 Literature Review

2.1 Domestic excess sludge

Large amounts of domestic excess sludge as an inevitable by-product are generated from wastewater treatment plants. As an example, production amount of excess sludge in Japan in 2015 was 2.27 million ton (dry weight), namely 16.4 kg per capita per year, while in 2013 dry production amount of excess sludge in China was 6.25 million ton (dry weight), namely 4.6 kg per capita per year. Obviously, sludge production of developed country is quite higher than developing countries. Excess sludge is usually classified into primary sludge and secondary sludge (**Figure 2.1**) and its composition is very complex (**Figure 2.2**), which contains bacteria, adsorbed matter, organic fibers, inorganic particle, and extracellular polymeric substances (EPS). Primary sludge mainly consists of settleable solid from raw sewage. Secondary sludge includes large amounts of microorganisms, EPS, recalcitrant organics (cell membrane and wall) and inorganics. Regardless of the type of sludge, its unstable biochemical properties cause it to be prone to decay, thus releasing various malodorous gases such as hydrogen sulfide, ammonia, methyl mercaptan and other volatile organic sulfur. In addition, excess sludge also contains many kinds of toxic substances, such as heavy metals, pathogenic bacteria, micro-pollutants, etc. As shown in **Table 2.1**, Alvarez *et al*. (2002) surveyed heavy metals in a wastewater treatment plant in south Spain. Heavy metals with relatively high contents included Al (8537-13999 mg/kg), Cu (204-326 mg/kg), Cr (54.4-439 mg/kg), Fe (6082-16794 mg/kg), Mn (191-364 mg/kg), Pb (179-23 mg/kg) and Zn (930-1636 mg/kg). Straub *et al*. (1993) summaried possible pathogenic microorganisms in wastewater and sludge, including bacteria, viruses, protozoa, helminths, yeast and fungi. Ben *et al*. (2018) detected 42 types of micr-pollutants including 30 pharmaceuticals and personal care products, and 12 endocrine disrupting chemicals in wastewater from 14 different domestic sewage treatment

plants of China (**Figure 2.3**). Obviously, excess sludge is a huge reservoir of pollutants and it should be treated and disposed properly before entering into environmental systems (waterbodies and soil) to reduce safety risks to ecological environment and human health.

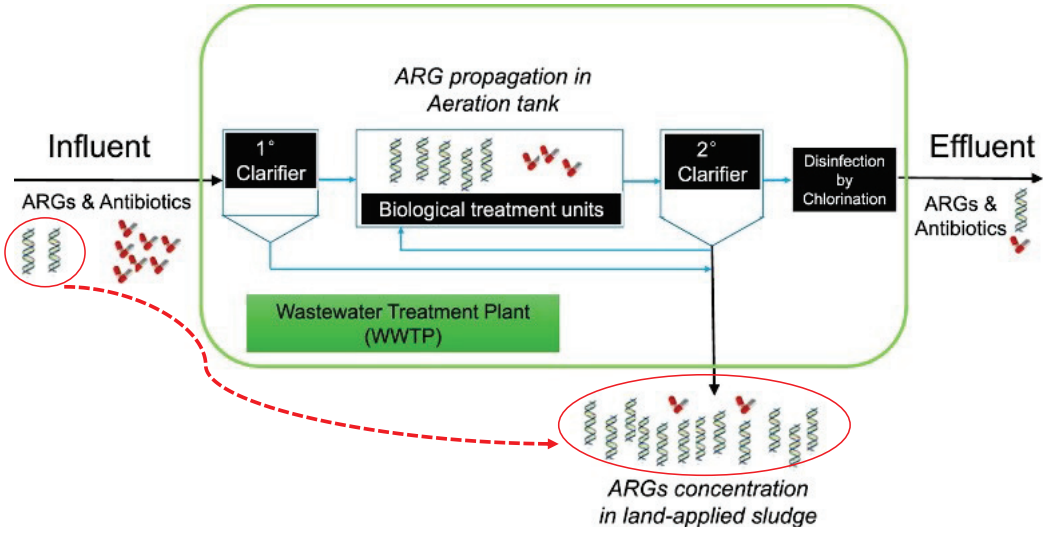

Figure 2.1 The dynamic of antibiotic resistance genes in a wastewater treatment plant (Mao *et al*., 2015). ARGs-antibiotic resistance genes.

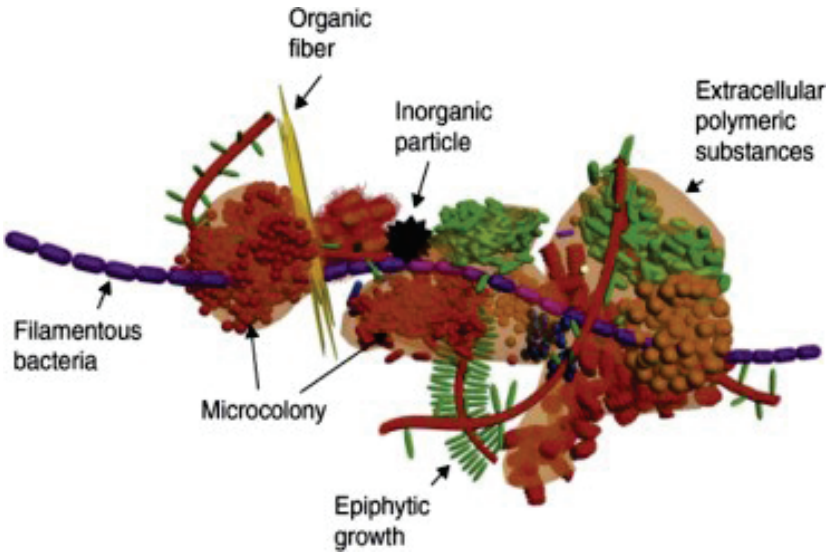

Figure 2.2 General **s**tructure and composition of activated sludge (Christensen et al., 2015).

Table 2.1 Concentrations (mg/kg, dry weight) of Al and heavy metals in various sludges (Alvarez *et al*., 2002)

	Primary sludge	Secondary sludge	Dewatered and digested sludge
Al	6442–11 002	4070–8683	8537–13 999
Cd	1.89–6.03	1.68–4.44	2.37–9.20
Co	1.99–5.49	1.37–3.16	3.54–7.08
Cu	131–256	145–278	204–326
Cr	36.1–239	23.2–245	54.4–439
Fe	4472–13 847	1851–10 666	6082–16 794
Mn	103–297	88.9–362	191–364
Hg	<DL	<DL	<DL
Mo	3.52–10.2	6.19–7.90	7.30–15.0
Ni	14.3–21.7	9.80–18.2	23.2–36.5
Pb	72.5–222	47.2–142	179–223
Ti	44.9–73.1	23.8–53.6	43.2–80.1
Zn	633–997	519–883	930–1636

DL: Detection limit.

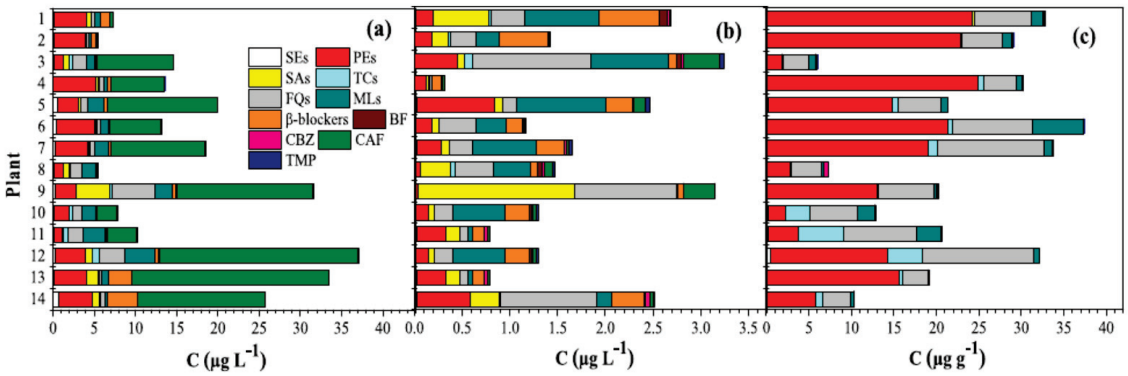

Figure 2.3 Compositions of target micro-pollutants in the influent (a), effluent (b), and excess sludge (c) for 14 wastewater treatment plants (Ben *et al.*, 2018). SEs-steroid estrogens, SAs-sulfonamides, FQs-fluoroquinolones, CBZ-carbamazepine, TMP-trimethoprim, PEs-phenolic estrogenic compounds, TCs-tetracyclines, MLs-macrolides, BF-bezafibrate, CAF-caffeine.

2.2 Antibiotic resistance genes

The overuse of antibiotics for preventing and treating animal and human diseases has caused the contamination of antibiotics and antibiotic resistance genes (ARGs) in the environment. In 2006, ARGs were classified as new environmental pollutants, which would pose more severe threat to ecosystem and human health than antibiotics itself (Zhang *et al*., 2018). The occurrence and dissemination of antibiotic resistance bacteria (ARB) and ARGs have attracted much concerns recently, and have been detected in many environmental medias including hospital effluents, wastewater treatment plants, and river systems. Most of antibiotics in the environment are derived from sewage, and are partially removed during wastewater treatment processes and finally enter surrounding environmental systems like water body and soil. Wastewater treatment plants are considered to be one of important reservoirs for various ARGs due to the existence of abundant microorganisms (Stalder *et al*., 2012). Corresponding antibiotics of these ARGs include fluoroquinolones, tetracycline, beta-lactam, sulfonamides, aminoglycosides, glycopeptides, chlormphenicols, and trimethoprim (Ezzariai *et al*., 2018).

The acquisition of resistance is mainly achieved through horizontal gene transfer, which is mediated by mobile gene elements such as plasmids, transposons, bacteriophages and integrons in three main ways (conjugation, transduction and transformation) (Stalder *et al*., 2012). Besides, heavy metals and other emerging contaminations could also pose selective pressures on the process of horizontal transfer of ARGs (Karkman *et al*., 2017). Main factors affectting the fate and behavior of ARGs in the environmental media include temperature, heavy metal, oxygen and light (Mao *et al*., 2015). High temperature showed an excellent attenuation in ARGs through decreasing the selective pressure from antibiotics. Anaerobic condition was comfirmed to be a better approach for attenuating ARGs in activated sludge through decreasing microbial activity as compared to aerobic digestion. Light also favors the

degradation of antibiotics, which indirectly attenuated the resistance genes including *tet*O, *tet*M, *tet*W and *tet*Q, hence decreasing its dissemination in the environment.

Quantitative polymerase chain reaction (q-PCR) is an efficient tool to quantify ARGs and integrase gene based on the specific primers. Recently, metagenomic sequencing is being widely used for broad-spectrum screening of ARGs due to which it can overcome the drawbacks of amplification-based methods (Guo *et al*., 2017). In addition, network analysis combined with high throughput sequencing technology can quickly obtain potential hosts of resistance genes to better clarify microbial mechanism for dissemination of ARGs.

2.3 Bio-treatment approaches

Composting and anaerobic digestion are widely adopted and applied methods to treat excess sludge in many countries. Here, we briefly describe them. Composting is a biochemical process where different functional microorganisms work together to transform organic wastes into more stable and nutrient-rich products by providing the ideal conditions (oxygen, carbon/nitrogen and water etc.). During the process, volatile organics are decomposed and many of the pathogens destoyed. It should be noted that malodorous gas released during the sludge composting process has become the main factor restricting the harmless production of compost. Malodors composition is complex and it is difficult to eliminate these gases. The study found that the differences in sludge composition and physical and chemical properties will affect the generation and emission of odor during composting, such as carbon to nitrogen ratio, pH value, water content, particle size, organic matter content and microbial biomass. Researchers have conducted research on the characteristics of ammonia emissions during sludge composting. Zhong Jia *et al*. studied the characteristics of ammonia emission during sludge composting process under different turn-over conditions. The results showed that ammonia emission factor under forced ventilation combined with

mechanical turn-over conditions is slightly lower than that under the mechanical turn-over condition.

Anaerobic digestion has become the most widely used method for treatment of excess sludge in the world due to its ability to recover energy and reduce environmental hazards. Anaerobic digestion of sludge refers to the process of decomposing biodegradable organic matter in sludge into carbon dioxide, methane and water by facultative bacteria and anaerobic bacteria under anaerobic conditions to stabilize the sludge. Therefore, it is one of the commonly used means of sludge reduction and stabilization. By direct anaerobic digestion or anaerobic digestion after pretreatment, organic matter content in sludge can be greatly reduced, and the stabilized sludge is used as an organic fertilizer for soil improvement and landscaping. At the same time, the biogas produced after purification can be widely utilized in grid-connected power generation and vehicles to reduce the consumption of traditional resources such as coal, oil and natural gas. According to the suitable temperature range of methanogens during anaerobic digestion, sludge anaerobic digestion can be divided into medium temperature (35~40 °C) and high temperature digestion process (50~60 °C). High temperature digestion has characteristics with fast speed, high load and small volume, thereby it is widely used in developed countries.

In recent years, many research has been carried out on the pretreatment technology of sludge to solve problems such as low digestion rate, long retension time (20~30d) and low processing efficiency. According to three-stage process of digestion, namely hydrolysis, acid fermentation and methanogenesis, hydrolysis process is a rate-limiting step in which particulate organic matter is transformed into soluble organic matter. Purpose of sludge pretreatment is to accelerate the hydrolysis efficiency by pretreatment methods like heat treatment, thermochemical treatment, alkali treatment and ultrasonic treatment.

2.4 Vermicomposting

The ancient Greek philosopher Aristotle once said that earthworms are the intestines of the earth" and digest all kinds of organic wastes. As an environmental manager of biosolid waste, earthworms have more than 60 million years of experience on this treatment. Waste treatment technology via earthworm is called vermicomposting or vermistablization in which waste is decomposed by the synergistic action of earthworms and microorganisms (**Figure 2.4**). Although microorganisms are mainly responsible for the degradation of organic matter, earthworm still exert key roles in promoting the process by drilosphere activity to increase the surface area for microbial colonization and change microbial activity, which thereby promote the efficiency of vermicomposting. Vermicomposting process usually is divided into two stages (Domínguez *et al*., 2017), as shown in **Figure 2.5**, including gut associated process (GAP) and cast associated process (CAP).

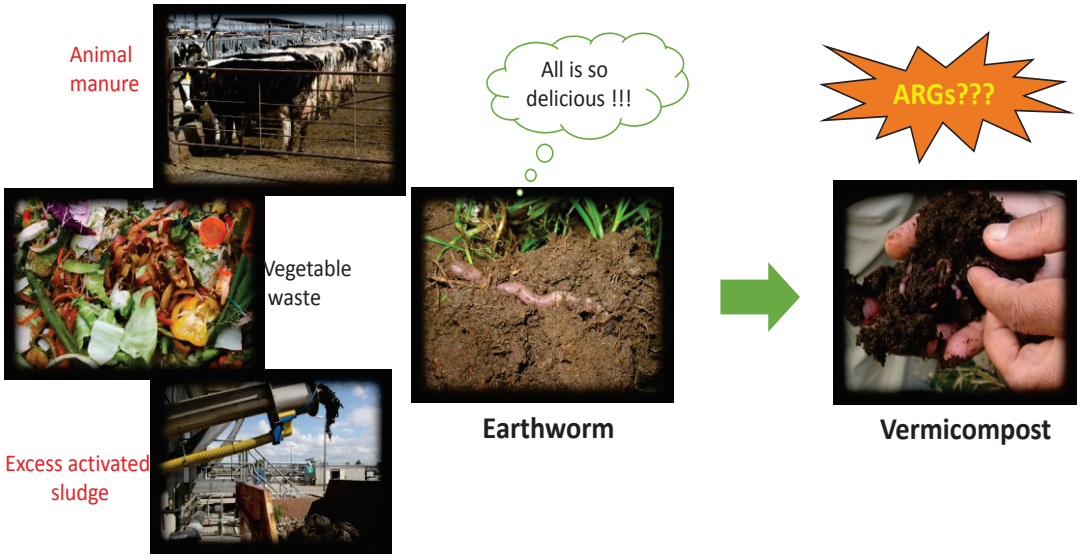

Figure 2.4 Schematic diagram of vermicomposting technology for treating various organic wastes. ARGs-antibiotic resistance genes.

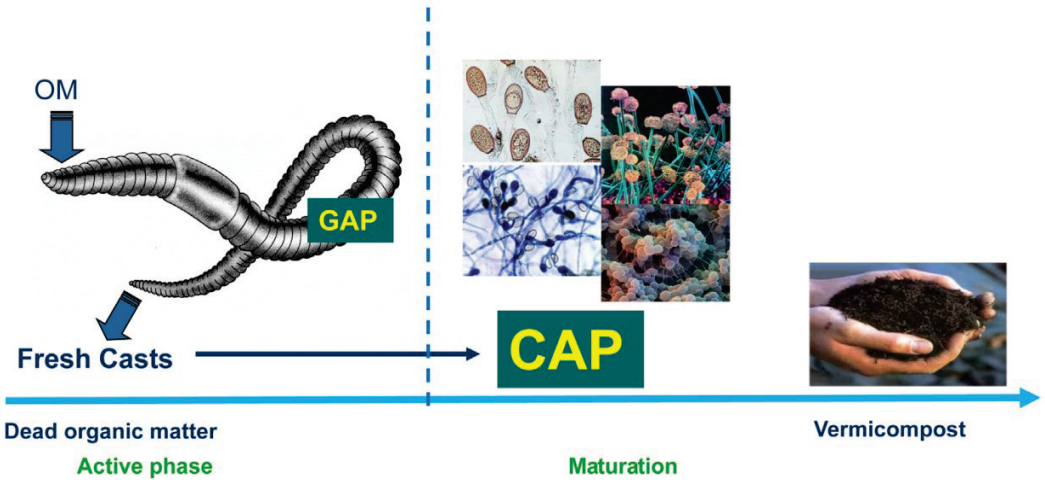

Figure 2.5 Vermicomposting process includes two different phases in relation to earthworm activity (Domínguez *et al*., 2017). GAP-gut associated process, CAP-cast associated process.

A large number of earthworms are harvested after vermicomposting (**Figure 2.6**), they are often used as feed for aquaculture due to high quality protein content (60-70%). At the same time, the final vermicomposting products contains abundant nutrients (NPK), are regarded as an excellent organic fertilizer and soil amendment (Bhat *et al.*, 2018). Vermicomposting technology due to its high processing efficiency, low investment and easy operation has been widely adopted for treatment of organic wastes such as livestock and poultry manure (Aira *et al.*, 2007; Lazcano *et al*., 2008; Domínguez and Gómez-Brandón *et al*., 2013), tannery sludge (Vig *et al*., 2011; Ravindran *et al*., 2015), Dairy sludge (Suthar, 2012), brewing sludge (Suthar and Singh, 2008), food industry sludge (Yadav and Garg, 2009), beverage industry sludge (Singh *et al*., 2010), agricultural waste (Fernández-Gómez, 2010; Bansal and Kapoor, 2010). On the other hand, from the perspective of environmental impact, current research mainly involves in several aspects including the effect of vermicomposting treatment on greenhouse gases (Lv *et al*., 2018), heavy metals (Liu *et al*., 2018), pathogenic bacteria (Procházková *et al.*, 2018), micro-pollutants (Liu *et al.*, 2018). These studies suggest that vermicomposting treatment has positive and benefical effects on the environmental system.

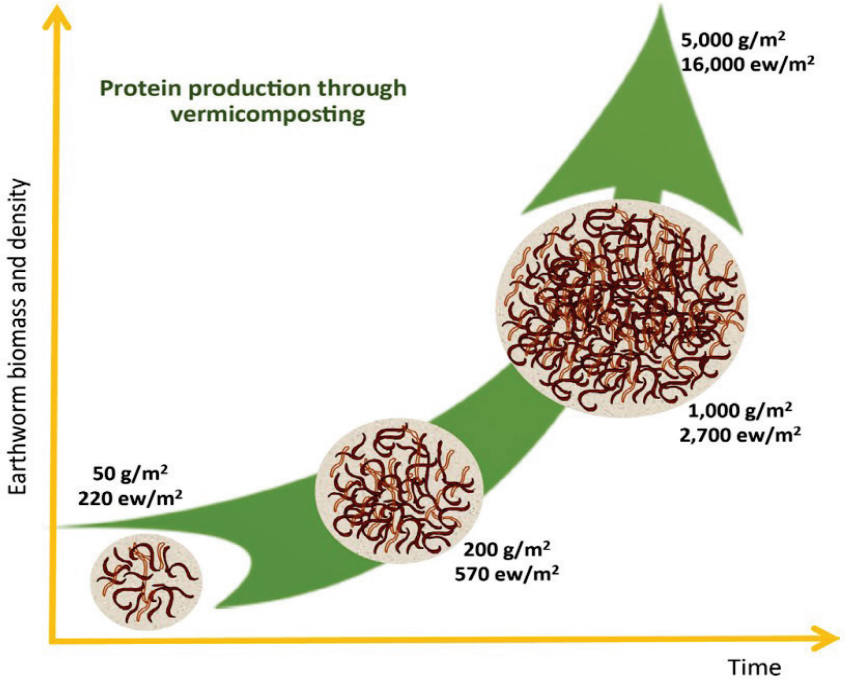

Figure 2.6 Production of earthworm biomass and evolution of earthworm density during vermicomposting of grape marc (Domínguez *et al*., 2017)

Vermicomposting technology is a potential biological treatment method, especially for agricultural wastes, fruit and vegetable wastes which would receive more and more attention in future. However, for municipal sludge, the instability of vermicomposting process (since high organic nitrogen content in sludge poses a threat to the survival of earthworms) and the environmental risks of final products (still containing pathogenic bacteria and micro-pollutants make it incompatible with agricultural application standards), probably narrowing its market space. For this, how to acurately evaluate and efficiently reduce the potential environmental risks caused from final product may be the main direction of vermicomposting of excess sludge.

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Chapter 3 Effect of earthworm density on antibiotic resistance genes during vermicomposting of domestic excess sludge

3.1 Introduction

The discharge of large amounts of pharmaceuticals into the environment has caused increases of bacterial resistance, one of the most concerned health threats in the world (UNEP, 2017). Anthropogenic compounds like antibiotics contained in wastewater can hardly be eliminated in biological wastewater treatment process. According to the documented report (Mao *et al*., 2015), many of the antibiotics are transferred from wastewater to the excess sludge, a major byproduct of the municipal wastewater treatment plants, and the abundant and diverse microorganisms in the sludge could facilitate the formation and spread of resistant bacteria. Consequently, excess sludge is becoming the hotspot and reservoir for the existence of antibiotic resistance genes (Stalder *et al*., 2012; Kaplan *et al*., 2013; Rizzo *et al*., 2013; Xu *et al*., 2015) and also for the spread of the genes to water and soil environments, thus causing adversary impacts on the environments and agricultural activities. Among the reported antibiotic resistance genes in excess sludge, quinolone resistance genes (*qnr*A and *qnr*S) have been widely detected due to the overuse of quinolones (Jia *et al*., 2012; Kaplan *et al*., 2013; Xu *et al*., 2015). Therefore, it is necessary to take proper measures to eliminate the harmful effects and risks posed by the quinolone resistance genes existed in excess sludge.

Biological treatment methods for sludge, such as anaerobic digestion, aerobic composting and biological drying, generally reveal a better performance in stabilizing excess sludge. Also, they may demonstrate significant influences on the fate and behavior of antibiotic resistance genes during the treatment process. For instance, the study of bio-dry for

sludge showed that many antibiotic resistance genes could be attenuated significantly by improving the aeration strategy. However, there are also many genes, like *erm*F, *sul*II and *tet*X genes, whose concentrations can get increased due to the enrichment of bio-copper (Zhang *et al*., 2016). Su *et al*. (2015) found that the abundance and diversity of resistance genes as well as the integrase genes increased significantly after the composting treatment of excess sludge. Comparatively, anaerobic digestion could significantly lower the abundance of tetracycline resistance genes in excess sludge, whereas, aerobic digestion showed only a slight effectiveness in eliminating the resistance genes (Diehl *et al*., 2010). Overall, these approaches have demonstrated their impacts on the fate of antibiotic resistance genes in excess sludge, depending on the types and abundance of the genes as well as the composition of microbial communities.

Vermicomposting is a traditional biochemical method for treating organic wastes through the joint action of earthworms and microorganisms (Bhat *et al*., 2013). Many types of organic wastes such as agricultural waste (Sharma and Garg, 2018), livestock manure (Monroy *et al*., 2009), fruit and vegetable wastes (Huang *et al*., 2017) as well as excess sludge (Suthar and Singh, 2008; Fu *et al*., 2015; Villar *et al*., 2016) have been studied for treatment by vermicomposting. As a green technology, vermicomposting is effective for treatment of excess sludge, and so far, many studies focusing mainly on the treatment efficiency under different conditions have been conducted, together with the studies for evaluation of the agricultural value of the final products when used as bio-fertilizers (Fu *et al*., 2015; Varma *et al*., 2015; Nigussie *et a*l., 2016; Villar *et al*., 2016; Malińska *et al*., 2017; Bhat *et al*., 2018). However, there are few studies that have attempted to evaluate the fate and behavior of antibiotic resistance genes in vermicomposting treatment of excess sludge.

It is well known that even in vermicomposting, microorganisms still involve as the main driving force for stabilization of organic wastes (Sen and Chandra, 2009; Ravindran *et al*., 2015). Earthworms could modify the bacterial abundance and diversity (Fu *et al*., 2015; Villar *et al*., 2016) and, at the same time, their competition for carbon source could also

reduce the growth and activity of microorganisms. The direct consumption of microorganisms by earthworms also adds to the reduction of microbial biomass at the end of the treatment of sludge (Aira and Domínguez, 2008; Villar *et al*., 2016). Accordingly, when the fate and behavior of antibiotic resistance genes in vermicomposting of excess sludge are concerned, specific attention should be paid to the microorganisms involved in the treatment, including their activity, abundance and diversity, although the involvement and effects of such factors as temperature, aeration, earthworm's metabolic products (cast and mucus) are also important.

In this charpt, targeting on *qnr*A and *qnr*S, two well-detected quinolone resistance genes in excess sludge, the changes of the resistance genes in vermicomposting treatment of excess sludge were investigated through vermicomposting experiment with reactors added with three different densities of earthworms. Meanwhile, by analyzing the time profiles of the microbial activity, bacterial abundance and diversity, the changes of microbial profiles in the vermicomposting treatment were also evaluated. Moreover, based on the results of redundancy analysis, for which the environmental factors (including pH, electrical conductivity, dissolved organic carbon, ammonium and nitrate) and the integrase gene that transmits resistance genes were also used as variables, the relationship of the quinolone resistance genes with the microbial profiles during vermicomposting treatment of excess sludge was also discussed.

3.2 Materials and methods

3.2.1 Earthworm and sludge

Eisenia fetida, a common epigic earthworm species used for vermicomposting, was purchased from Agricultural Management Institute, Co. Ltd., Japan. Earthworms with an individual weight of 0.3 - 0.4 g were selected for use in the experiment. Before inoculating into excess sludge, all earthworms were washed with sterilized water and then cleaned with

wet filter papers inside petri dishes. Filter papers were replaced every 8 hours until no intestinal excrement was observed (normally two days were required).

Excess sludge was collected from a wastewater treatment plant that treats municipal wastewater from Gifu-city, Japan using the conventional anaerobic/aerobic biological treatment process. In order to reduce water content, the collected fresh sludge was immediately centrifuged at 3500 rpm for 10 min after transported to the laboratory. The settled sludge after centrifuging was spread out under room temperatures (**Figure 3.1**) and was turned over every 8 hours for further reduction of the water content. This took two days for the sludge to reach the expected water content of about 85% for direct use as the substrate for vermicomposting treatment. The physicochemical properties of the sludge after reduction of the water content are shown in **Table 3.1**.

Table 3.1 Physicochemical properties of initial municipal excess sludge.

Parameters	Initial municipal excess sludge
Water content (%)	88.3 ± 0.53
Organic matter (%)	78.1 ± 0.40
pH	6.38 ± 0.05
Dissolved organic carbon (g/kg)	24.2 ± 0.33
Electrical conductive (mS/m)	80.0 ± 1.73
Total carbon (g/kg)	280 ± 0.15
Total nitrogen (g/kg)	68.0 ± 0.06
Ammonium (mg-N/kg)	0.88 ± 0.24
Nitrate (mg-N/kg)	22.9 ± 0.1
Dehydrogenase activity (g-TF/kg/h)	1.36 ± 0.17

Values are represented as Mean ± SD (n=3).

3.2.2 Vermicomposting process

Vermicomposting treatments and the control treatment (treatment without earthworms) were established simultaneously. Plastic bins with a size of 22 cm × 15 cm × 9 cm for each were used as the reactors. As for the vermicomposting treatments, earthworms with designated numbers were released into three reactors to make the earthworm density as 50, 100, 150 earthworms/kg wet sludge (427, 854, 1281 earthworms/kg dry sludge), respectively based on previous studies (Aira *et al*., 2002; Fu *et al*., 2015). These three reactors are referred hereafter as EL, EM and EH, respectively. To these three reactors and the reactor used for the control treatment (referred hereafter as E0), 400 g of the sludge was added into each reactor. All these treatments were prepared in triplicate and were operated in dark by covering all reactors with a dark plastic film in an incubation room with the temperature varying in the narrow range of 20-22 °C for totally 31 days. The operation under the relatively constant temperature was mainly for the purpose to eliminate the effects from temperatures. For each reactor, samples (40 g for each) were collected at day 7, 14, 21 and 31. In order to maintain a constant earthworm density inside each reactor before and after sampling, 2, 4 and 6 earthworms were taken out respectively at each sampling time from the vermicomposting treatments with three different earthworm densities (EL, EM and EH). For all samples, one part was directly used for determination of water content, organic matter and dehydrogenase activity, and the remaining part was dried in a freeze-vacuum dryer before used for analysis of other physicochemical and microbial parameters.

3.2.3 Physicochemical parameters

Water content of each sample was determined by drying in an oven at 105 °C for 8 hours. Organic matter was determined by igniting the sample in a muffle furnace (Yamato, Japan) at 600 °C for 4 hours. pH and electrical conductivity (EC) were measured as follows. A designated amount of dried sample was added to deionized water in the ratio of 1/50

(mass/volume) and was then shaken at 20 °C for 2 hours. The resulting aqueous solution was subjected to the measurement for pH and EC with pH and conductivity meters (TOA-DDK, Japan). The aqueous solution was further filtered through cellulose acetate membrane with a pore size of 0.45 μm and the filtrate was then used for determination of dissolved organic carbon (DOC) by a total organic carbon analyzer (Shimadzu, Japan), ammonium and nitrate by an ion chromatograph (Shimadzu, Japan), and the dehydrogenase activity (DHA) by the triphenyl tetrazolium chloride (TTC) reduction method by reference to Fu et al. (2015). This method includes the reaction of each sample with the TTC solution in a water bath shaker at 37 °C in dark for 30 minutes. The resulting red solution was extracted with acetone solution and the extract was measured with a spectrophotometer at the designated wavelength of 485 nm.

Figure 3.1 Water evaporation for centrifuged excess sludge

3.2.4 Bacterial community composition

Dried samples were used to extract total genomic DNA with the DNA extraction kit (MOBIO, USA) based on manufacturer's manual. The bacterial 16S rDNA gene fragment was amplified by PCR (TP800, Takara, Japan). The bacterial primer pairs are presented in **Table 3.2**. The PCR amplification conditions consisted of three steps of thermal cycle: 95 °C for 5 min, 35 cycles (95 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min) and 72 °C for 10 min. The amplification reaction solution contained: 1 μL DNA template, 5 μL 10 × Taq buffer, 4 μL dNTP Mixture, 0.5 μL (20 μM) GC341F, 0.5 μL (20 μM) 907R, 1 μL bovine serum albumin and 37.75 μL sterile water. The obtained PCR product was checked by electrophoresis with the agarose gel concentration of 1.2%.

Table 3.2 Primer pairs for target genes and PCR amplification conditions

Genes	Primer sequence (5'-3')	Annealing temperature	Reference
*qnr*A gene	*qnr* AF:ATT TCT CAC GCC AGG ATT TG *qnr* AR:GCA GAT CGG CAT AGC TGA AG	54 °C	Di Cesare *et al*. (2016)
*qnr*S gene	*qnr* SF:GAC GTG CTA ACT TGC GTG AT *qnr* SR:TGG CAT TGT TGG AAA CTT G	54 °C	Di Cesare *et al*. (2016)
*intl*1 gene	*intl* 1F:GGC TTC GTG ATG CCT GCT T *intl* 1R:CAT TCC TGG CCG TGG TTC T	57 °C	Luo *et al*. (2010)
16S rDNA gene for qPCR	Com1: CAC CAG CCG CGG TAA TAC Com2: CCG TCA ATT CCT TTG AGT TT	50 °C	Fu *et al*. (2016)
16S rDNA gene for conventional PCR	GC341R: CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG CAC GGG GGG CCT ACG GGA GGC AGC AG 907F: CC GTC A AT TCC TTT GAG TTT	54 °C	Fu *et al*. (2016)

F: forward; R: reverse

Denaturing gradient gel electrophoresis (DGGE) for the assessment of bacterial community structure was performed by a Dcode™ system (BIO-RAD, USA) using 6% polyacrylamide gel with the denaturing gradient of 40%-60%. Electrophoresis was run at 80

V for 16 hours. The resulting gel after electrophoresis was stained with SYBR Green I Nucleic Acid Gel Stain (Takara, Japan) for 15 minutes and then photographed using a gel imager (BIO-RAD, USA) for microbial diversity analysis. The representative bands from the DGGE gel were excised with a disinfected knife and kept in ultrapure water for one night. PCR amplification with the same primers and DGGE were conducted to obtain single bands. The obtained PCR products were purified with a Gel and PCR Clean-up kit (MACHEREY-NAGEL, Germany) according to the instruction and were then submitted for sequencing using a Genetic Analyzer (ABI 3100, Applied Biosystems, USA). The obtained sequencing data were then used for species identification by the BLAST of NCBI database.

3.2.5 *qnr*A, *qnr*S, *intl*1 and 16S rDNA genes

The abundances of the resistance genes, integrase gene and bacterial 16S rDNA gene were determined by the quantitative PCR instrument (TP800, Takara, Japan). Detailed information on the primers used in the quantitative PCR is presented in **Table 3.2.** The PCR reaction solution was prepared by including 12.5 μL of TB Green Premix Ex Taq (Takara, Japan), 0.5 μL of each primer, 2 μL of DNA template and 9.5 μL of sterile water. For quantitative PCR amplification, denaturing was conducted at 95 °C for 30 s, followed by 40 cycles (95 °C for 15 s, annealing temperature for 30 s, 72 °C for 30 s) and finally dissociation at 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s.

The standard curve of 16S rDNA gene was constructed using known concentration of DNA containing *Escherichia coli* 16S gene (NBRC, 13965), based on a previous report (Huang *et al.*, 2017). The standard curve for *qnr*A, *qnr*S and *intl*1 genes constructed by referring to the TA cloning method of TAKARA as follows (Takara, Japan). Firstly, the target gene fragment was amplified by non-quantitative PCR (LifePro, China). The obtained PCR product was purified with a Gel and PCR Clean-up kit (MACHEREY-NAGEL, Germany). The ligation reaction of the target gene fragment was performed with the pMD20-T vector

(Takara, Japan). The resulting product was transformed into *E. coli* DH5α competent cells (Takara, Japan) and then cultured overnight in the LB selection medium (LB + Amp + X-gal + IPTG). After that, white colonies were picked for PCR amplification to confirm the insert of the target DNA fragment. The confirmed colonies containing the desired plasmid were then cultured overnight with the LB + Amp medium. Finally, the plasmid DNA was purified using a Plasmid EasyPure kit (MACHEREY-NAGEL, Germany) and subjected to sequence analysis. The obtained sequencing results for *qnr*A, *qnr*S and *intl*1 genes were deposited in the Genbank with the accession numbers of MH511634, MH511635 and MH511636, respectively. The validated plasmids were then used as the standard for quantitative PCR after their concentration was measured with a DNA concentration meter (Promega, USA). The results showed that the correlation coefficient (r) of the standard curves obtained for all four genes was above 0.99, and that the amplification efficiency for all samples was stable in the range of 80-110%, thus indicating the quantitative analysis requirements were satisfied.

3.2.6 Statistical analysis

Statistical analysis was carried out by using Statistica 10.0 software. One-way analysis of variance (ANOVA) was performed between different treatments in the confidence level of $p < 0.05$. Shannon diversity of the bacterial community was analyzed and calculated by using Quantity One® 1-D software based on the obtained DGGE fingerprinting image. Redundancy analysis (RDA) of the relationship of the targeted resistance genes with the indexes of microbial profiles (16S rDNA abundance, DHA and Shannon index), integrase gene and the major environmental factors reflecting the degree of the stabilization of the vermicomposting process (pH, EC, DOC, NH_4^+-N, NO_3^--N) was conducted by using Canoco 4.5 software with all data after normalization treatment. OM was not considered in the RDA because it is in strong association with the index of DOC.

3.3 Results and discussion

3.3.1 Physicochemical analysis

The changes of physicochemical parameters of the excess sludge before and after composting treatment are summarized in **Table 3.3**. Dissolved organic carbon (DOC) is the most unstable portion in sludge that can be easily metabolized by microorganisms and is thus frequently used for characterizing the degree of stabilization of the composting/vermicomposting products (García-Sánchez *et al*., 2017). Compared to the control, the content of DOC in the vermicomposting treatments was lower at the end of the experiment ($p < 0.05$), with the lowest level of DOC being found for the treatment with the highest earthworm density (EH). The marked decreases of DOC from 27.79 g/kg for the control treatment to 9.81-17.78 g/kg for the vermicomposting treatments with three different earthworm densities (EL, EM and EH) clearly demonstrated that the presence of earthworms significantly promoted the stabilization of the excess sludge, which supports the results of previous studies (Xing *et al*., 2015; García-Sánchez *et al*., 2017). The observed effect with the presence of earthworms could be probably explained from the following two aspects based on the results of previous studies (Mougin *et al*., 2013; Huang and Xia, 2018): (1) the gut of earthworms facilitated the transformation of complex organic matter in the sludge into the available forms for easier utilization by microorganisms, and (2) the mucus of earthworms stimulated microbial activity and thus promoted the degradation of organic matter in the sludge.

The magnitude of electrical conductivity (EC) reflects the mineralization degree of organic wastes in composting/vermicomposting systems (Bhat *et al*., 2017). Compared to the EC value of the final product with the control treatment (166.7 mS/m), the EC values of the final products with the three vermicomposting treatments (187.3-252.3 mS/m) were obviously higher ($p < 0.05$). For the three vermicomposting treatments, by increasing the density of

earthworms increased from 50 to 150 earthworms/kg-wet sludge, the value of EC of the final product increased by about 35%, indicating clearly that increasing the density of earthworms further promoted degradation and mineralization of organic matter in the excess sludge. The promotion was due probably to the enhanced degradation and mineralization of the organic matter into the soluble salts, such as ammonium, nitrate and phosphate as reported (Kaviraj and Sharma, 2003; Hait and Tare, 2011; Xing *et al.*, 2015; Lv *et al*., 2018).

Table 3.3 Chemical properties of initial municipal excess sludge, final products of control (without earthworm) and vermicomposting

Parameters	Initial sludge	Final products			
		Without earthworm	Earthworm density		
			Low	Medium	High
Organic matter (%)	78.1 (0.40)	71.2 (0.66)	69.1 (0.06)	68.9 (0.21)	68.2 (0.24)
Dissolved organic carbon (g/kg)	24.2 (0.33)	27.8 (3.24)	17.8 (3.53)	11.1 (1.33)	9.81 (1.24)
pH	6.38 (0.05)	6.52 (0.01)	6.74 (0.05)	6.14 (0.08)	5.85 (0.02)
Electrical conductivity (mS/m)	80.0 (1.73)	167 (1.53)	187 (1.15)	206 (2.08)	252 (3.51)
Ammonium (mg-N/kg)	0.88 (0.24)	13.8 (1.43)	14.4 (2.03)	11.0 (0.79)	8.12 (1.20)
Nitrate (mg-N/kg)	22.9 (0.1)	55.8 (0.1)	60.0 (1.5)	402 (21.2)	1400 (118)

Values are represented as Mean (SD) (n=3).

Nitrification is a typical feature of vermicomposting treatment of excess sludge (Yang et al., 2014; Fu et al., 2015). For NH_4^+-N, compared to the concentration in the initial excess sludge (0.88 mg-N/kg), the concentrations in the final products after treatment were much higher (13.83 mg-N/kg for the control, and 14.37, 10.94 and 8.12 mg-N/kg for the vermicomposting treatments of EL, EM and EH, respectively) ($p < 0.05$). This indicated that mineralization and ammonification were significantly promoted by composting with or without the introduction of earthworms. For NO_3^--N, the highest concentration (1400.5 mg-N/kg) appeared in the final product of the treatment with the highest earthworm density (EH), which was 25 times as high as the control (55.8 mg-N/kg), thus demonstrating clearly

that earthworms played a tremendous role in promoting the process of nitrification during vermicomposting treatment of excess sludge. The enhanced nitrification was probably one of reasons leading to the more obvious decreases of pH noticed for the vermicomposting treatments with higher densities of earthworms.

3.3.2 Absolute abundance of *qnr*A, *qnr*S and *intl*1 genes

Plasmid-mediated resistance genes play a more important role in transmitting quinolone resistance (Kaplan *et al*., 2013). In the present study, two typical quinolone resistance genes, *qnr*A and *qnr*S that have been frequently detected in wastewater treatment system (Kaplan *et al.*, 2013; Marti and Balcázar, 2013), were targeted and quantified. The changes of the absolute abundance of the resistance genes during the whole vermicomposting process are given in **Figure 3.2**. The magnitudes of the relative abundance of the genes are presented in **Figure S7** as supplementary material because they demonstrated trends very similar to the trends of the absolute abundance. In the initial excess sludge, the absolute abundance of *qnr*A gene (1.67 E+04 copies/g dry sample) was significantly higher than that of *qnr*S gene (3.32 E+03 copies/g dry sample) ($p < 0.05$). The reason may be associated with the more abundant existence of the genotypes of *qnr*A than *qnr*S in the environment (Jacoby et al., 2008). Compared to the initial excess sludge, the absolute abundance of the resistance genes *qnr*A and *qnr*S in all vermicomposting treatments on day 7 increased, whereas the absolute abundance in the control for both the genes remained unchanged. The observed increases of the two genes were probably in association with the observed increases of the bacterial abundance as a result of the increment of DOC since a positive correlation between the resistance genes and the bacterial 16S rDNA gene were revealed (to be shown later in **Figure. 5**).

After 7 days, both resistance genes in the control and vermicomposting treatments turned into a declining trend that lasted until the end of the experiment. However, compared to the control treatment, the decline for the vermicomposting treatments was more obvious.

Complete removal of *qnr*A gene was observed in the treatment with the highest density of earthworms (EH). The different behavior noticed for the two resistance genes in this study may suggest that these two genes were carried by different host bacteria that required different amino acids to encode their proteins (Strahilevitz *et al*., 2009). In addition, compared to the sludge before treatment, the absolute abundance of the *qnr*A gene in the sludge after treatment in the control and vermicomposting treatment systems decreased by 66.7% and 85.6-100%, respectively. For the *qnr*S gene, its abundance in the control and the three vermicomposting treatments was found to decrease by 79.3% and 92.3-95.3%, respectively.

The abundance decreases of *qnr*A and *qnr*S in the control treatment may suggest that the quinolone resistance genes can be attenuated under natural environmental condition due probably to the lysis of the host bacteria involved in carrying the resistance genes. The occurrence of the lysis of the host bacteria in the control treatment of this study was likely since a previous study has reported that the endogenous respiration of microbes in the dewatered sludge could occur under natural environment condition, thus leading to reductions in their number and/or activity (Hao *et al*., 2010). For the treatments with earthworms, however, the improvement of aerobic condition in the sludge due to earthworm's burrowing behavior was also a possible reason that probably contributed to the effective attenuation of quinolone resistance genes because some of the host microbes of quinolone resistance genes are reported to be anaerobic ones (Xu *et al*., 2015). From the perspective of applications, it is reasonable to infer that increasing earthworms' density is probably a feasible measure to enhance the attenuation efficiency for quinolone resistance genes in the excess sludge by vermicomposting.

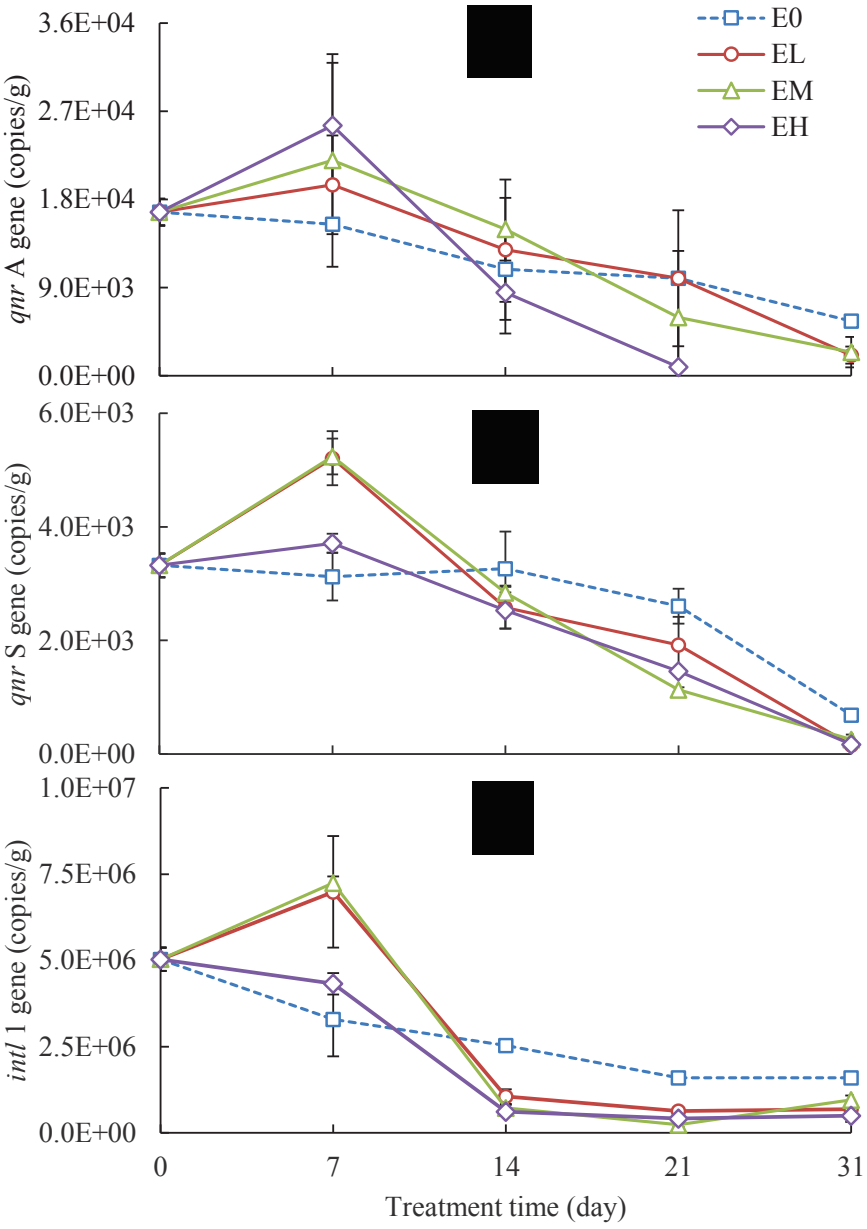

Figure 3.2 Changes in the absolute abundance of *qnr*A gene (a), *qnr*S gene (b) and *intl*1 gene (c) during the vermicomposting of excess sludge. Data are represented as mean and standard deviation (n=6). E0-the treatment inoculated without earthworm, EL-the treatment inoculated with earthworms at the low density, EM-the treatment inoculated with earthworms at the medium density, EH-the treatment inoculated with earthworms at the high density.

Class 1 integrase gene (*intl*1) is an important gene element that transmits bacterial resistance through capturing special sites to integrate most drug resistance genes (Ma *et al.*, 2011), and has been commonly found in multidrug-resistant bacteria in the environment (Diehl and LaPara, 2010). As shown in **Figure 3.2 (c)**, the absolute abundance of the *intl*1 gene in the initial sludge (5.03 E+6 copies/g dry sample) was much higher than that of both the resistance genes discussed above, implying that the excess sludge may possess a great potential in disseminating bacterial resistance. On day 7, the abundance of the *intl*1 gene in the treatment with earthworms increased to levels significantly higher than the control ($p < 0.05$), and the observed increase was particularly obvious for the two treatments with low and medium densities of earthworms (EL and EM). After that, the abundance of the gene decreased rapidly to day 14 and then remained only slight changes until the end of the experiment within the range of 5.0-9.6 E+5 copies/g dry sample. This thus implied that earthworms could significantly facilitate the attenuation of the Class 1 integron in the excess sludge during the vermicomposting treatment. However, a relatively higher concentration of *intl*1 gene remaining in the final products of the vermicomposting treatments of this study suggests the dissemination risk of the resistance gene may still exist after vermicomposting treatment, which needs special caution when the final products are used as bio-fertilizers or soil amendments.

3.3.3 Changes in microbial profiles

(1) Abundance of bacterial 16S rDNA gene

The bacterial abundance in the excess sludge displayed a slight increase from 1.21 E+8 copies/g dry sample before treatment to 1.33-1.46 E+8 copies/g dry sample after vermicomposting treatment for 7 days. At the confidence level of 95%, no significant differences existed among the vermicomposting treatments with three different earthworm densities, as could be seen from **Figure 3.3 (a)**. The bacterial abundance revealed an increase

in the earlier stage of the treatment process, which could be explained by the ready release of easily-degradable organic matter in the excess sludge through the gut-associated process of earthworms (Binet *et al*., 1998; Domínguez *et al*., 2010; Huang and Xia, 2018). This is in accordance with the finding of a previous study on vermicomposting treatment of sewage sludge and cattle dung conducted by Lv *et al.* (2018). In another previous study, using pig manure as the substrate, Aira *et al*. (2006) found that the increase of the microbial biomass in the early stage of vermicomposting was closely linked with the activation of four enzymes (β-glucosidases, cellulase, phosphatase and protease). In addition, the existence of abundant carbon source easy for use by microbes in the mucus of earthworms may also become one of the likely reasons contributing to the observed increase of the microbial biomass. After day 7, all treatments showed a continuous reduction in the bacterial abundance. Particularly, for the treatment with the highest earthworm density (EH), the extent of reduction was the largest ($p < 0.05$), probably due to the rapid depletion of nutrients caused by the intensified digestion by the larger number of earthworms (Aira *et al.*, 2002). In the final stage of vermicomposting treatment of pig manure, Domínguez (2004) also observed a significant decrease of microbial biomass and the decrease was inferred to be attributed to the depletion of the nutrients, particularly the depletion of organic carbon used as the important food source for the growth of earthworms (Aira *et al*., 2006). A statistically significant positive correlation between the bacterial abundance and DOC was shown existent during vermicomposting of the excess sludge ($p < 0.05$) (**Figure 3.4**). The results further confirmed that the stabilization process of excess sludge by vermicomposting is associated with the abundance reduction of bacteria.

(2) Microbial activity

Dehydrogenase activity (DHA) is a parameter reflecting the microbial activity and it has thus been used for evaluation of the extent of stabilization of various organic wastes in composting or vermicomposting (Benitez *et al*., 1999). The changing profiles in DHA during vermicomposting of the excess sludge are shown in **Figure 3.3 (b)**. DHA increased in the

treatments with low and medium earthworm densities (EL and EM) till day 7 as compared to the treatment with the highest earthworm density (EH) and the control treatment (E0) where consistent DHA decreases were observed till the end of the experiment. DHA was significantly lower as compared to the final product of the control treatment without earthworms ($p < 0.05$), even though marked differences among the three vermicomposting treatments were not revealed. This thus indicated that the presence of earthworms could lead to a final product with higher stability through composting treatment of the excess sludge. Lv *et al.* (2018) also found lower values of DHA in the final products of vermicomposting of sewage sludge and cattle dung. The present study suggested that earthworms can enhance the degradation of organic matter in the excess sludge through simultaneously increasing the abundance and activity of bacteria in the earlier stages of vermicomposting, thereby prompting the stabilization of excess sludge. This finding coincided with the results observed in vermicomposting of cattle manure (Lazcano *et al.*, 2008) and vegetable and fruit wastes (Huang *et al.*, 2013).

(3) Bacterial diversity and composition

The results of PCR-DGGE could reflect the microbial diversity in vermicomposting. **Figure 3.5** shows that compared to the value of the Shannon index in the control treatment, the values the vermicomposting treatments at the 7th day of the experiment were higher. This suggests that earthworms increased the diversity of the bacterial community in the initial stage of the vermicomposting treatment of the excess sludge. According to Liu *et al.* (2012), such an increase was probably attributed to the abundant bacterial diversity in the gut of earthworms. At the end of the experiment, the Shannon index was 2.65 for the control treatment (E0) and were 2.86, 3.11 and 3.10 for the vermicomposting treatments with three different earthworm densities (EL, EM and EH), respectively. This indicated that the final products of vermicomposting had a bacterial community richer than that of the conventional composting. The results were similar with the results of previous studies on vermicomposting

of olive-mill waste (Vivas *et al*., 2009) and vegetable and fruit wastes (Huang *et al*., 2013). Recently, Chen *et al*. (2018) observed distinct differences with the Shannon-Wiener diversity index in their vermicomposting systems with different earthworm densities for treatment of medicinal herbal residues through the analysis with the high throughout sequencing technology. In the present study, the treatment with higher density of earthworms also showed richer bacterial community diversity than the control treatment without earthworms, indicating the capability of earthworms in facilitating the degradation of organic matter in the excess sludge and thus the production of vermicompost with higher stability.

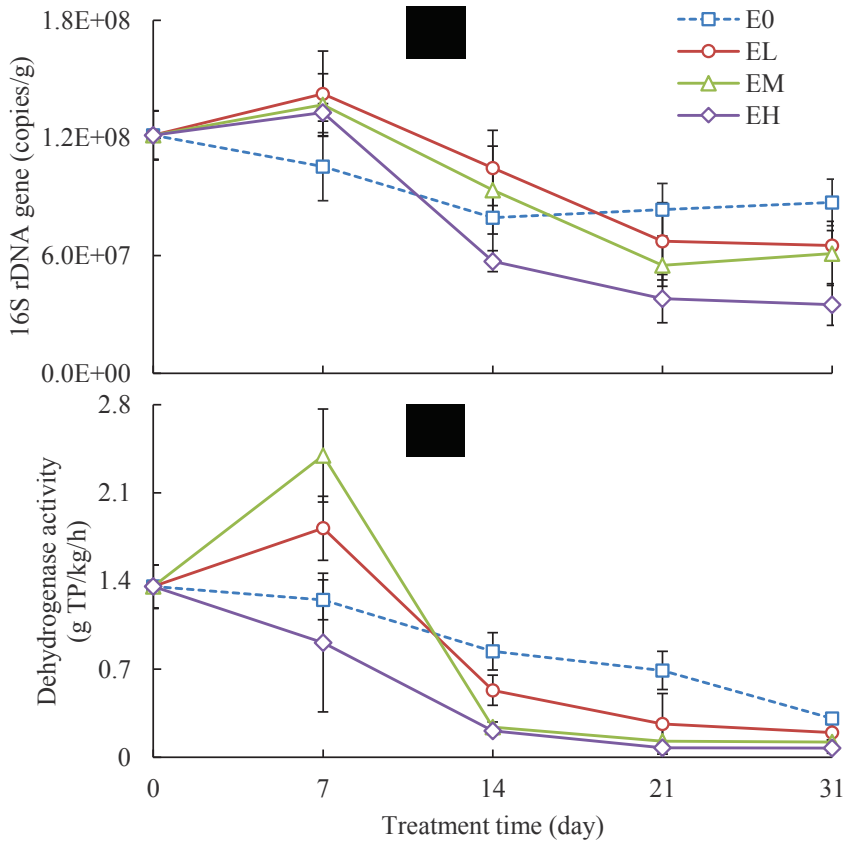

Figure 3.3 Changes in abundance of bacteria (a) and dehydrogenase activity (b) during the vermicomposting of excess sludge. Data are represented as mean and standard deviation (For abundance of bacteria, n=6; for dehydrogenase activity, n=3).

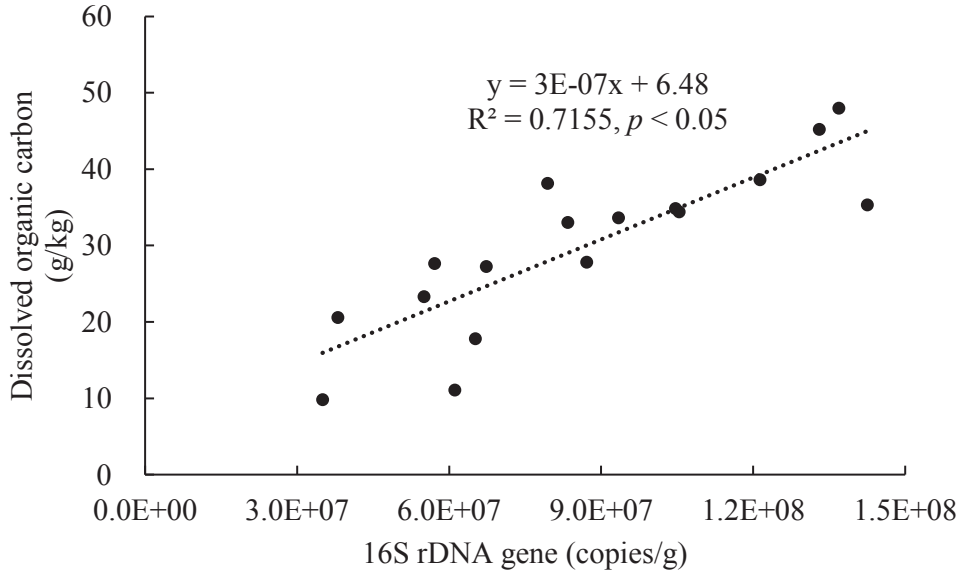

Figure 3.4 Correlation between the bacterial abundance and dissolved organic carbon during the vermicomposting of excess sludge

The sequencing results of the representative bands of the DGGE image are summarized in **Table 3.4**. The dominant bacteria in the vermicomposting treatments were identified as *Flavobacterium* sp. (band 1), uncultured *Saprospiraceae* bacterium (band 3), uncultured *Rhodocyclaceae* bacterium (band 6), *Dechloromonas* sp. (band 8), uncultured gamma proteobacterium (band 10), uncultured *Anaerolineaceae* bacterium (band 11) and uncultured *Kouleothrix* sp. (band 13). At the phylum level, the above bacteria are affiliated to Proteobacteria, Bacteroidetes, Chloroflexi and Chlorobi. Proteobacteria, Bacteroidetes and Chloroflexi were reported to possess relatively strong degradation capability for organic wastes (Zhang *et al.*, 2017), and were also found in the aeration tank of wastewater treatment plants by previous researchers (Cydzik-Kwiatkowska and Zielińska 2016; Zhang *et al*., 2017). It is noteworthy that the uncultured *Flavobacteriales* bacterium (band 7) and the uncultured *Anaerolineaceae* bacterium (band 11) only existed in the final products of vermicomposting treatments with the higher earthworm density (EM and EH). The uncultured *Flavobacteriales*

bacterium was probably excreted from the gut of earthworms (Horn *et al*. 2006). Additionally, compared to the control, the relatively weaker intensity of the bands related to the bacterial species of *Flavobacterium* sp. R046 (band 1), the uncultured *Flexibacter* sp. (band 4), uncultured gamma proteobacterium (band 10) and uncultured bacterium (band 12) indicated that the growth of these bacterial species was probably affected in the later stage of vermicomposting. Previous studies have reported that most gamma proteobacterium species are pathogenic ones, such as *Salmonella* spp, *Escherichia coli* and *Vibrio cholera* (Williams *et al*., 2010). It is thus reasonable to infer that the presence of earthworms might have inhibited the growth of these species, a result probably related to the specific environment in the earthworm's gut (Monroy *et al.*, 2009; Lv *et al.*, 2018).

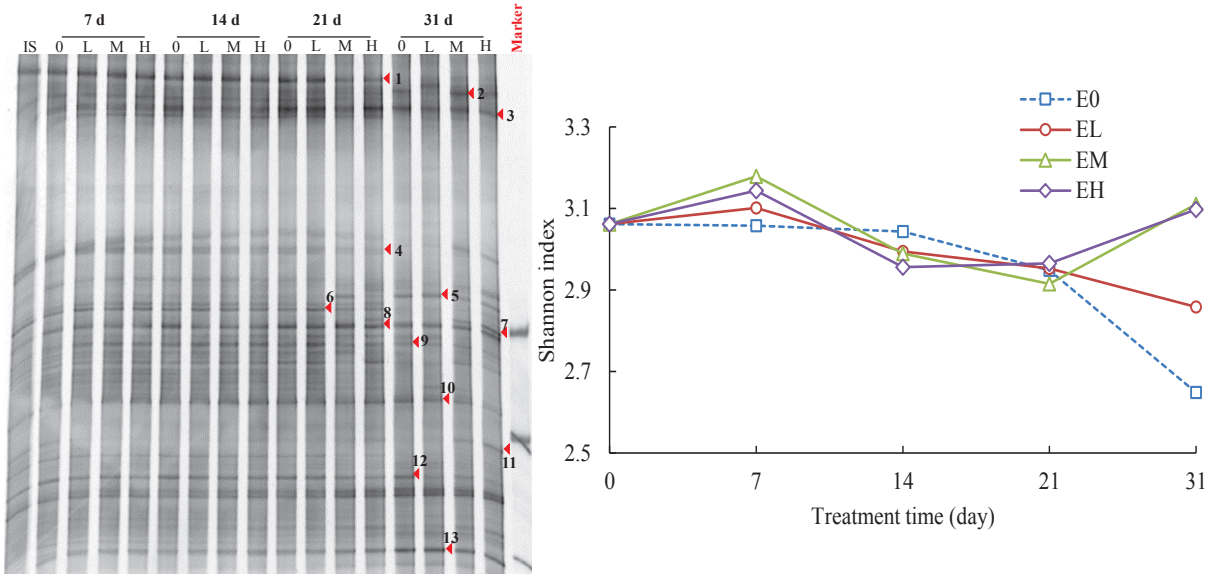

Figure 3.5 DGGE image of bacterial 16S rDNA gene fragment and Shannon index during the vermicomposting of excess sludge. IS-initial sludge, 0-the treatment inoculated without earthworm, L-the treatment inoculated with earthworms at the low density, M-the treatment inoculated with earthworms at the medium density, H-the treatment inoculated with earthworms at the high density. 7d, 14d, 21d and 31d represent sampling days. Arabic numbers on the DGGE image present excised bands for sequence analysis.

3.3.4 Relations among microbial profiles, environmental factors and resistance genes

In order to further clarify how microbial profiles and environmental factors in vermicomposting affected the fate and behavior of quinolone resistance genes in the excess sludge, the redundancy analysis was conducted. As shown in **Figure 3.7**, the first and the second axis explain 68.9% and 3.6% of the selected variables, respectively. For both the targeted resistance genes, the influential extent of the microbial profiles and environmental factors in the process of vermicomposting was found to follow the order of 16S rDNA > DHA > Shannon index, and NH_4^+-N > pH > EC > DOC > NO_3^--N, respectively. This may imply that quinolone resistance genes were greatly affected by the degradation of organic matter during vermicomposting of excess sludge. Moreover, vermicomposting of excess sludge was found to be a process accompanied with the attenuation of *qnr* genes since excess sludge is mainly comprised of abundant and diverse microorganisms. Similar findings have been reported regarding the microbial community succession as a main mechanism explaining the changes of antibiotic resistance genes in the composting process of sludge and manure (Su *et al*., 2015; Zhang *et al*., 2015; Yin *et al.,* 2017). However, through pig manure vermicomposting with housefly larvae, Wang *et al*. (2017) reported that the reduction of bacterial diversity was accompanied by the decrease of certain resistance genes, which differs with the results of the present study. The difference may be due to the different properties of the raw substrates, worm species and target resistance genes used in the experiment. The *qnr*S gene showed a stronger correlation with either DHA or the bacterial abundance as compared to the *qnr*A gene, probably implying that the host bacteria encoding both resistant genes were different. Furthermore, positive correlations were revealed between the *intl*1 gene and *qnr* genes (**Figure. 3.6**), which may indicate that the *qnr* genes were probably mediated by the integrase gene. This finding was in accordance with the result of a recent study on anaerobic digestion of cattle farm wastewater that the integrase genes (*intl*1 and *intl*2) displayed positive correlations with *qnr* genes (Sun *et al*. 2018). Moreover, the negative correlations of NH_4^+-N

with *qnr* genes and *intl*1 gene appeared for all the treatments may suggest that ammonification of excess sludge could enhance the elimination of quinolone resistance genes. Similarly, in a pig manure composting system, Yin *et al.* (2017) observed the existence of correlations between NH_4^+-N and certain resistance genes and pointed out that the host bacteria of some resistance genes were affected by NH_4^+.

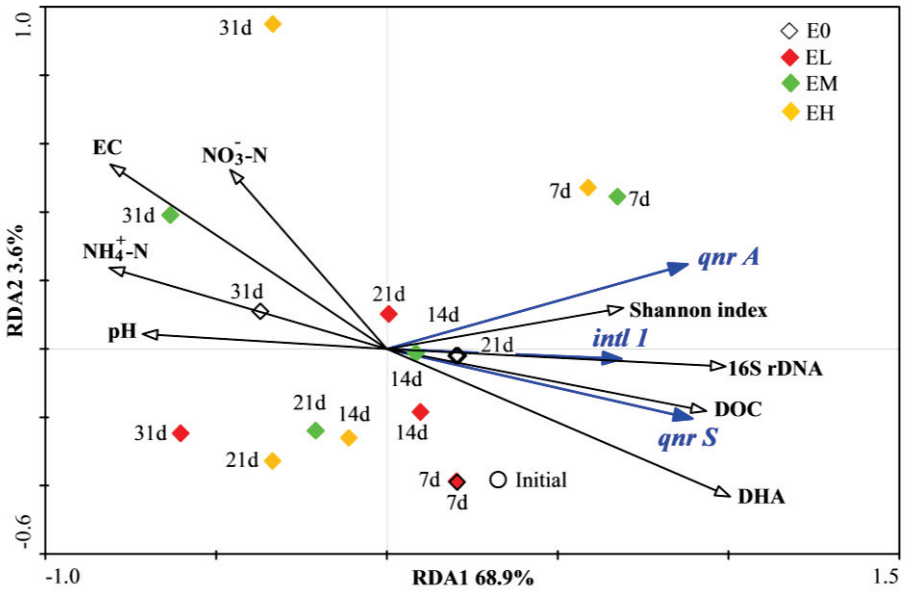

Figure 3.6 Redundancy analysis (RDA) of the relationship among resistance genes, integrase gene, microbial profiles and environmental factors during the vermicomposting of excess sludge. DOC-dissolved organic carbon, EC-electrical conductivity, DHA-dehydrogenase activity.

A significant attenuation for quinolone resistance genes (*qnr*A and *qnr*S) in the excess sludge was found during the process of vermicomposting. However, the involved major mechanisms, especially the occurrence and behavior of the host bacteria of the quinolone resistance genes, need to be clarified for optimization of the vermicomposting process. For this purpose, high throughput sequencing technology should be considered as the first choice for application in coming studies because it can generate more information for better

understanding the complex bacterial communities involved in the vermicomposting treatment process for excess sludge.

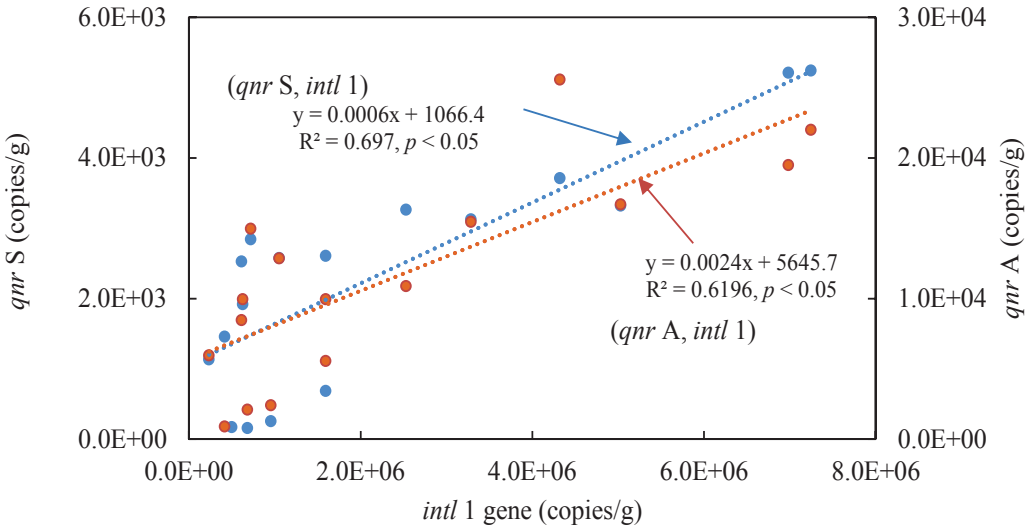

Figure 3.7 The correlation between the absolute abundance of *qnr* genes (A, S) and the absolute abundance of *intl* 1 gene during vermicomposting

Table 3.4 Sequencing results of excised bands from the DGGE gel

Band	Closest related bacteria	Accession number	Similarity (%)	Phylum
1	*Flavobacterium* sp. R046	KC252875	100	Bacteroidetes
2	Uncultured *Sphingobacteriales* bacterium	FJ536881	99	Bacteroidetes
3	Uncultured *Saprospiraceae* bacterium	EU177727	93	Bacteroidetes
4	Uncultured *Flexibacter* sp.	KT182555	97	Bacteroidetes
5	*Clostridium tertium*	MH282454	93	Firmicutes
6	Uncultured *Rhodocyclaceae* bacterium	KF956489	99	Proteobacteria
7	Uncultured *Flavobacteriales* bacterium	LC016552	98	Bacteroidetes
8	*Dechloromonas* sp.	KY117473	96	Proteobacteria
9	Uncultured *Chlorobi* bacterium	JF808803	95	Chlorobi
10	Uncultured gamma proteobacterium	KT336037	85	Proteobacteria
11	Uncultured *Anaerolineaceae* bacterium	KU000219	97	Chloroflexi
12	Uncultured bacterium	MF104541	99	-
13	Uncultured *Kouleothrix* sp.	AB980162	97	Chloroflexi

3.4 Summary

Vermicomposting resulted in significant decreases of *qnr*A and *qnr*S genes. The extent of decreases at the final stage of vermicomposting was more obvious for treatment introduced with higher densities of earthworms. The behavior of the resistance genes was correlated more closely with the microbial activity and abundance than diversity.

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Chapter 4 Effect of temperature on antibiotic resistance genes during vermicomposting of domestic domestic excess sludge

4.1 Introduction

ARGs are newly emerging environmental micro-pollutants. Excess activated sludge, an inevitable by-product of domestic wastewater treatment plant, is enriched with a large number of ARGs (Xu *et al*., 2015; Cui *et al.*, 2018; Huang *et al.*, 2018), such as tetracycline resistance gene (*tet*), sulfonamide resistance gene (*sul*), and quinolone resistance gene (*qnr*). Excess sludge usually needs to be properly treated before entering into environmental system in order to minimize the adverse effects of toxic substances on human health and ecological environment. For this purpose, conventional treatment methods such as thermophilic composting and anaerobic digestion have been widely adopted. Various studies have shown that both methods could significantly affect the antibiotic resistance genes in the excess sludge at different extents. By using high-throughput quantitative PCR (polymerase chain reaction), Su *et al.* (2015) observed that composting significantly increased the abundance and diversity of ARGs in the excess sludge. Anaerobic digestion showed remarkable decline in the abundance of tetracycline and sulfonamide resistance genes (*tet*, *sul*) in the excess sludge (Diehl *et al*., 2010). Collectively, the fate of antibiotic resistance genes in the excess activated sludge varied depending on the selected treatment methods and the types of detected resistance genes.

Vermicomposting is a traditional and eco-friendly method to transform organic wastes into nutrients-rich products by the joint action of earthworms and microorganisms (Bhat *et al*., 2017). Recent studies have shown that vermicomposting treatment of excess sludge had a

good reduction effect on tetracyclines and quinolone resistance genes (Huang *et al.*,2018; Cui *et al.*, 2018), and higher inoculating density of earthworms could further improve the removal efficiency (Cui *et al*., 2018). From the application perspective of vermicomposting, temperature is a more easily controlled environmental prameter, and an appropriate increase in temperature can contribute to the improvement of stabilization of vermicomposting. Garg and Gupta (2011) observed that there was higher earthworm activity in winter than in summer, and the final vermicomposting product was more abundant in nutrients (N, P and K). Earthworms *Eisenia fetida* showed a good adaptability for pelletized sludge at the range of 15~25°C (Edwards and Lofty, 1977). Additionally, microbial population as a critical driver of decomposition of pollutants in wastewater treatment plants, its structure is significantly correlated with ambient temperature (Zhang *et al*., 2018). Also, the recent studies had confirmed that the antibiotic resistance genes and intermediates of their dissemination are positively linked to the bacterial populations during biological-treatment of organic wastes (Ma *et al*., 2011). Therefore, we can speculate that antibiotic resistance genes would have specific responses to vermicomposting of excess sludge at different temperature conditions, and from application viewpoint of vermicomposting, and it would be possible to reduce the antibiotic resistance genes in the excess sludge by optimizing temperature conditions.

The objective of this chapter was to investigate the effect of temperature on the antibiotic resistance genes of domestic excess sludge during vermicomposting. For this, the vermicomposting of excess sludge were established at three different temperatures (15°C, 20°C and 25°C). Abundance of the target genes including tetracycline resistance genes (*tetG*, *tet*M and *tet*X), sulfonamide resistance gene (*sul*1), quinolone resistance genes (*qnr*A and *qnr*S) as well as integrase gene (*intl*1) were determined by quantitative PCR with the specific primers. Moreover, in order to clarify the response mechanism of the selected resistance genes to vermicomposting at different temperatures, bacterial community composition was analyzed by utilizing high throughput sequencing. The relationship between the resistance genes and the bacterial community were also discussed by applying the redundancy analysis.

4.2 Materials and methods

4.2.1 Earthworm and sludge

Earthworm *Eisenia fetida* widely adopted in vermicomposting system was used for this research. Dewatered excess sludge was taken from the Qilihe-Anning Wastewater Treatment Plant in Lanzhou City, China. Referring to a previous method (Fu *et al*., 2015), dewatered sludge was made into granular sludge with a size of 5 mm. Physicochemical property of inital sludge is presented in **Table 4.1**.

Table 4.1 Property of initial sludge and final vermicompost

Parameter	Initial sludge	Final vermicompost		
		15°C	20°C	25°C
Organic matter (%)	68.0 ± 7.7	55.0 ± 0.01	51.7 ± 0.02	49.8 ± 0.01
Dissolved organic carbon (g/kg)	16.7 ± 2.32	12.9 ± 1.66	14.0 ± 2.31	7.60 ± 1.29
Electrical conductivity (μS/cm)	573 ± 8.49	1035 ± 2.04	1275 ± 6.94	2100 ± 16.3
pH	6.77 ± 0.11	6.53 ± 0.09	6.77 ± 0.12	7.01 ± 0.11
Ammonium (mg-N/kg)	7.36 ± 0.08	65.0 ± 0.76	107 ± 0.23	296 ± 2.90
Nitrate (mg-N/kg)	10.3 ± 2.0	220 ± 45.2	285 ± 9.10	1389 ± 47.1
Phosphate (mg/kg)	2.61 ± 0.12	3.05 ± 0.15	3.65 ± 0.22	4.75 ± 0.36
Microbial biomass carbon (g/kg)	105 ± 10.6	15.6 ± 0.98	8.50 ± 0.18	6.71 ± 1.08
Dehydrogenase activity (mg-TF/g/h)	28.1 ± 1.34	0.49 ± 0.08	0.49 ± 0.02	0.27 ± 0.01

Means ± deviation (n=3).

4.2.2 Vermicomposting process

Vermicomposting was conducted in lab was repeated in triplicate. 100 adult earthworms of approximately 1.0 g per earthworm was inoculated into each reactor (stainless steel pot) with 4 kg of pelletized sludge. All reactors were covered with a plastic film and placed in constant temperature incubators at 15°C, 20°C, and 25°C, respectively. Vermicomposting

experiment was conducted for 60 days and the sampling interval was 10 days. The fresh sample was used for measuring the organic matter, microbial biomass carbon (MBC) and dehydrogenase activity (DHA). Another part of sample was air-dried, ground and sieved through 100-mesh. The remaining sample was frozen-dried and stored at -40°C for ARGs and bacterial analysis.

4.2.3 Physicochemical analysis

Organic matter (OM) content was determined by burning sample in a muffle furnace at 600°C. Dissolved organic carbon (DOC) content was measured following oxidizing method by following oxidizing method with sulfuric acid-potassium dichromate. In detail, dry sample was dissolved in distilled water at a ratio of 1:10 (mass: volume), and obtained mixture was filtered through membrane with a pore size of 0.45 μm. After filtration, filtrate was oxidized with potassium sulfate-dichromate at 200°C -220°C and then titrated with ferrous sulfate solution. Electrical conductivity (EC) was measured by using conductivity meter. Ammonium nitrogen (NH_4^+-N) content was analyzed by spectrophotometry after sample was dissolved with potassium chloride solution. Nitrate nitrogen content was determined using phenol disulfonic acid colorimetric method. Microbial biomass (MBC), was analysed by using chloroform fumigation-potassium dichromate volumetric method reported by Wu (2006). DHA was referred to the TTC reduction method reported by Fu *et al*. (2015).

4.2.4 Microbial analysis

Total genomic DNA was extracted by using the Powersoil DNA Extraction Kit (MOBIO, USA) according to the manual instructions. Bacterial abundance was analyzed by a fluorescent quantitative PCR instrument (Takara, Japan). Primer information used for amplification is shown in **Table 4.2**. 25 μL PCR reaction solution included 12.5 μL SYBR

Green, 0.5 μL primers with concentration of 10 μM, 2 μL DNA template, and the rest is supplemented with steriled water. PCR amplification condition consisted of pre-denaturation 95°C for 3 min, 40 thermal cycles (95°C for 5 s, 60°C for 30 s). Bacterial quantitative PCR standard curve was made with known concentrations of standards of *E. coli*.

Bacterial composition of sample was analyzed by using high-throughput sequencing technology with the primer pair of 341F-806R referring to a previous report by Huang *et al*. (2018).

Table 4.2 Primers information and PCR amplification conditions

Target genes	Primer sequence (5'-3')	Annealing temperature	Reference
*qnr*A	F:ATT TCT CAC GCC AGG ATT TG R:GCA GAT CGG CAT AGC TGA AG	54°C	Di Cesare *et al*. (2016)
*qnr*S	F:GAC GTG CTA ACT TGC GTG AT R:TGG CAT TGT TGG AAA CTT G	54°C	Di Cesare *et al*. (2016)
*intl*1	F:GGC TTC GTG ATG CCT GCT T R:CAT TCC TGG CCG TGG TTC T	57°C	Luo *et al*. (2010)
*sul*1	F:CAC CGG AAA CAT CGC TGC A R:AAG TTC CGC CGC AAG GCT	55°C	Luo *et al*. (2010)
*tet*G	F:TTA TCG CCG CCG CCC TTC T R:TCA TCC AGC CGT AAC AGA AC	55°C	Apley *et al*. (2012)
*tet*M	F:ACA GAA AGC TTA TTA TAT AAC R:TGG CGT GTC TAT GAT GTT CAC	45°C	Apley *et al*. (2012)
*tet*X	F:CAA TAA TTG GTG GTG GAC CC R:TTC TTA CCT TGG ACA TCC CG	60°C	Ng *et al*. (2001)
16S rDNA	Com1: CAC CAG CCG CGG TAA TAC Com2: CCG TCA ATT CCT TTG AGT TT	50 °C	Fu *et al*. (2016)

4.2.5 Determination of ARGs and *intl*1 gene

Detailed information on the primers used in the quantitative PCR is presented in **Table 4.2**. The amplification condition of quantitative PCR is described in Chapter 3.

4.2.6 Statistical analysis

One-way ANOVA of physicochemical parameters before and after vermicomposting of excess sludge was conducted by using SPSS software at a significance level of $p < 0.05$. Principal component analysis (PCA) of parameters regarding stabilization of vermicomposting was carried out by Canoco 4.5 statistical software. Redundancy analysis (RDA) of correlation between resistance genes and physicochemical parameters as well as bacterial community was analyzed by using Canoco 4.5. Pearson correlation between bacteria (genus level) and resistance genes as well as integrase gene was assessed by using SPSS software at a significance level of $p < 0.01$.

4.3 Results and Discussion

4.3.1 Stabilization process of vermicomposting

DOC is regarded to be readily utilized by microorganisms, its change can well reflect the extent of stabilization in environmental system. As shown in **Table 4.1**, DOC decreased after vermicomposting, with the lowest and highest concentrations at 25°C and 15°C, respectively. The result indicates that the highest stability of the final vermicomposting product was at 25°C. Dehydrogenase activity in the final vermicomposting product at 25°C was significantly lower than that at 15°C and 20°C, which also suggests that the extent of stabilization of the final product at 25°C was relatively higher. High stability was mainly due to the high earthworm activity at this temperature (Grag *et al*., 2018), thereby enhancing the decomposition of organic matter in excess sludge by microorganisms.

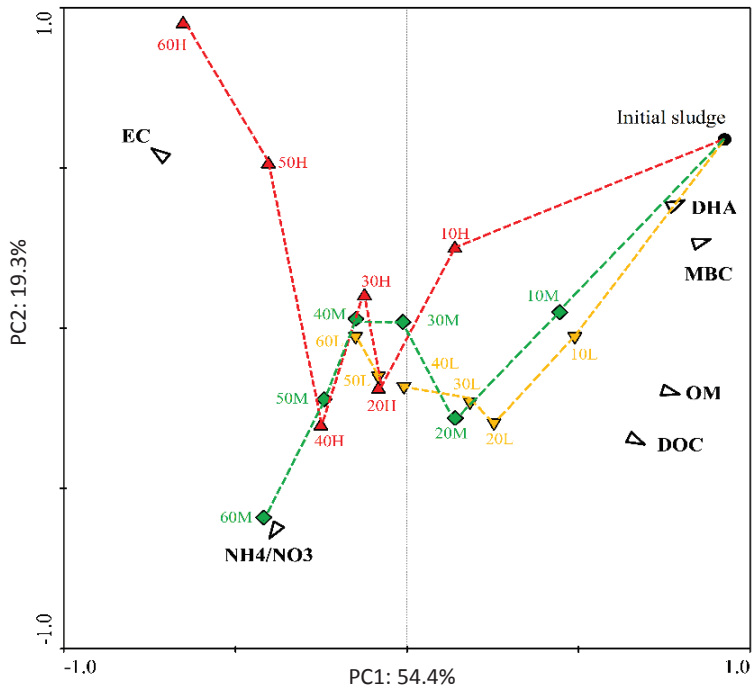

Figure 4.1 Principal component analysis of stabilization process of vermicomposting at different temperatures. L, M and H represent treatments at 15°C, 20°C and 25°C. DHA-dehydrogenase activity, EC- electrical conductivity, DOC-dissolved organic carbon, MBC-microbial biomass carbon, OM-organic matter, NH_4/NO_3-the ratio of ammonium to nitrate.

In order to further comprehensively investigate the effect of temperature on the stability of sludge treated by vermicomposting, we performed principal component analysis (PCA) of parameters closely related to the stabilization of the vermicomposting process (as shown in **Figure 4.1**). The first principal component can explain the characterization associated with the biodegradation and mineralization of organic matter during vermicomposting. The second principal component primarily characterized the conversion of mineral nitrogen. According to the stabilization path of samples during vermicomposting process under three different temperature conditions, we can obtain related information about the progress of vermicomposting of excess sludge. Vermicomposting at 15°C mainly performed the biodegradation of organic matter, and vermicomposting at 20°C not only accelerated the

degradation process of organic matter, but also obviously promoted the ammonification process. It is worthy to note that intensive nitrification may also occur at the end of the vermicomposting process after degradation and ammonization of organic matter at 25°C, which can be confirmed by the high content of NO3- in the final products of vermicomposting.

4.3.2 Dynamics of ARGs and *intl1* gene

Dynamics of absolute abundance of three types of resistance genes and integron gene in the vermicomposting process are shown in **Figure 4.2**. For the initial excess sludge, the abundance of sulfonamide and tetracycline resistance genes with order of magnitude of E8-9 was higher than quinolone resistance genes with order of magnitude of E5-7, whereas the integrase gene (*intl*1) had the highest absolute abundance with magnitude of E10. The results indicated that domestic excess sludge not only contained abundant antibiotic resistance genes but also had high horizontal transfer potential for resistance genes.

Effect of vermicomposting process on the resistance genes was closely associated with the type of selected resistance genes. The abundances of two quinolone resistance genes (*qnr*A and *qnr*S) were significantly declined during the whole vermicomposting process. However, this resistance gene rebounded at the end of vermicomposting at 25°C. Vermicomposting process did not have a significant effect on the *sul* resistance gene, and temperature difference was not very obvious. There was a remarkable decrease in *tet*M gene, but *tet*G and *tet*X genes did not present significant changes in the abundance, and they also rebounded in the later stage of vermicomposting at 25°C. In addition, vermicomposting at 25°C favored the reduction of *intl*1. Overall, vermicomposting at 25°C did not show significant attenuations in the abundance of resistance genes in comparison to that of 15°C and 20°C, which is not in coincidence with the stabilization result of vermicomposting. The reason behind this

difference may be related to the discrepancy of community structure of bacteria hosting the above mentioned resistance genes under different temperature conditions.

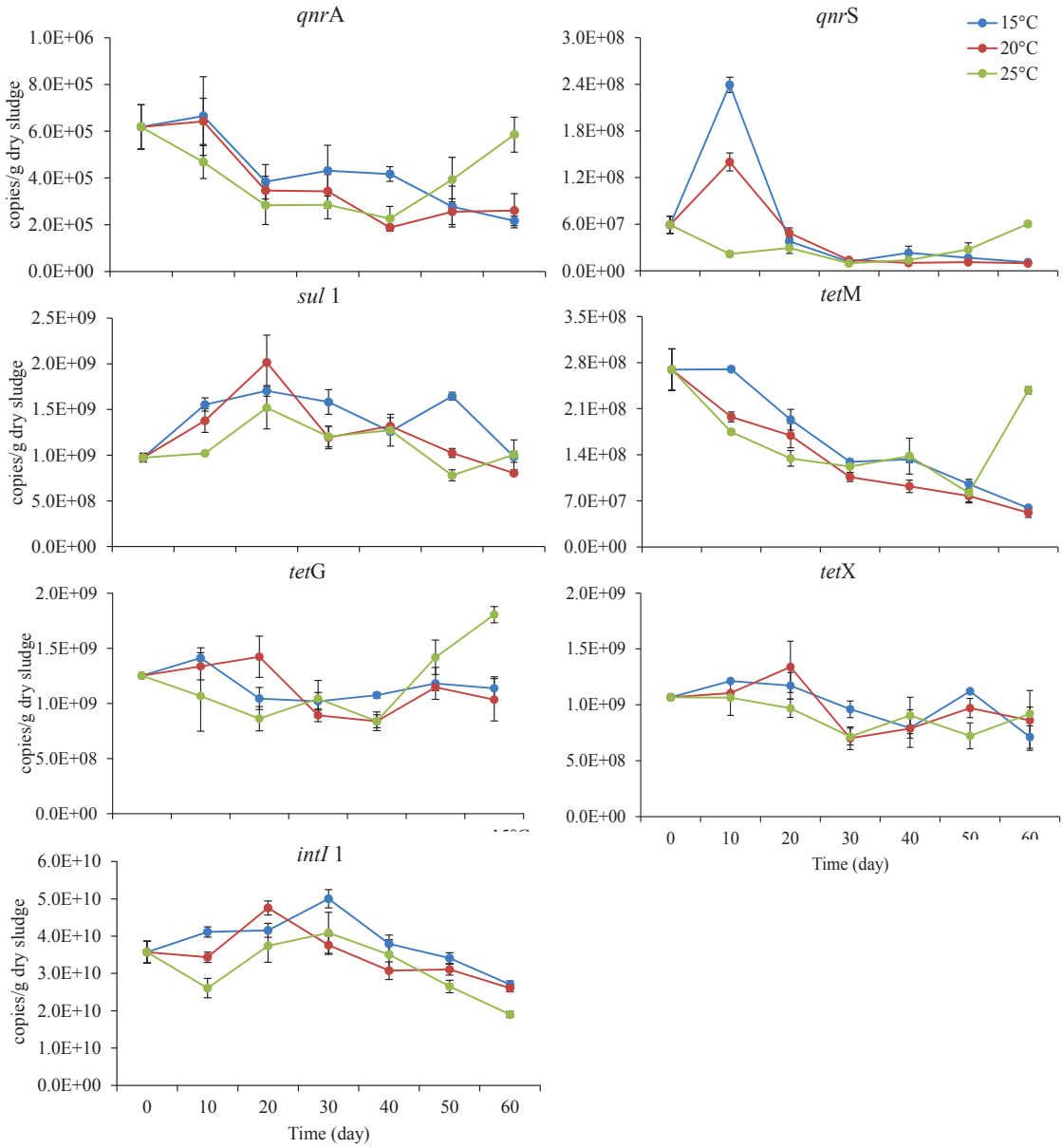

Figure 4.2 Changes of absolute abundance of the target genes during vermicomposting of domestic excess sludge at 15°C, 20°C and 25°C

Relative abundance (ARGs/16S rDNA) of resistance genes is directly associated with dynamics of the host bacteria. Changes of relative abundance of the resistance genes in the vermicomposting process are shown in **Figure 4.3**. *Qnr*A, *qnr*S and *tet*M gene were

significantly reduced in the relative abundance, whereas other genes did not significantly change. It suggests that vermicomposting had significant removal efficiency for these resistance genes, and exhibited selectivity for the reduction of three types of ARGs. It is probably related to a fact that cell-free DNA existed in the excess sludge is more susceptible to be degraded compared to intracellular DNA, thereby the corresponding carried resistance genes would exhibit different degradation characteristics.

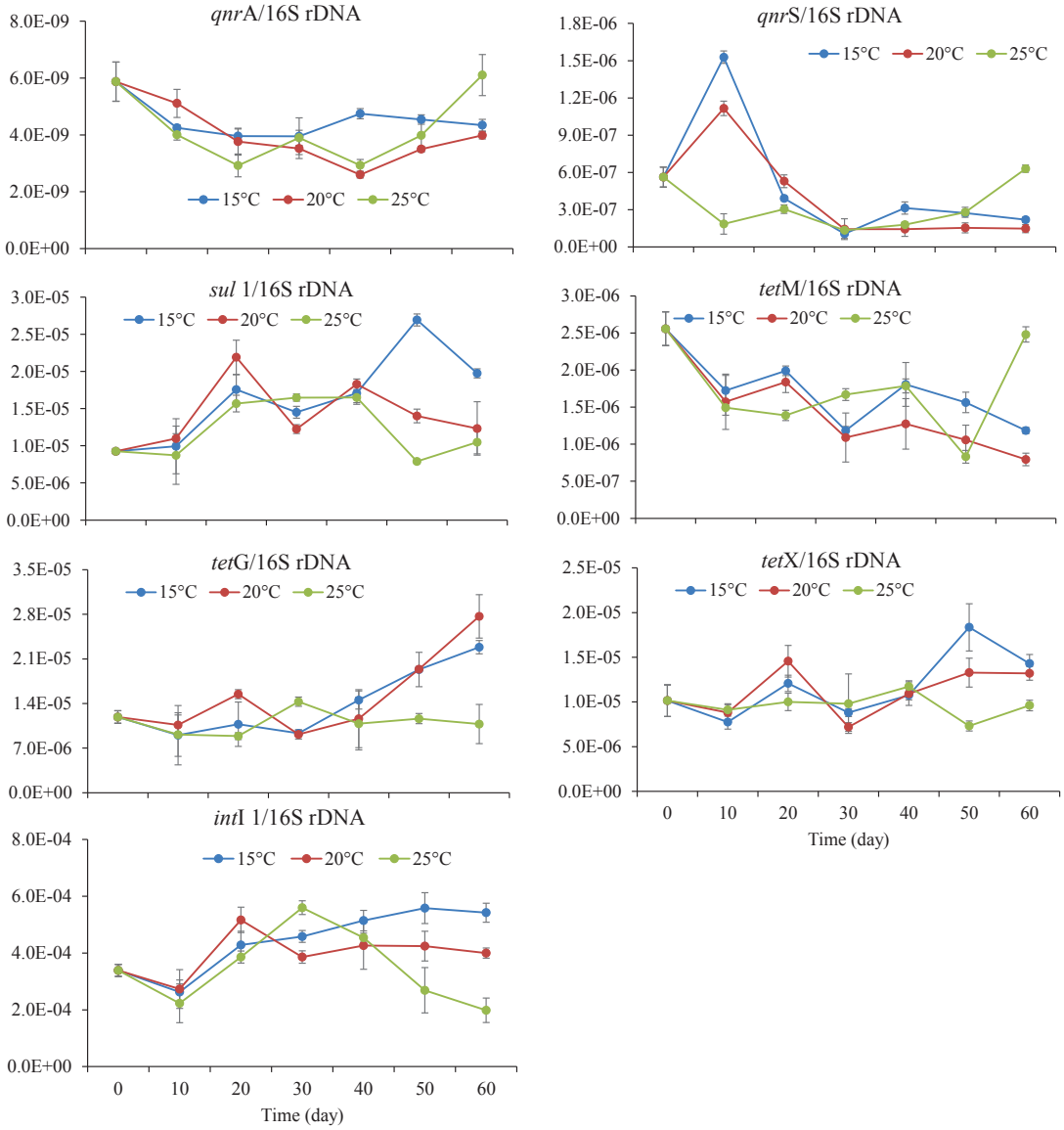

Figure 4.3 Changes of relative abundance of ARGs and integrase gene during vermicomposting of domestic excess sludge at 15°C, 20°C and 25°C

4.3.3 Bacterial community

Shannon diversity index in the vermicomposting process was in the range of 4.78-5.91 (**Table 4.3**), and this index for the final products under three different temperature conditions were 5.05, 5.91, and 4.78, respectively, suggesting that bacterial diversity was also affected by temperature. The lowest bacterial diversity observed in the final product at 25°C was in consistent with the OTU (Operational taxonomic unit) results obtained through Venn image in **Figure 4.4.**

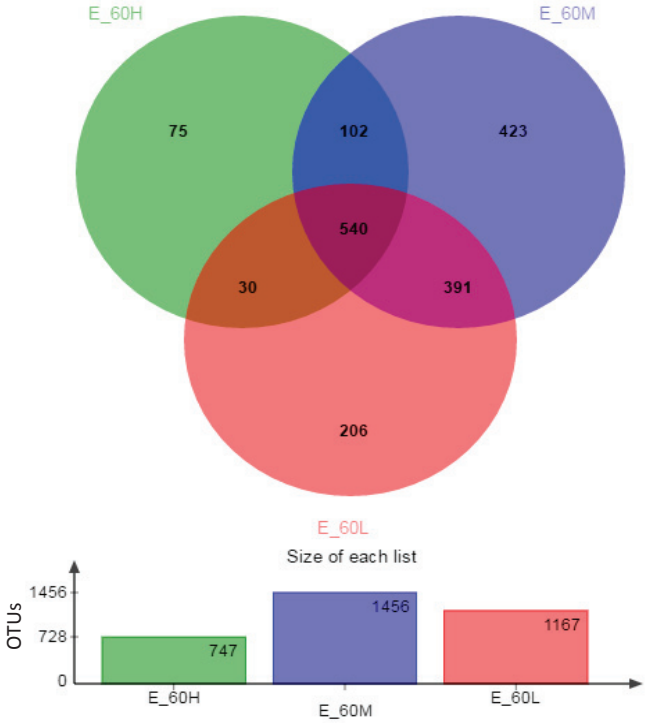

Figure 4.4 Venn diagram of bacterial community for the final vermicomposting products. L, M and H represent treatments at 15°C, 20°C and 25°C, respectively. Numbers in pie and pillar graphs mean the number of operational taxonomic units (OTUs).

Figure 4.5 shows the dynamics of bacterial composition at the phylum level during vermicomposting, and is mainly composed of Proteobacteria, Bacteroidetes, Actinobacteria, Chloroflexi, Firmicutes and Acidobacteria. From the results of cluster analysis, the samples of

50H, 60M and 60H were affiliated into an independent group, which is significantly different from the samples. It indicates that the changes of bacteria in the vermicomposting of excess sludge at the early and middle stages were not obvious. At the end of vermicomposting, temperature had a significant effect on the bacterial composition. In detail, relative abundance of bacteria including Proteobacteria, Actinobacteria, Chloroflexi and Firmicutes was observed to be lower at 25°C compared to that at 20°C, while relative abundance of Bacteroides at 25°C was higher than that at 20°C. The reduced abundance of Proteobacteria and Firmicutes suggests that contaminants in the excess sludge may undergo more complete degradation under higher temperature conditions. Firmicutes belongs to a facultative anaerobic bacterium (Wang *et al*., 2016). Higher earthworm predation activity and intestinal activity at 25°C degrees can improve aerobic environment of excess sludge, probably leading to lower abundance of Firmicutes. Bacteroidetes can decompose compounds such as chitin and cellulose, and the observed highest abundance at 25°C indicate that the stability of vermicompost under this temperature condition was the best compared to that at 15°C and 20°C.

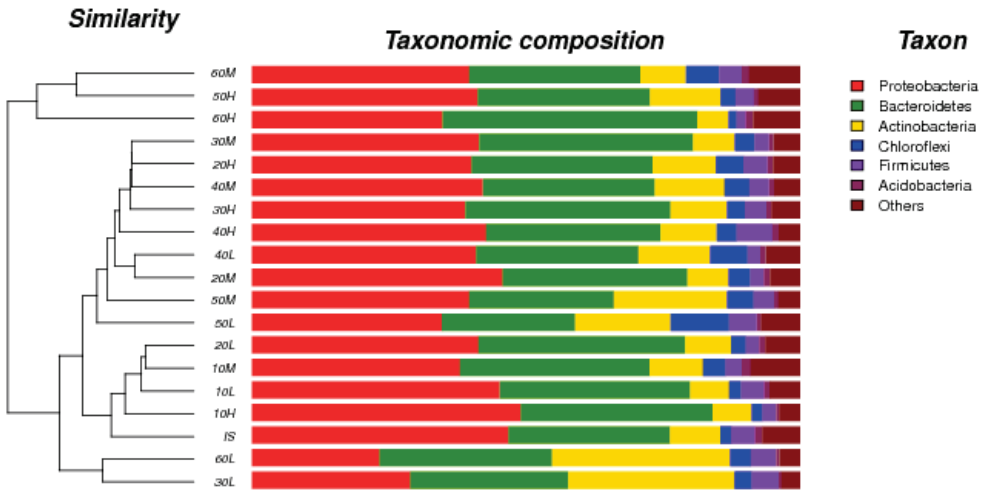

Figure 4.5 Bacterial community (Phylum) during vermicomposting of domestic excess sludge. IS-initial sludge. L, M and H represent treatments at 15°C, 20°C and 25°C. The

number represent sampling time (day). The number at the front of L, M and H represent sampling time (day).

In addition, dynamics of bacteria at the genus level during vermicomposting were also analyzed, as shown in **Figure 4.6**. Bacterial communities are clustered into two groups, especially for three genus of *Arenibacter*, *Halomonas* and *Niabella* grouped together, which was different from other bacteria. These bacterial abundances increased at the end of vermicomposting stage ranging from 0.00% to 0.41, 0.16, 10.68% for *Arenibacter*, from 0.02% to 0.18, 0.21, 1.67% for *Halomonas*, from 0.01% to 0.09, 0.51, 2.05% for *Niabella*. *Arenibacter* is strictly aerobic, and the optimum growth temperature is 25°C, and catalase and oxidase are positive (Jeong *et al*., 2013). *Niabella* is affiliated to a genus of bacteroidetes, strictly aerobic bacteria, and its optimum growth temperature is at the range of 25-30°C (Kim *et al.*, 2007). Aerobic state in the final vermicomposting product and the optimal temperature condition were responsible for the remarkable increase in abundance of these bacteria. Moreover, *Zoogloea*, *Sediminibacterium*, *Trichococcus*, *Longilinea* and *Macellibacteroides* were significantly decreased in abundance at 25°C in comparison to that at 20°C and 15°C. *Zoogloea* plays an important role in formation of activated sludge flocs by absorbing nutrients from the environment. Significant decrease in abundance at 25°C indicates that the floc structure of the excess sludge has been destroyed. *Sediminibacterium* is an organic degrading bacterium under strict aerobic conditions, and its optimal growth temperature is in range of 22-28°C, therefore, it was able to exert the maximum degradation ability for pollutants at 25°C. *Trichococcus* has been found to be capable of transforming complex organic matter into small molecular compounds such as lactate, acetate, formate and ethanol that can be readily utilized by other microorganisms (Song *et al.*, 2018; Dai *et al*., 2017). Therefore, significant decrease in the abundance of the above-mentioned bacteria at 25°C confirms that the excellent stability of the domestic excess sludge treated by vermicomposting can be reached.

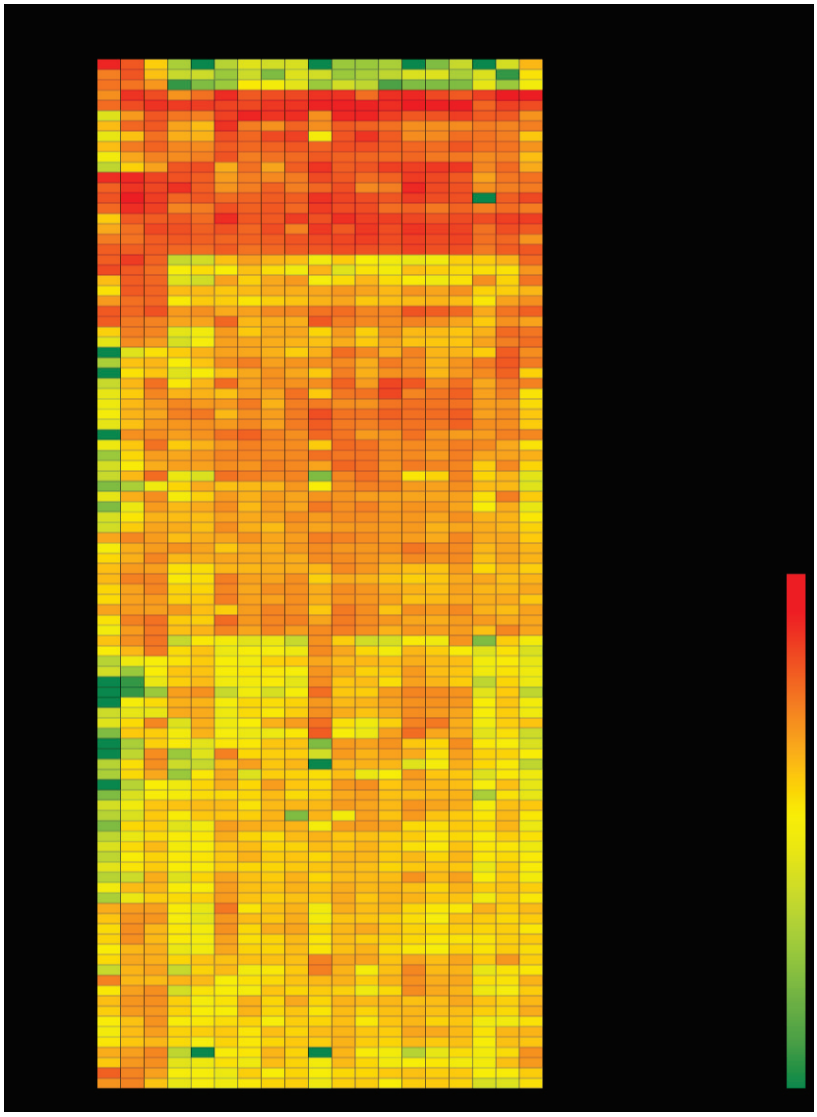

Figure 4.6 Heat map of bacterial community (genus level) with cluster analysis during vermicomposting of municipal excess sludge. The color of box presents the relative abundance of bacteria in total bacterial community. IS-initial sludge. L, M and H represent treatments at 15°C, 20°C and 25°C. The number at the front of L, M and H represent sampling time (day).

4.3.4 Correlation analysis

Physicochemical factors can influence the abundance of antibiotic resistance genes in environmental samples through indirectly altering their microbial communities. As shown in

Figure 4.7, based on the length of arrow in the principal component analysis, parameters including OM, NH_4-N, NO_3-N and EC had significant effects on the resistance genes and integrase gene. Of these, NH_4-N, NO_3-N and EC had significantly negative correlations with the *sul*1 and *intl*1 genes. The difference caused by temperature treatments started from day 10 until the end of the vermicomposting process. The final product at 25°C was significantly different from the temperature treatments of 15°C and 20°C. Significant increase of nitrate nitrogen at 25°C was probably a main reason of rebound of *qnr* and *tetG* genes at the end of vermicomposting. During thermophilic composting of dung manure, Yin *et al*. (2017) also found that the dynamics of multiple resistance genes and integrase gene (*intl*1) were significantly associated with the content of nitrate nitrogen. Additionally, OM had positive correlations with almost of resistance genes (except *tet*G), indicating that the resistance genes were decreased with the stabilization of excess sludge by vermicomposting. Negative correlations between EC and resistance genes also imply that the host bacteria carrying these resistance genes will be decomposed during vermistabilization of excess sludge.

Correlation between resistance genes and bacteria community (genus level) under different temperature conditions were investigated by redundancy analysis as shown in **Figure 4.8**. The results showed that the first and second components explained 72.8% of the total selected variables. Remarkable discrepancy of relationship between bacterial population and resistance genes was observed during vermicomposting of excess sludge under three different temperature conditions. At the beginning of vermicomposting (10d), the scores of samples from three temperature groups were positive on the RDA1 axis, with order of 15°C >20°C >25°C. However, the scores of three temperature groups from 20d to 60d were almost negative, and the final vermicomposting product at 25°C got positive score on the RDA1 axis and the highest score on the RDA2 axis. This means that the rebound of resistance genes occurred at 25°C, which was consistent with the increase in the abundance of the resistance genes *tet*G, *tet*M, *qnr*A and *qnr*S on day 60. This increase was attributed to relatively higher abundance of *Aeromonas* and *Chitinophagaceae*. It has been reported that

Aeromonas hydrophila was not only indigenous to the earthworm *E. fetida* (Toyota and Kimura, 2000), the genus *Aeromonas* also carried integrase gene and plasmid, and was confirmed to be an important host bacteria of quinolone resistance genes (Huddleston *et al*., 2006; Cattoir, 2008; Jacobs and Chenia, 2017). Besides, suitable temperature close to the recorded optimal growth temperature (22°C-37°C) may be an important cause of sudden increase in the abundance of these resistance genes in the final product. Zhu *et al*. (2017) have pointed out that *Chitinophagaceae* was a major host strain of tetracycline resistance genes. Bernard *et al*. (2012) confirmed that Chitinolytic organisms were strongly affected by earthworm activity, and the increase in earthworm activity under relatively higher temperature conditions could promote a significant increase in the abundance of this bacterium.

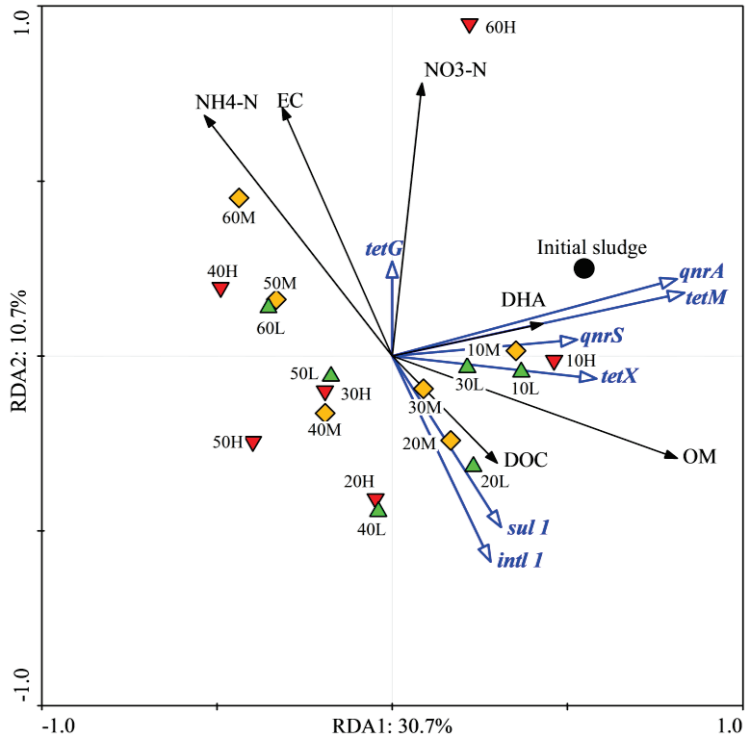

Figure 4.7 Redundancy analysis of relationship between ARGs and physicochemical properties. IS-initial sludge. L, M and H represent treatments at 15°C, 20°C and 25°C. The number at the front of L, M and H represent sampling time (day).

Table 4.4 Pearson correlations between ARGs and bacterial community (genus level). Asterisk (*) represent significant difference at the level of $p < 0.01$.

	*intl*1	*qnr*A	*qnr*S	*sul*1	*tet*G	*tet*M	*tet*X
Aeromonas	*-0.593			*-0.593			
Arenibacter	*-0.544						
Comamonas					*0.572		
Dechloromonas						*0.600	
Desulfocapsa		*-0.669				*-0.534	
Desulfomicrobium		*-0.540					
Ferribacterium						*0.625	*0.609
Filimonas	*-0.638						
Fluviicola	*-0.543			*-0.619		*-0.700	*-0.545
Halomonas	*-0.561						
Lysinimonas						*-0.606	
Macellibacteroides	*0.548						
Niabella	*-0.632						
Pedobacter	*-0.538			*-0.555			
Pseudoxanthomonas	*-0.655			*-0.567			
Rhodanobacter	*-0.614						
Rhodobacter		*-0.726				*-0.705	
Steroidobacter	*-0.555						
Thauera							*0.538
Thermomonas				*-0.544			
Zoogloea		*0.706	*0.622			*0.784	*0.640
Bacteroidetes_vadinHA17		*-0.591					
Acidimicrobiaceae	*-0.630						
Caldilineaceae		*-0.540				*-0.568	
Chitinophagaceae	*-0.559	*0.584					
Draconibacteriaceae	*0.619			**0.576			
OPB56	*-0.557						
ST-12K33	*0.526						
WCHB1-69		*-0.605					
Obscuribacterales		*0.657				*0.633	
Xanthomonadales	*-0.571						

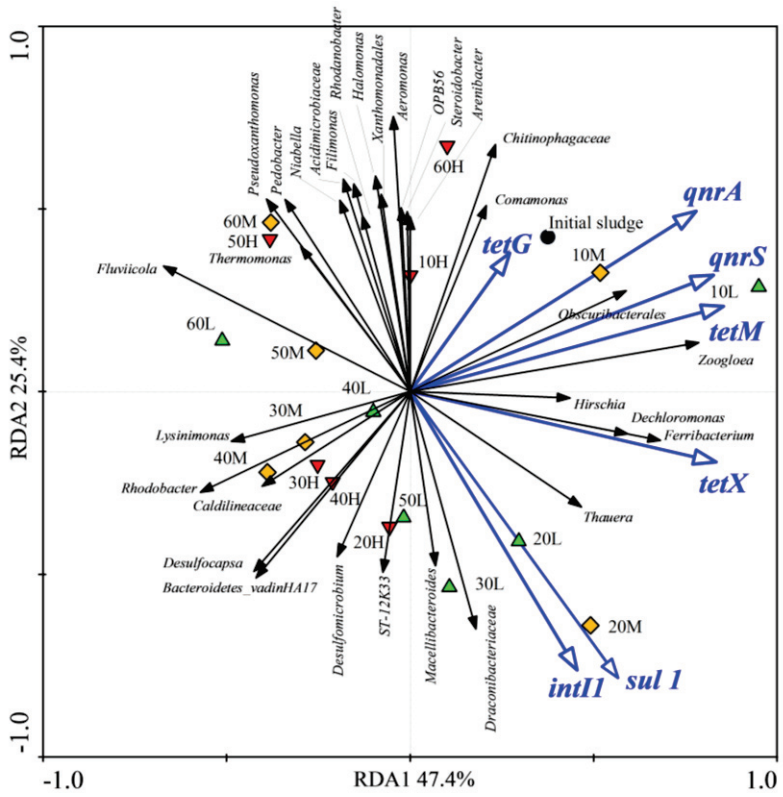

Figure 4.8 Redundancy analysis of relationship between ARGs and bacterial community. IS-initial sludge. L, M and H represent treatments at 15°C, 20°C and 25°C. The number at the front of L, M and H represent sampling time (day).

Pearson correlation analysis was carried out to clarify possible host bacteria of the resistance genes in the vermicomposting process. As given in **Table 4.4**, *Ferribacterium* was a possible host bacteria of other resistance genes except for *tet*G, and *Zoogloa* and *Obscuribacvterale*s were possible host bacteria of resistance genes except for *sul*1 and *tet*G. *Thauera* may carry resistance genes including *sul*1, *tet*M and *tet*X. In addition, bacteria that can carry integrase genes included *Dechloromonas*, *Ferribacterium*, *Macellibacteroides*, *Thauera*, *Draconibacteriaceae*, *ST-12K33*. This finding suggests that there are many different types of bacteria that were able to carry *intl*1 gene in the domestic excess sludge, which is consistent with the report from Huang *et al*. (2018). Possible host bacteria of *qnr*A and *qnr*S are mostly same, except Dechloromonas that may only carry the *qnr*A gene. Its possible

reason may be attributed to different types of amino acid coding both resistance (Strahilevitz *et al*., 2009). Potential host bacteria of the three *tet* genes were quite different. Host bacteria was also affected by the type of resistance genes. In fact, this can be explained by the proven mechanism of tetracycline resistance, namely efflux pumps for *tet*G gene (Martinez *et al*., 2009), ribosome-protected proteins for *tet*M gene, encoding an enzyme that modified or inactivated tetracycline for *tet*X gene (Roberts, 2005; Sui *et al*., 2017).

4.4 Summary

This chapter was conducted to explore the effect of temperature on ARGs in domestic excess sludge in the vermicomposting process. Several findings were obtained as follows. Vermicomposting at 25°C did not show significant attenuations for resistance genes in comparison to the vermicomposting at either 15°C or 20°C. Higher removal efficiency for *qnr*A, *qnr*S and *tet*M genes was identified during vermicomposting. Bacterial diversity was affected by temperature during vermicomposting of excess sludge, with the lowest Shannon index of 4.78 at 25°C compared to that at 15°C and 20°C. Potential host bacteria of the three *tet* genes were quite different. The significant increase of nitrate nitrogen at 25°C was probably a main reason that contributed to the rebound of *qnr* and *tet*G genes at the end of vermicomposting.

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Chapter 5 Effect of earthworm gut digestion on antibiotic resistance genes in domestic excess activated sludge

5.1 Introduction

Excess activated sludge is a reservoir of various pollutants and pathogens contained in wastewater. As a newly environmental pollutant, ARGs have been highly concerned in recent years due to their adversary effects to human health and natural ecosystems (Baquero *et al*., 2008; Bondarczuk *et al*., 2016; Ju *et al*., 2016; Karkman *et al*., 2018; Urra *et al*., 2019). ARGs detected in the excess activated sludge mainly contain tetracycline resistance genes, sulfide resistance genes and quinolone resistance genes (Diehl and LaPara, 2010; Ma *et al*., 2011, Narciso-da-Rocha *et al*., 2018; Karkman *et al*., 2018), due to the overuse of their corresponding antibiotics for the treatment and prevention of bacterial infection in humans and animals. In order to reduce the adversary effects of excess sludge on the environment, sustainable biological treatment methods such as anaerobic digestion and composting, have been widely adopted. According to the previous studies, both methods (anaerobic digestion and composting) are effective in stabilizing excess activated sludge, however, their removal efficiency for ARGs is greatly affected by various factors such as temperatures and sludge properties (Diehl and LaPara, 2010; Zhang *et al.*, 2015; Zhang *et al*., 2016). Additionally, compared to conventional composting method, vermicomposting was confirmed to be more effective in alleviating toxic chemicals (antibiotics and heavy metal) through the joint actions of earthworms and microorganisms (Domínguez *et al*., 2017; Bhat *et al*., 2018; Mougin *et al*., 2013; Ravindran and Mnkeni, 2017; Cao *et al*., 2018). Recently, preliminary studies have

shown that vermicomposting could effectively reduce the tetracycline and quinolone resistance genes in the domestic excess sludge, by affecting the possible host bacteria that encode these genes and integrase gene (Huang *et al.*, 2018; Cui *et al.*, 2018). However, the underlying mechanisms on how earthworm behaviors such as gut digestion and excretion affect ARGs were still not understood well, because they are considered to play critical roles in vermicomposting process.

Vermicomposting of solid organic wastes mainly consists of gut associated process (GAP) and cast associated process (CAP) (Domínguez *et al.*, 2017). For the GAP, the ingested feed are suffered to intensively complicated biochemical reaction such as razing, digestion, metabolism, which lead to intensive changes in microbial profiles (activity, number and community structure) (Domínguez *et al*., 2017). Horn *et al*. (2003) and Brown *et al*. (2000) found that neutral pH, high moisture and suitable temperature in the earthworm gut favor the growth of some bacteria. Anaerobic environment in the hindgut of earthworm also poses a threat to the survival of some facultative bacteria (Pedersen and Hendriksen, 1993). Huang and Xia (2018) observed that the bacterial activity and diversity was significantly increased when earthworm mucus was added to the excess sludge, which can be attributed to the rich nutrients contained in the mucus that can favor bacterial growth. Consequently, it is reasonable to speculate that gut digestion of earthworm may directly/indirectly affect the fate and behavior of ARGs hosted bacterial cell existed in excess activated sludge. In addition, previous study also confirmed that swine manure vermicomposting via housefly larva could significantly reduce the concentration of various antibiotic residuals and their resistance genes. The study concluded the worm intestinal bacterial community played a critical role in attenuating the resistance genes by means of high-throughput sequencing technique coupled with network analysis (Wang *et al*., 2017).

ARGs disseminate between different bacteria when mediated to mobile gene elements such as plasmids, integron and transposon, through horizontal transfer methods (conjugation, transformation and transduction). For transformation process, extracellular DNA carrying

ARGs directly enters competent bacterial cells (*Neisseria*, *Bacillus*, *Streptococcus*, and *Haemophilus*) in the environment (Lorenz and Wackernagel, 1994). Nagler *et al*. (2018) observed that extracellular DNA not only maintains the structural stability of biofilms, but also promotes the horizontal dissemination of genetic information. Excess activated sludge that has different properties from livestock manure comprises a large number of viable bacteria and dead bacteria in decline phase. DNA released from dead bacteria can be adsorbed on sludge particles, and still has potential to transform between different bacteria. Therefore, different forms of ARGs in excess activated sludge would show different behaviors under stress of gut digestion.

The objective of this study is to investigate the response of gut digestion of earthworm to ARGs in excess activated sludge and the relationship between ARGs and bacterial community. Cell-associated resistance genes and integrase gene were quantified by using fluorescence quantitative PCR after propidium monoazide (PMA) pretreatment. Bacterial community composition was analyzed by using high throughput sequencing. Pearson correlation analysis was used to clarify the relationship between bacterial community and ARGs.

5.2 Materials and methods

5.2.1 Earthworm and excess sludge

Eisenia fetida species of earthworms purchased from Agricultural Research Co., Ltd, Japan was used for the experiment and feed with domestic dewatered excess sludge in the laboratory for two years. Excess sludge was collected from a domestic sewage treatment plant of Gifu, Japan and was naturally settled for one night in the laboratory. The settled sludge was centrifuged at 3500 rpm for 10 minutes to further reduce the water content of excess sludge. The obtained sludge (hereafter called as initial sludge) was adopted for the earthworms gut digestion experiment.

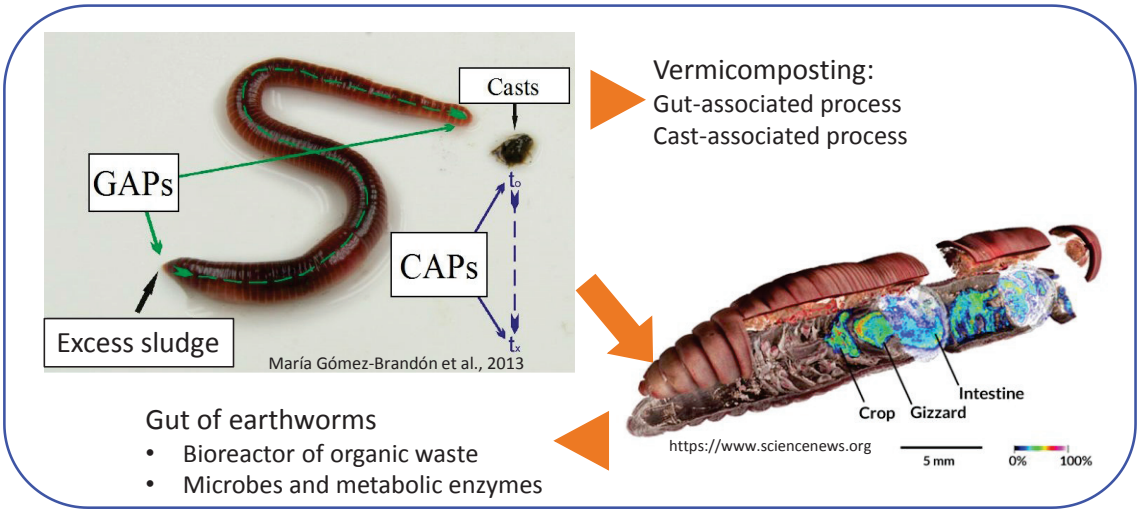

Figure 5.1 Earthworm gut digestion process (Domínguez and Gómez-Brandón, 2012)

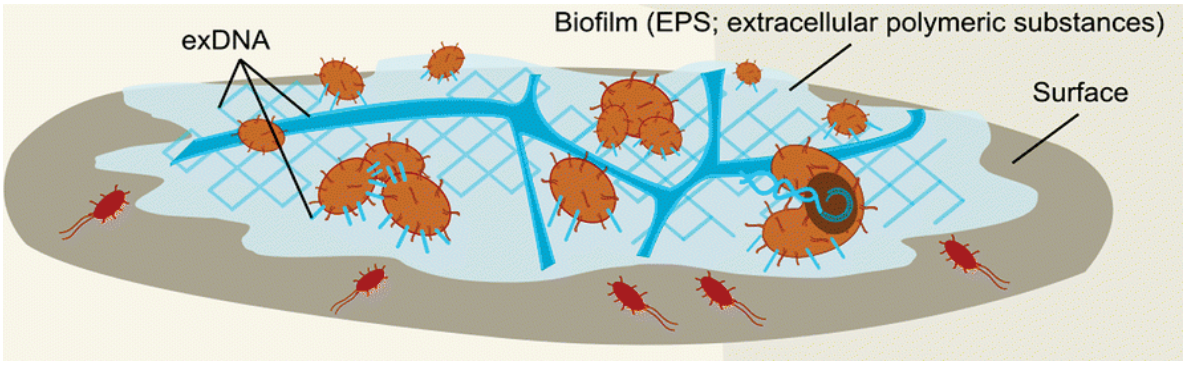

Figure 5.2 Distribution of extracelluar DNA in biofilim (Nagler *et al*., 2018)

5.2.2 Gut digestion process

Before the experiment, the contents in the earthworm gut were firstly emptied to reduce the effects of residual food on the experimental results prior to digestion experiment (**Figure 5.3**). Earthworms were cleaned with ultrapure water. Discharging of earthworms' gut contents was conducted in a petri dish with sterile filter paper for 24 hours. Finally, the gut digestion was started through adding the centrifuged excess sludge and cleaned earthworms in a sterilized petri dish. Such digestion treatment was conducted in triplicate. After digestion for 8 hours, the earthworms were taken out and emptied for 8 hours in a petri dish with sterilized

filter paper, and were returned to the previous excess sludge. The collection of casts (cast2 and cast3) were repeated for 2 times following the protocol mentioned before. The initial sludge (IS) and the three collected casts were subjected to PMA pretreatment-DNA extraction and direct DNA extraction, respectively. PMA pretreatment and extraction DNA were elucitated as follow. The extracted DNA samples were stored at -25°C for the ARGs and bacterial community analysis.

5.2.3 PMA pretreatment and DNA extraction

The PMA pretreatment process is referred to the previous study (Nocker *et al*., 2007; van Frankenhuyzen *et al*., 2011). Sample of 0.0500g was added into 1.5 mL centrifuge tube with 1mL of phosphate buffer solution. The mixture solution was mixed completely with a pipette, and divided into two same parts. One part was used for DNA exctration after centrifuged at 5000 rpm for 5 min. The other one was added with 3 μL PMA with concentration of 25 μM, and placed on ice for 5 min, and then placed in a photolysis apparatus for photolysis reaction for 20 min. The photolysis product is centrifuged for DNA extraction. The entire experimental flow from PMA pretreatment to ARGs and bacterial analysisis are shown in **Figure 5.4**.

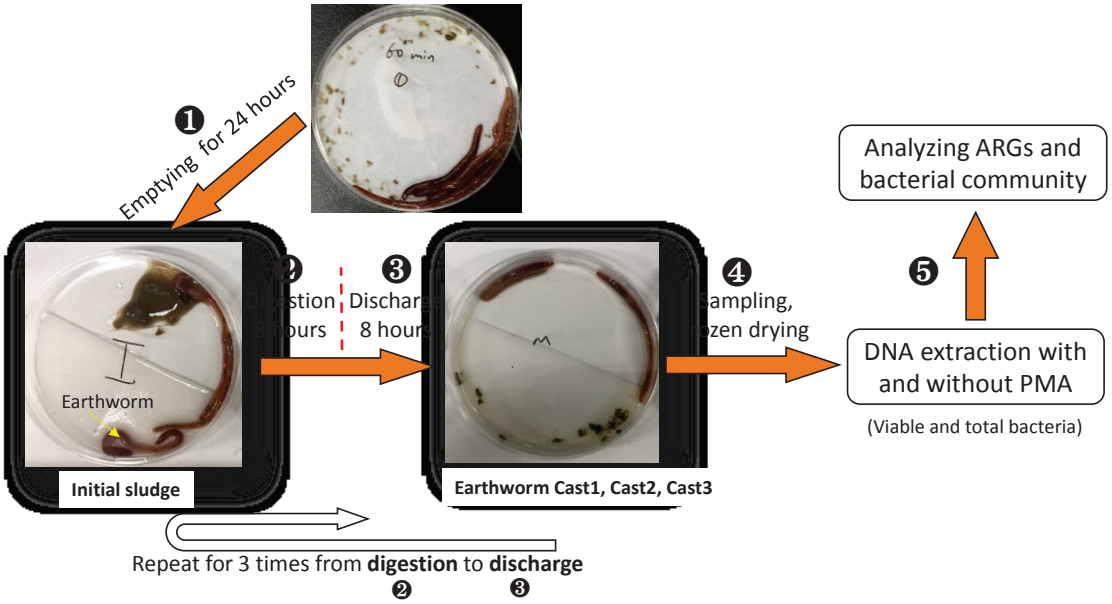

Figure 5.3 Experimental flow for the gut digestion of earthworm

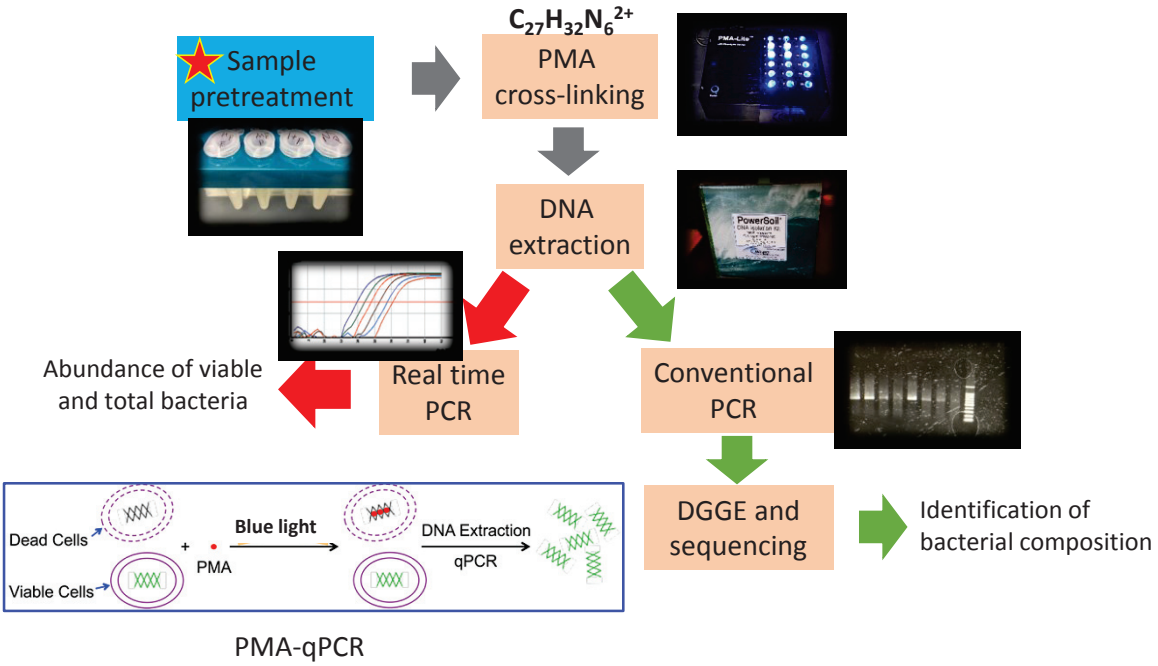

Figure 5.4 Experimental flow from PMA pretreatment to ARGs and bacterial analysisis

5.2.4 High-throughput sequencing of bacterial community

Bacterial composition of sample was analyzed by using high-throughput sequencing technology with the primer pair of 341F-806R referring to a previous report by Huang *et al.* (2018).

5.2.5 ARGs and *intI*1 gene

Methods for determining the abundance of the resistance genes and integrase gene are explained in Chapter 4.

5.2.6 Data analysis

Principal component analysis (PCA) of ARGs and integrase gene before and after digestion was performed by using Canoco 5 software (Wageningen University & Research,

Netherlands). Sequence analysis were carried out by using Uparse software (http://drive5.com/uparse/), and representative sequences were submitted for the annotation of taxonomic information with Silva database (https://www.arb-silva.de/). Alpha diversity indices including Observed-species, Chao1, Shannon, Simpson and ACE, were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3). Beta diversity on weighted unifrac was calculated by QIIME software (Version 1.7.0). PCA of bacterial community was conducted using the FactoMineR package and ggplot2 package in R software (Version 2.15.3). Spearman correlation between bacterial community and ARGs and *intl*1 gene was evaluated and the significance was tested by corr.test in the psych package from R software.

5.3 Results and Discussion

5.3.1 Abundance of ARGs and *intl*1 gene before and after gut digestion

To gain insight into the efficiency of the antibiotic resistance genes and *intl*1 gene (hereafter called as target genes) and their potential host bacteria through earthworm gut digestion, the absolute and relative abundances of the target genes in the excess activated sludge before and after earthworm gut digestion were examined, as shown in **Figure 5.5**. For the initial sludge, the cell-associated and total target genes were respectively detected, and their difference in abundance depended on the types of the tested genes. The genes with the high abundance included *intl*1, *tet*X and *tet*G with 10 orders of magnitude, followed by *sul*1 with 9 orders of magnitude and finally tetM, *qnr*A and *qnr*S genes with 5-6 orders of magnitude. The significant difference in gene abundance was associated with the discrepancy in types and used frequency of antibiotics. Xu *et al*. (2015) and Liu *et al*. (2017) detected abundant tetracycline resistance genes in domestic excess activated sludge due to the selective pressure from the use of large quantities of tetracycline in clinic and livestock and poultry breeding. The property of activated sludge totally differed from that of other substrates used

for vermicomposting like livestock manures. Activated sludge primarily consists of abundant and diverse microbes, whereas livestock manures are rich in abiotic organics such as cellulose and lignin. Therefore, activated sludge was readily enriched ARGs as compared to manures even though the latter contained large amount of antibiotics. It should be not neglected that the total genes in the initial sludge jointly comprised cell-associated and cell-free genes, and their ratios greatly varied with the types of used genes in absolute or relative abundance. The finding indicated that a high potential of horizontal gene transfer of the resistance genes still existed in the excess activated sludge because cell-free resistance genes are proved to exert a crucial role in disseminating genetic information (Nagler *et al*., 2018).

The response of the cell-associated and total genes in the activated sludge to earthworm gut digestion was decreased at different degrees (**Figure 5.5**), with the reduced rates of *qnr*S *tet*G*, tet*X and genes exceeding 90%, and *qnrA*, *sul1* and *tetM* genes ranging from 31-73% (**Figure S12**). It indicates that earthworm gut digestion exhibited differential performances in attenuating these resistance genes of excess activated sludge, which is similar to the result of excess sludge vermicomposting performed by Huang *et al*. (2018) and Cui *et al*., (2018). Early reports have pointed out that these *tet* genes had different antibiotic resistance mechanisms including efflux pump (*tet*G), enzyme modification (*tet*X) and ribosome protection (*tet*M) (Roberts, 2005), which was likely responsible for the different fate of these genes. Earthworm gut is regarded as a bioreactor, where the ingested substrates were suffered a remarkable variations in microbial profiles and physicochemical properties (Gómez-Brandón *et al*., 2011; Domínguez and Gómez-Brandón, 2012), thus probably leading to the changes of resistance genes harbored by microorganisms. On the other hand, anaerobic environment in the intestine of earthworm makes it difficult for many aerobic bacteria in the excess sludge to survive. Although earthworm's gut digestion exhibited excellent attenuations in most of resistance genes, potential environmental risk caused by these resistance genes in the cast should not be underestimated due to considerably high abundance of these resistance genes in the initial sludge.

The relative abundance of ARGs could descript the ratio of ARGs to the total bacterial gene, therefore their variations are probably correlated with the changes of host bacteria harboring these resistance genes or horizontal gene transfer element like *intl* gene. Compared to the initial sludge, the casts exhibited lower relative abundances of the total genes *qnr*S, *tet*G and *tet*X ($p < 0.05$), which was identical to the pattern of corresponding absolute abundances. The result reveals that the essential reductions of the resistance genes in the excess activated sludge were achieved via earthworm gut digestion and. However, other genes (*qnr*A, *sul*1 and *tet*M) were increased or remained no changes in relative abundance after earthworm gut digestion. The collective results suggest that earthworm gut digestion performed the selective effects on the potential host bacteria carrying these resistance genes. It may be ascribed into the specific gut environment, where some bacteria rapidly proliferate due to the simulation of mucus that contains abundant and readily degradable nutrients, while other bacteria were significantly eliminated because of earthworms' predation and deficiency of oxygen in the gut. Furthermore, the equivalent reductions or increments of almost all resistance genes (except *sul*1) harbored in total and viable bacterial cells were observed, indicating that earthworm gut digestion had analogous influences on the hosts of most of cell-free and cell-associated resistance genes. It is worthy to note that the total *sul*1 gene was increased in the relative abundance after gut digestion, whereas the cell-associated *sul*1 gene was decreased, which indicated that the cell-free *sul*1 gene was more difficult to be removed compared with the cell-associated *sul*1 gene through earthworm gut digestion, hence probably leading to a higher horizontal transfer potential of these resistance genes.

As displayed in **Figure 5.5**, the occurrence of considerable amount of *intl*1 gene in the excess activated sludge provided a high possibility of horizontal transfer of the resistance genes between different bacteria. After earthworm gut digestion, *intl*1 gene in the absolute abundance was significantly decreased by 83.3% and 82.1%, respectively, and its relative abundance also showed similar patterns. It is apparent that earthworm gut digestion can significantly eliminate the integron as a horizontal gene transfer element existed in the excess

activated sludge, which is basically in agreement with the previous studies in terms of the effect of vermicomposting on integrase gene in the excess sludge (Cui *et al*., 2018; Huang *et al*., 2018).

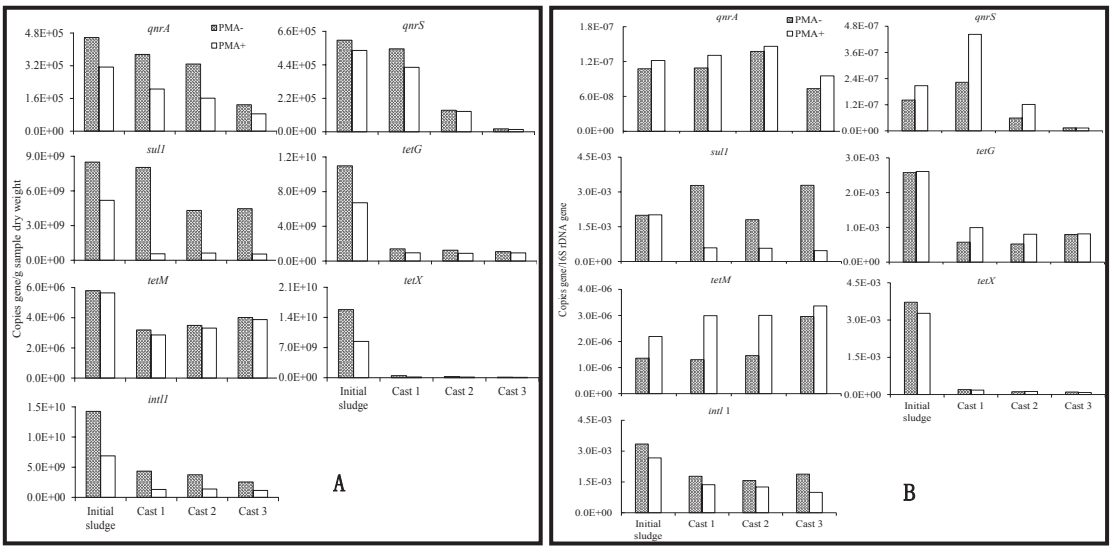

Figure 5.5 Absolute (A) and relative (B) abundances of ARGs and gene before and after earthworm gut digestion of excess activated sludge. PMA+ and PMA- represent the samples treated with and without PMA.

5.3.2 Correlation analysis of the target genes

Principal component analysis (PCA) of the target genes was constructed to better understand the effect of earthworm gut digestion on these genes. As presented in **Figure 5.6**. Positive correlations between the *intl*1 gene and all resistance genes ($p < 0.05$) were observed, implying that integron plays an important role in the horizontal transfer of these resistance genes. Although all casts treated with and without PMA were not clustered together, most of them located in the second and third quadrants. The result indirectly elucidated that earthworm gut digestion could significantly attenuate the abundance of the target genes in the excess activated sludge. Moreover, the PMA-treated casts were in the lower left side of the

casts without PMA treatment, which further implies that the reduction of the extracellular target genes during earthworm gut digestion process had a critical contribution to the reduction of the total target genes. The reduced was the result of the combined effects including physical grinding, chemical digestion, and biological digestion in earthworm gut.

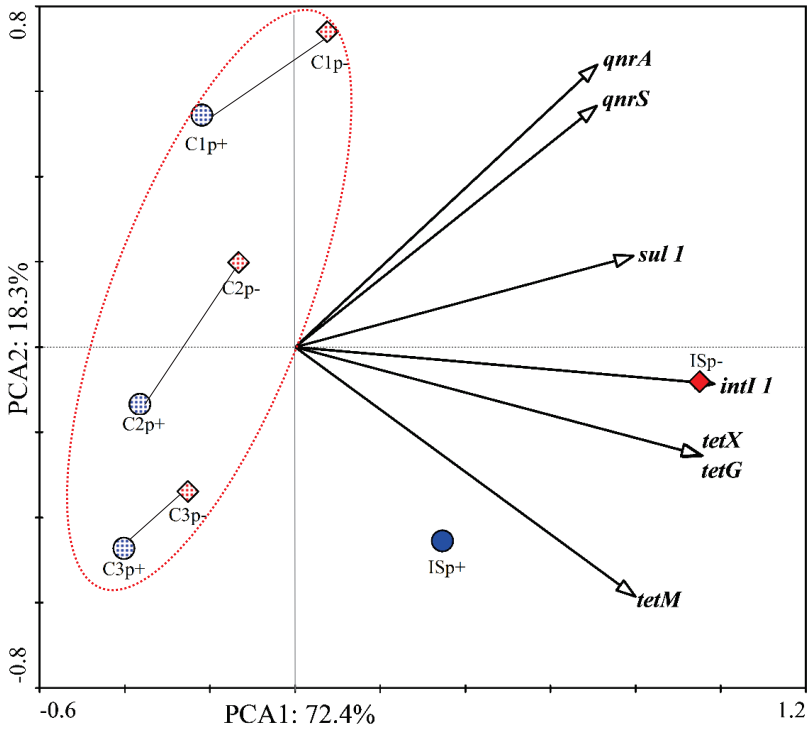

Figure 5.6 Principal component analysis (PCA) of relationship among 3 types of ARGs during earthworm gut passage digestion of excess sludge. IS, C1, C2 and C3 indicate initial sludge, cast 1, cast 2 and cast 3, respectively. p+ and p- indicate samples were pretreated with and without PMA, respectively.

5.3.3 Bacterial community composition before and after gut digestion

Due to the complexity of micro-environment of earthworm gut, high throughput sequencing was applied to clarify the dynamic of bacterial community before and after earthworm gut digestion of excess activated sludge. OTU in rarefaction curve (**Figure S13**) based on the bioinformatics analysis, maintained stable with sequences number, implying that

sequencing analysis of present study was reasonable. Venn graph was constructed to the distribution of OTU from different samples. As shown in **Figure 5.7**, approximately 33% (179/592, 199/562, 180/562) of OTU numbers in the initial sludge with PMA treatment was shared with the responding cast samples accounting for approximately 87% (179/204, 199/228 and 180/209) of each OTU number. In other words, most of viable bacteria in the cast originated from the initial sludge. However, the OTU number in the cast treated with PMA accounted for approximately 54% of OTU number in the cast treated without PMA, and 75.7% of the latter OTU number originated from the initial sludge treated with PMA. The result suggests that the almost half of bacteria in the cast was dead bacteria and probably stemmed from the viable bacteria in the initial sludge. Therefore, it reveals that earthworm gut digestion significantly sharped the ratio of viable to total bacterial community in the excess activated sludge. Large amount of dead bacteria existed in vermicompost likely rise the environmental risk of dissemination of resistance genes by means of transformation when applied as bio-fertilizer in agriculture.

Table 1 Bacterial community richness and diversity before and after earthworm gut digestion
Note: IS, C1, C2 and C3 indicate initial sludge, cast 1, cast 2 and cast 3, respectively. P indicates samples were pretreated with PMA.

Sample	IS	IS.P	C1	C1.P	C2	C2.P	C3	C3.P
Chao1	665	610	404	261	419	303	400	291
Observed species	610	562	368	204	378	228	364	209
Shannon	6.21	6.19	5.49	4.01	5.67	4.30	5.41	3.95

Alpha community diversity was greatly affected by earthworm gut digestion, as shown in **Table 1**. Parameters like observed species and Chao 1 which characterize the community richness in an environmental system showed significant reduction in the casts compared with

the initial sludge regardless of whether the samples was pretreated with PMA or not. Similarly, Shannon diversity index also decreased from 6.21 for the initial sludge to 5.52 for the cast. The decreased community richness and diversity due to earthworm gut digestion was consistent with the previous findings through vermicomposting of activated sludge (Fu *et al*., 2015; Huang *et al*., 2018; Lv *et al*., 2018). An important reason regarding the decreased alpha community diversity is that the anaerobic micro-environment in earthworm gut (Wüst *et al*, 2009), does not favor the growth and reproduction of the predominant aerobic/facultative bacteria habiting in the activated sludge. Additionally, in earthworm gut, abundant and diverse digestive enzymes (β-glucosidase protease, urease, phosphatase and cellulose) (Wüst *et al*., 2009), can promote the decomposition of dead bacteria from the activated sludge, thereby probably leading to the poor amount and diversity of viable bacteria in the cast. Although earthworm gut mucus that contains large amount of readily degradable organic carbon (monosaccharides, low-molecular-weight amino acid, and glycoproteins, enables to improve the proliferation of indigenous bacteria habited in the activated sludge, the mucus effect appears to be incapable of resisting the decreased trends of the bacterial richness and diversity in the excess activated sludge.

Principal component analysis (PCA) of beta diversity was employed to comprehensively evaluate the effect of earthworm gut digestion on the bacterial community. As presented in **Figure 5.8**, the first and second principal components explained 22.92% and 49.84% of the selected variances, respectively. The distance between the initial sludge samples treated with and without PMA was relatively closer, which suggests that the initial sludge mainly consisted of viable bacteria community. The cast samples treated with and without PMA were located in the second and fourth quadrants of PCA plot, respectively. It indicated that the abundant dead bacteria was contained in the cast. Additionally, regardless of whether the samples were pretreated with PMA or not, three cast samples were clustered into one group, suggesting that the possibility of the existence of sampling error caused by the instability of

microenvironment in earthworm gut could be excluded and that the rationality of the experimental sampling was also confirmed.

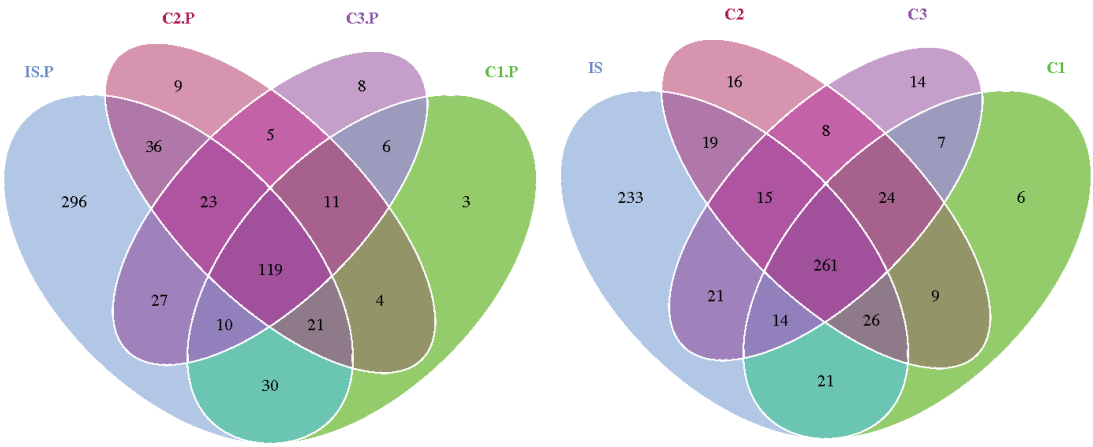

Figure 5.7 Venn graph of OTU (operation taxonomy unit) number for the initial sludge and cast. IS, C1, C2 and C3 indicate initial sludge, cast 1, cast 2 and cast 3, respectively. P indicates samples were pretreated with PMA.

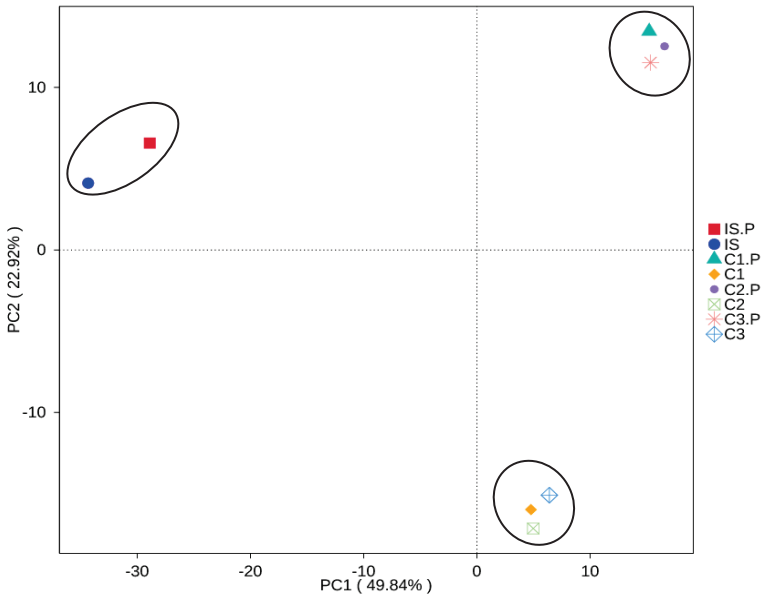

Figure 5.8 Principal component analysis of beta diversity of samples before and after earthworm gut digestion of sludge. IS, C1, C2 and C3 indicate initial sludge, cast 1, cast 2 and cast 3, respectively. P indicates that samples were pretreated with PMA.

The dominant bacterial community composition (phylum level) before and after earthworm gut digestion of activated sludge included Firmicutes, Proteobacteria, Actinobacteria and Bacteroidetes, which was similar to the results of previous studies (Guo *et al*., 2017; Huang *et al*. 2018). As shown in **Figure 5.9**, the relative abundance of Firmicutes in the casts was significantly enriched relative to the initial sludge, especially for the viable bacteria. Many members of Firmicutes belong to intestinal bacteria, which may be a possible reason responsible for their enrichment in the casts. Additionally, the relative abundance of total Actinomycetes increased after earthworm gut digestion. Similarly, other researchers observed the high abundance of Actinomycetes after vermicomposting of organic wastes (Paul *et al*., 2011; Singh and Suthar, 2012; Negi and Suthar, 2013; Devi and Prakash, 2015). Neutral pH, high moisture and ideal temperature conditions in the gut of *Eisenia foetida* provide excellent conditions for the growth of Actinomycetes (Jyotsna *et al*., 2010). The abundant Actinomycetes was generally considered to be an important indicator of matured compost (Xiao *et al*., 2011). Although relative abundances of Actinomycetes was significantly increased after activated sludge passing through earthworm gut, it was very low in the casts treated by PMA. The finding indicated that earthworm gut digestion exerted a significant inhibitory effect on the viable Actinomycetes, which was probably associated with its feeding effect (Yuan *et al*., 2017) and the strong competition of microbes involved in the earthworm gut (Lazcano *et al*., 2008). Additionally, earthworm gut digestion notably decreased the relative abundances of Proteobacteria and Bacteroidetes from 30.6% to 23.3% and 30.6% to 0.25%, respectively. Both phylum play important roles in the degradation of organic pollutants in sewage, and the significant reduction of Bacteroidetes suggested that the amount of degradable organics in the initial sludge was greatly reduced. Wang *et al*. (2017) observed a substantial reduction in the relative abundance of swine manure-borne Bacteroidetes after vermicomposting via housefly larvae.

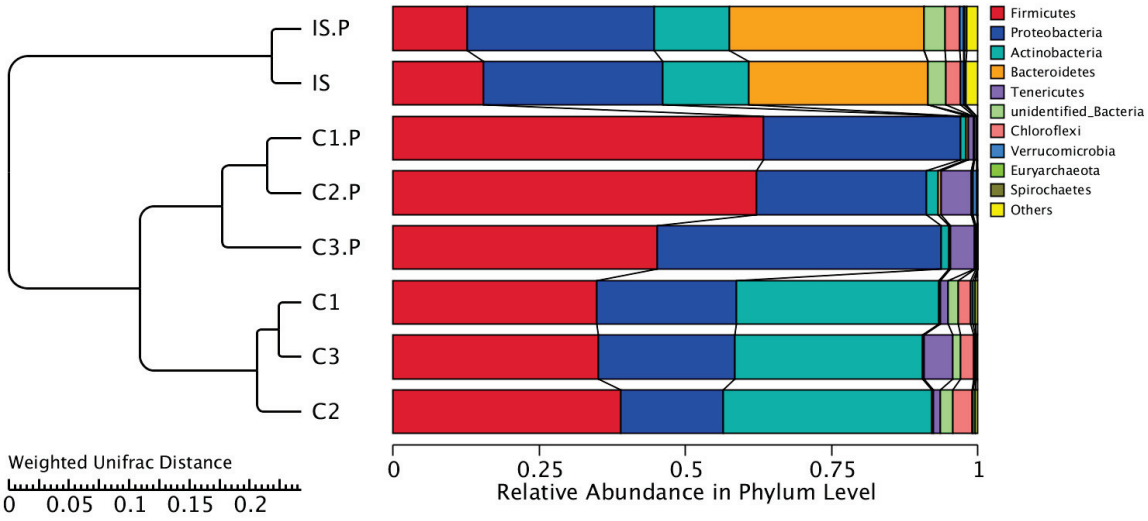

Figure 5.9 Bacterial community composition (phylum level) with cluster analysis. IS, C1, C2 and C3 indicate initial sludge, cast 1, cast 2 and cast 3, respectively. P indicates samples were pretreated with PMA.

5.3.4 Correlation analysis between the target genes and bacterial community

Pearson correlation analysis between target genes and bacteria community was conducted to explore the potential host bacteria of resistance genes in the activated sludge. Almost target genes were observed to remarkably correlate with alpha diversity indexes including the observed species, Shannon, Simpson, Chao1 and ACE (**Figure 10a).** The decreased alpha diversity caused by earthworm gut digestion (**Table 1**) might weaken the possibility of dissemination of resistance genes to the environment through horizontal gene transfer. In fact, microbial profile succession is considered to be a core driver of behavior of antibiotic resistance genes during other biological approaches (thermophilic composting and anaerobic digestion) of livestock manures or excess sludge.

On the other hand, 14 of 35 different classes with highly relative abundance were observed to be positively linked with most of the target genes ($p < 0.05$), as shown in **Figure 10b**. These 13 classes affiliated to 11 phyla including Acidobacteria, Euryarchaeota, Ignavibacteriae, Fibrobacteres, Deinococcus-Thermus, Gemmatimonadetes, Spirochaetes,

Euryarchaeota, Firmicutes, Proteobacteria and Chloroflexi. Although potential host bacteria of the target genes had lower relative abundance, their diversities were considerably abundant, indicating that the risk of dissemination of antibiotics resistant in the excess activated sludge have considerably server. Especially, *Bacilli* and *Deltaproteobacteria* that belong to the phylum of Firmicutes and Proteobacteria, were also confirmed to be potential hosts in other biological reactors of excess sludge. *Bacilli* contains several well-known pathogens such as *Bacillus anthracis* (the cause of anthrax). It implies that earthworm gut digestion not only attenuated the host bacteria of the target genes in the excess sludge, but also reduced the environmental risk of human pathogen. On the other hand, The *Erysipelotrichia* are a subclass of bacteria of the phylum Firmicutes and its species are known to be common in the gut microbiome, likely resulting in the increments of abundance of the target genes in the casts compared with the initial sludge. The *Clostridia* are obligate anaerobes and oxygen is toxic to them. Anaerobic environment in the earthworm gut might contribute to the raised abundance of the target genes found in the casts.

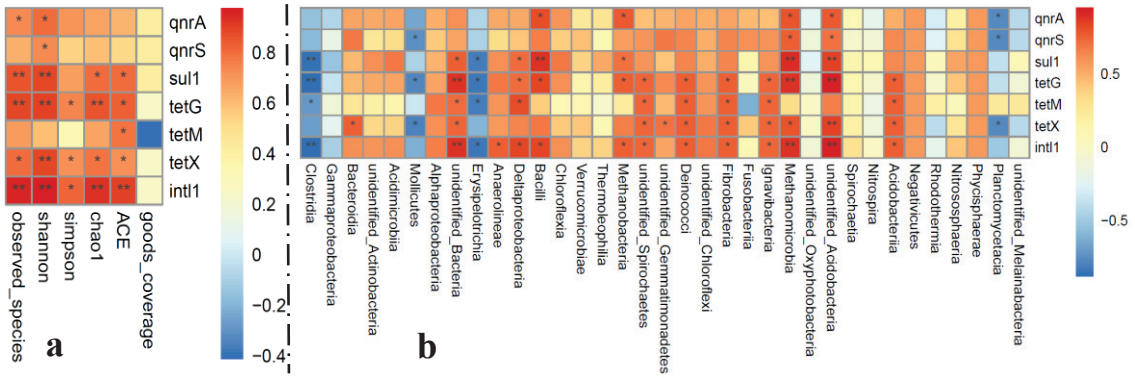

Figure 10 Heat map of Pearson correlation analysis between the target genes (ARGs and *intl1*) and community composition (phylum level) (A) as well as Alpha diversity indexes (B). Asterisks * and ** indicate statistical differences of $p < 0.05$ and $p < 0.01$, respectively. ACE represents abundance-based coverage estimator.

5.4 Summary

This chapter investigated the effect of earthworm gut digestion on the ARGs in excess activated sludge. Significantly reduction in abundance of the resistance genes, however, the reduction rate greatly varied with the tested gene types. The dynamics of cell-free resistance genes had an important contribution to the reduction of the total resistance genes. High throughput sequencing revealed that earthworm gut digestion significantly altered the ratio of viable to total bacterial community in the excess activated sludge. 13 classes affiliated to 11 phyla including Acidobacteria, Euryarchaeota, Ignavibacteriae, Fibrobacteres, Deinococcus-Thermus, Gemmatimonadetes, Spirochaetes, Euryarchaeota, Firmicutes, Proteobacteria and Chloroflexi may be the potential host.

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Chapter 6 Conclusions

This study was performed to investigate the fate and behavior of antibiotic resistance genes and their relation with microbial profiles during vermicomposting of domestic excess sludge. Main conclusions are given as follows.

The effect of earthworm density on ARGs during vermicomposting of excess sludge were studied with reactors introduced with *Eisenia fetida* at three different densities. The results showed that earthworms could significantly reduce the absolute abundance of quinolone resistance genes in the excess sludge, with a reduction ratio of 85.6-100% for *qnr*A and 92.3-95.3% for *qnr*S, respectively. Redundancy analysis revealed clearly that the *qnr* genes had positive correlations with the targeted indexes of microbial profiles, with the correlations with the bacterial abundance and dehydrogenase activity being more statistically significant than the bacterial diversity ($p < 0.05$). The results of this study suggested that earthworms cold promote the attenuation of quinolone resistance genes in the excess sludge through lowering the bacterial abundance and activity, and the promotion effect could be enhanced by increaing the density of earthworms.

The effect of temperature on antibiotic resistance genes during vermicomposting of domestic excess sludge was investigated. Vermicomposting systems for treating excess sludge were established at three different temperatures (15, 20 and 25°C). Domestic excess sludge not only contained abundant antibiotic resistance genes, expecially sulfonamide and tetracycline resistance genes, but also had high horizontal transfer potential for resistance genes. Vermicomposting at 25°C did not show significant reductions in the abundance of resistance genes in comparison to that 15°C and 20°C. NH_4-N, NO_3-N and EC had significantly negative correlations with the *sul*1 and *intl*1 genes. The rebound of resistance genes (*tet*G, *tet*M, *qnr*A and *qnr*S) on day 60 at 25°C was attributed to the emergence of

Aeromonas and *Chitinophagaceae*. *Ferribacterium* was a possible host bacteria of other resistance genes except for *tet*G.

The effect of gut digestion of earthworms on ARGs in domestic excess sludge was investigated. The gut digestion of earthworms significantly reduced the abundance of the target genes, however, the reduction rates varied greatly with the types of resistance genes. Furthermore, the reduction of cell-free resistance genes had an important contribution to the reduction of the total resistance genes. High throughput sequencing revealed that the gut digestion of earthworms significantly altered the characteristics (live and dead) of bacterial community in the excess sludge, especially for alpha diversity, which was closely related to the reduction of the target genes in the sludge after digestion. Although earthworm's gut digestion exhibited excellent attenuations in most of resistance genes, potential environmental risk of these resistance genes in the cast should not be ignored due to their high abundance genes in the initial excess sludge.

From the application viewpoint, further research on other influencing factors such as sludge property, bulking agents and ARGs types should be considered to better understand underlying manchnisms regarding how vermicomposting affects ARGs profiles in excess sludge, and then to reduce the environmental risk of final products as soil conditioner or biogical fertilizer.

Supplymentary materials

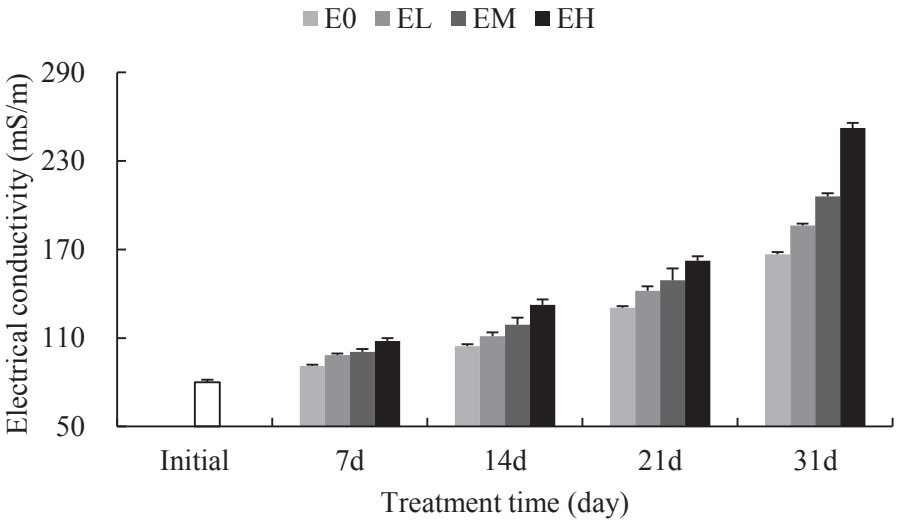

Figure S1 Change of electrical conductivity during vermicomposting at different earthworms' density. E0-the treatment inoculated without earthworm, EL-the treatment inoculated with earthworms at the low density, EM-the treatment inoculated with earthworms at the medium density, EH-the treatment inoculated with earthworms at the high density. Error bar was displayed based on the mean of the triplicates (the same as follows).

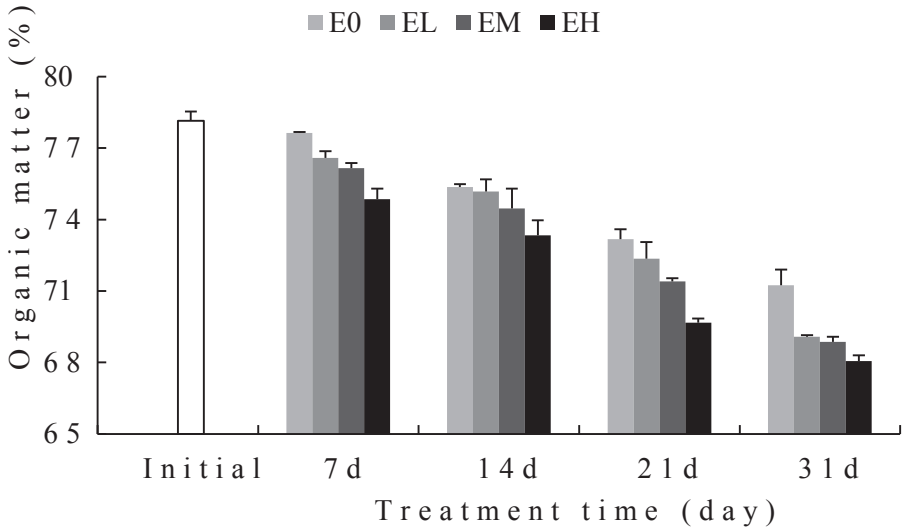

Figure S2 Change of organic matter during vermicomposting at different earthworms' density

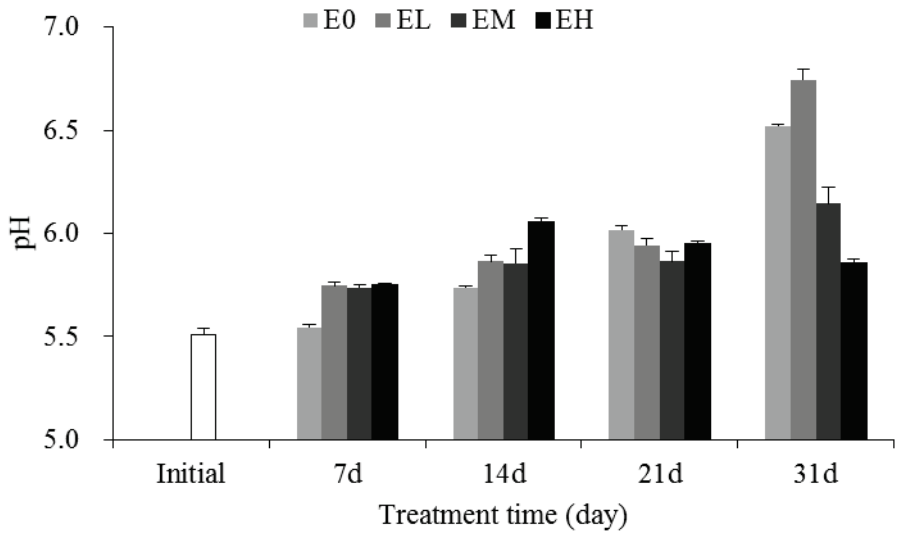

Figure S3 Change of pH during vermicomposting at different earthworms' density

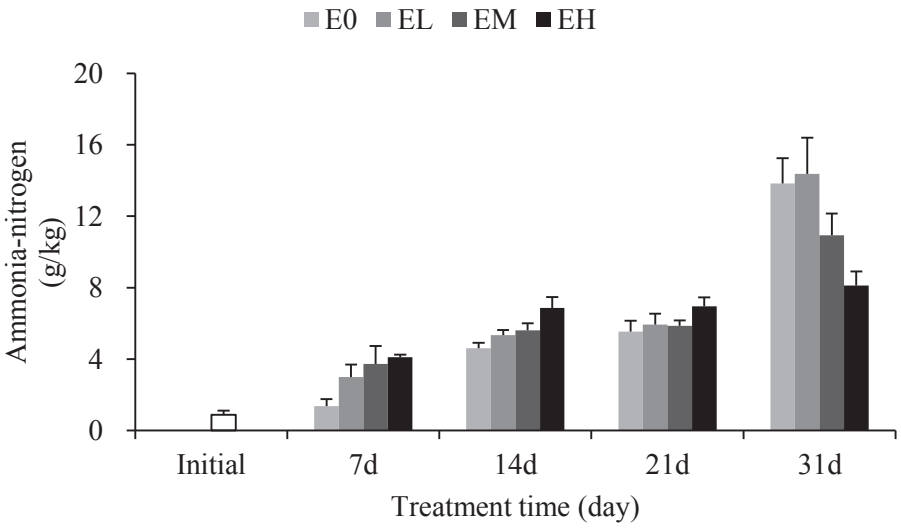

Figure S4 Change of ammonia-nitrogen during vermicomposting at different earthworms' density

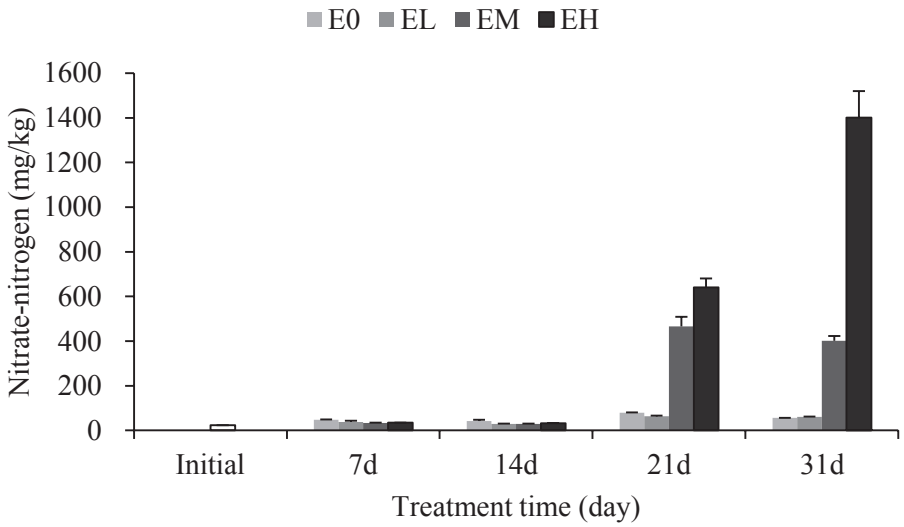

Figure S5 Change of nitrate-nitrogen during vermicomposting at different earthworms' density

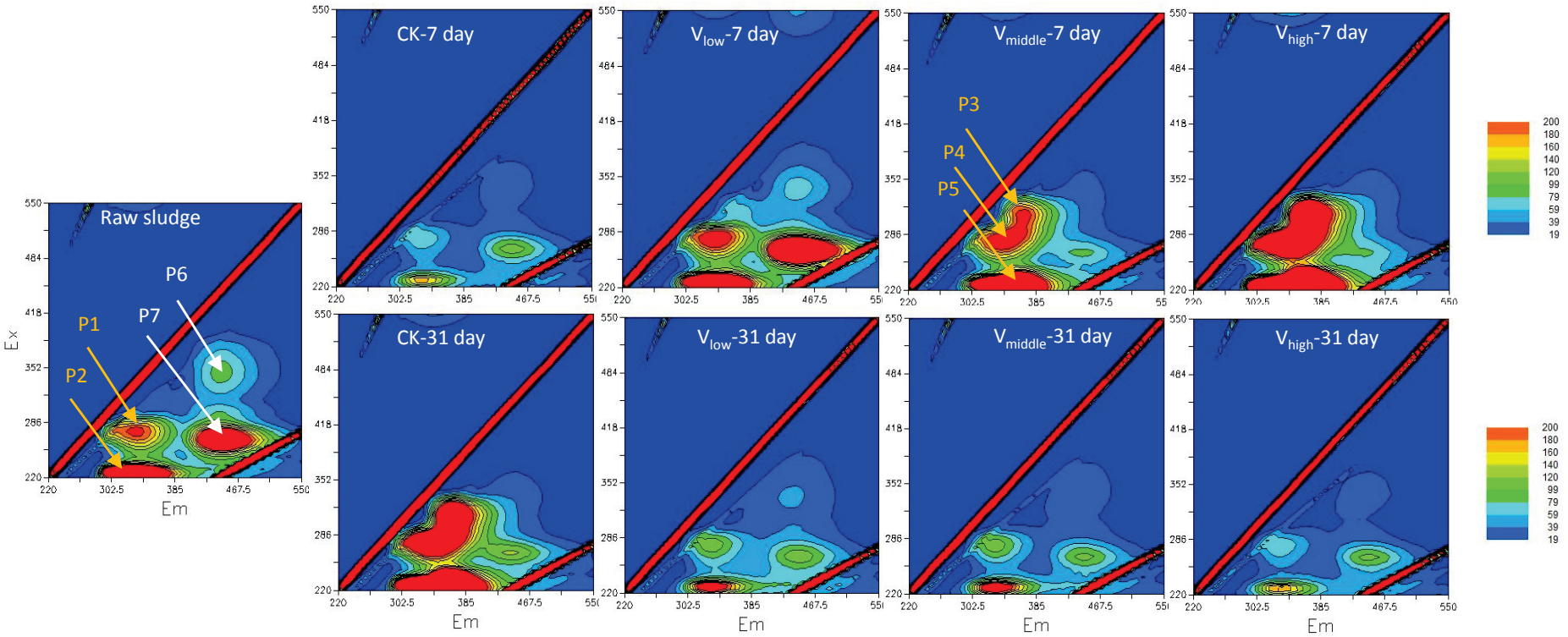

Figure S6 Fluorescence EEM of substances released to water from samples at the beginning day, 7 day and 31 day under different earthworms' density conditions. CK-control; V_{low}-low earthworms' density; V_{middle}-middle earthworms' density; V_{high}-high earthworms' density. P1 (335, 275), P3 (370, 310) and P4 (350, 280) represent soluble microbial product; P2 (330, 250) and P5 (355, 225) represent aromatic proteins; P6 (445, 345) and P7 (445, 265) represent humic acid.

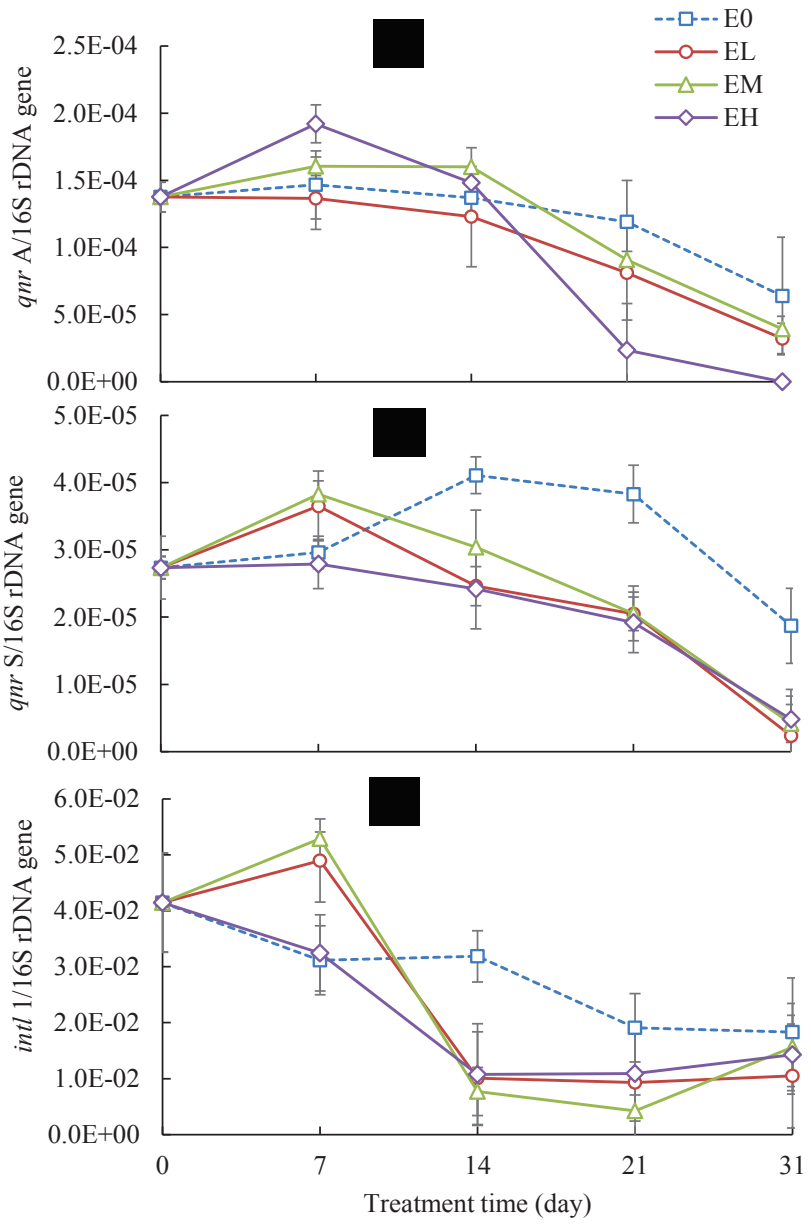

Figure S7 Changes in relative abundance of *qnr*A gene (a), *qnr*S gene (b) and *intl*1 gene (c) during the vermicomposting of excess sludge. Data are represented as mean and standard deviation (n=6). E0-the treatment inoculated without earthworm, EL-the treatment inoculated with earthworms at the low density, EM-the treatment inoculated with earthworms at the medium density, EH-the treatment inoculated with earthworms at the high density.

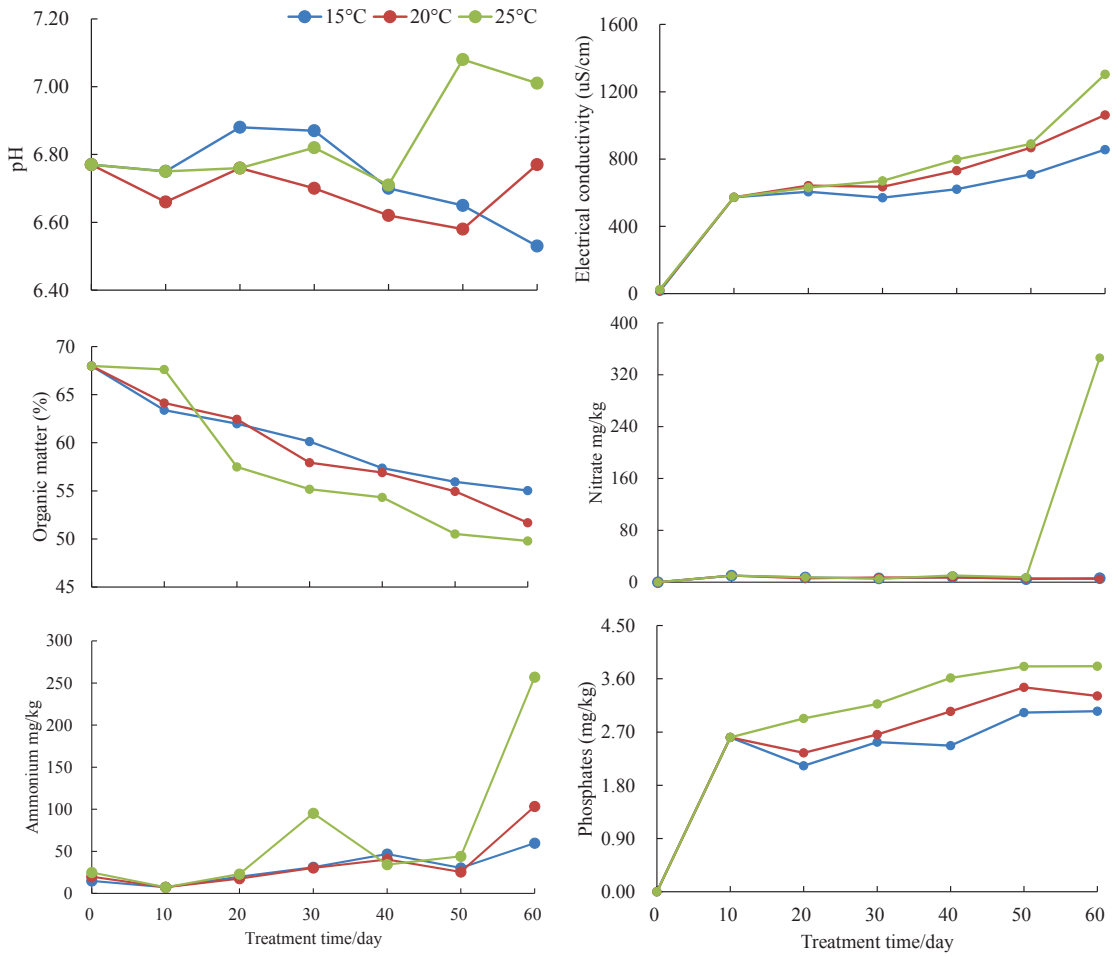

Figure S8 Changes of physicochemical parameters during vermicomposting of municipal excess sludge at three different temperatures

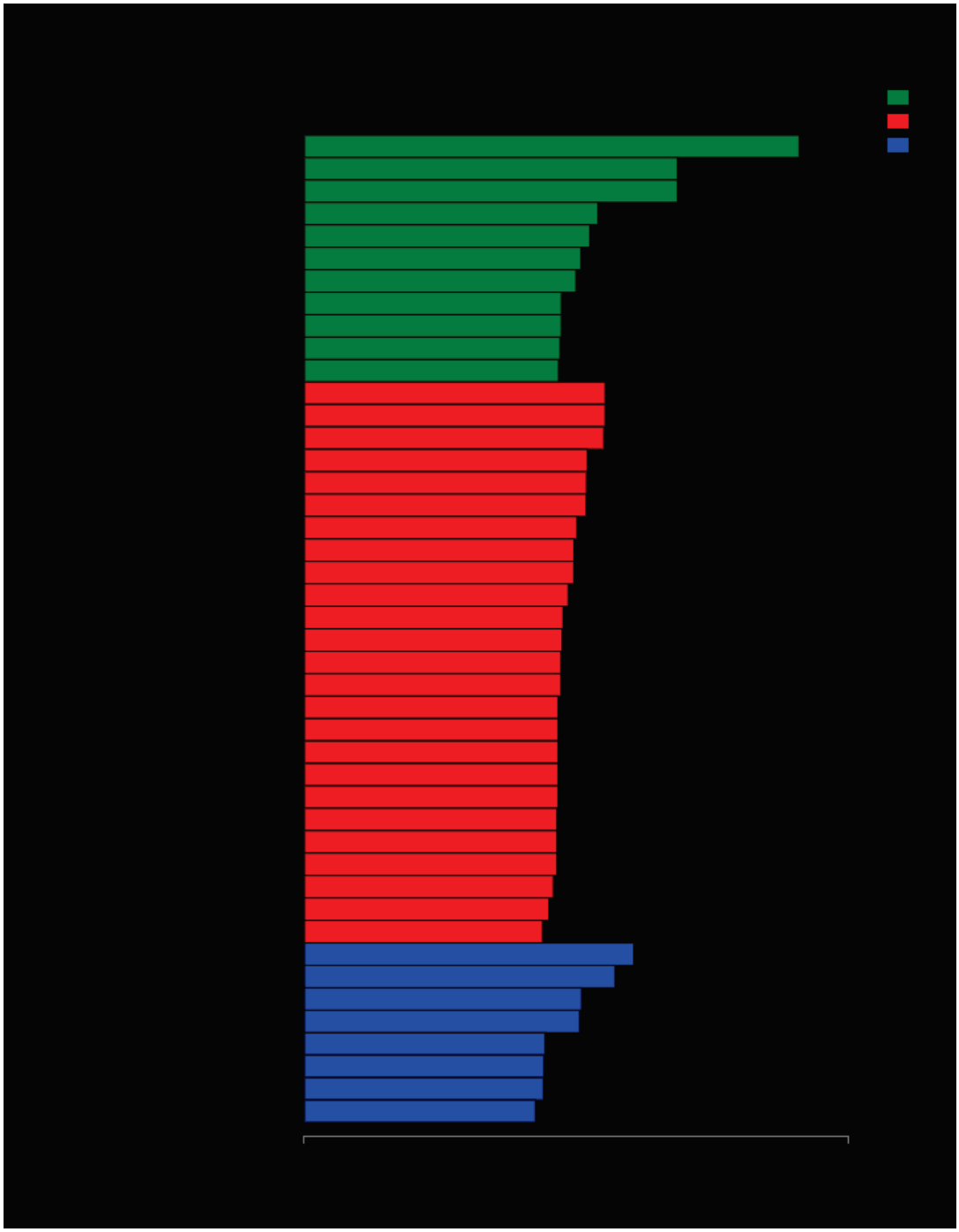

Figure S9 Linear discriminant analysis effect size (LEfSe) for the final vermicomposts at different earthworms' density. L, M and H represent treatments at 15°C, 20°C and 25°C.

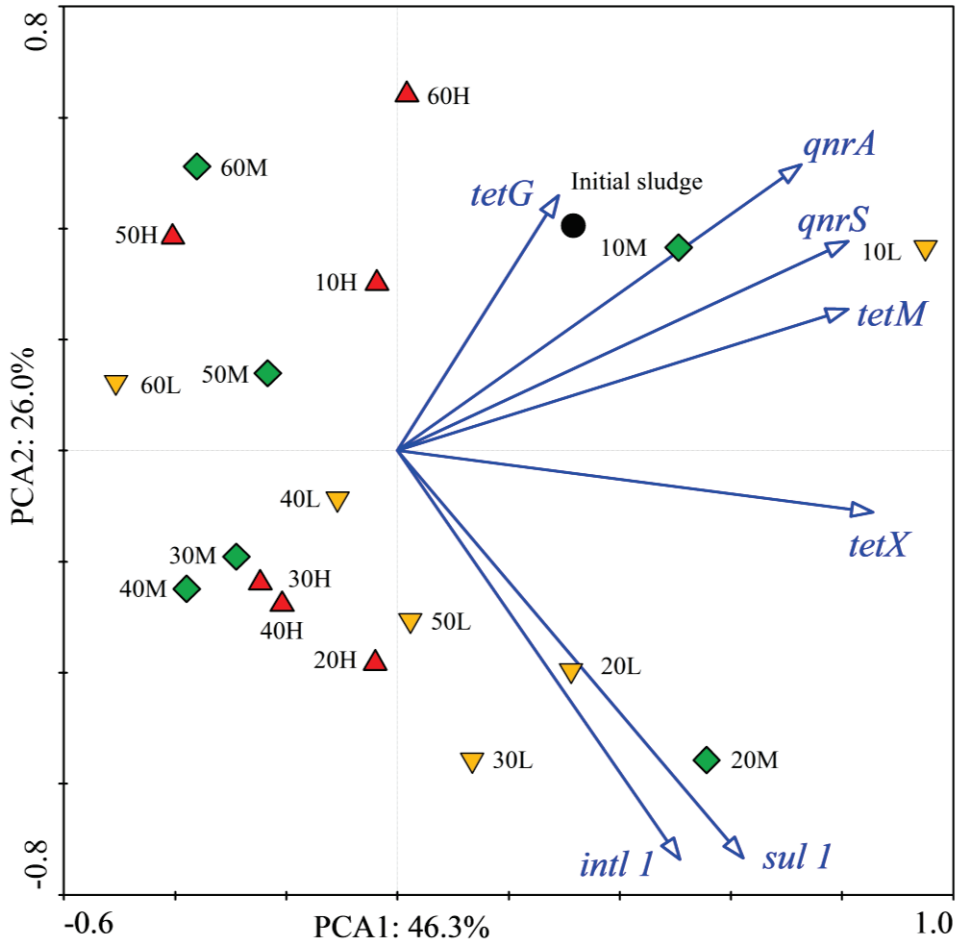

Figure S10 Principal component analysis for ARGs during vermicomposting of excess sludge at different earthworms' density. IS-initial sludge. L, M and H represent treatments at 15°C, 20°C and 25°C. The number represent sampling time (day). The number at the front of L, M and H represent sampling time (day).

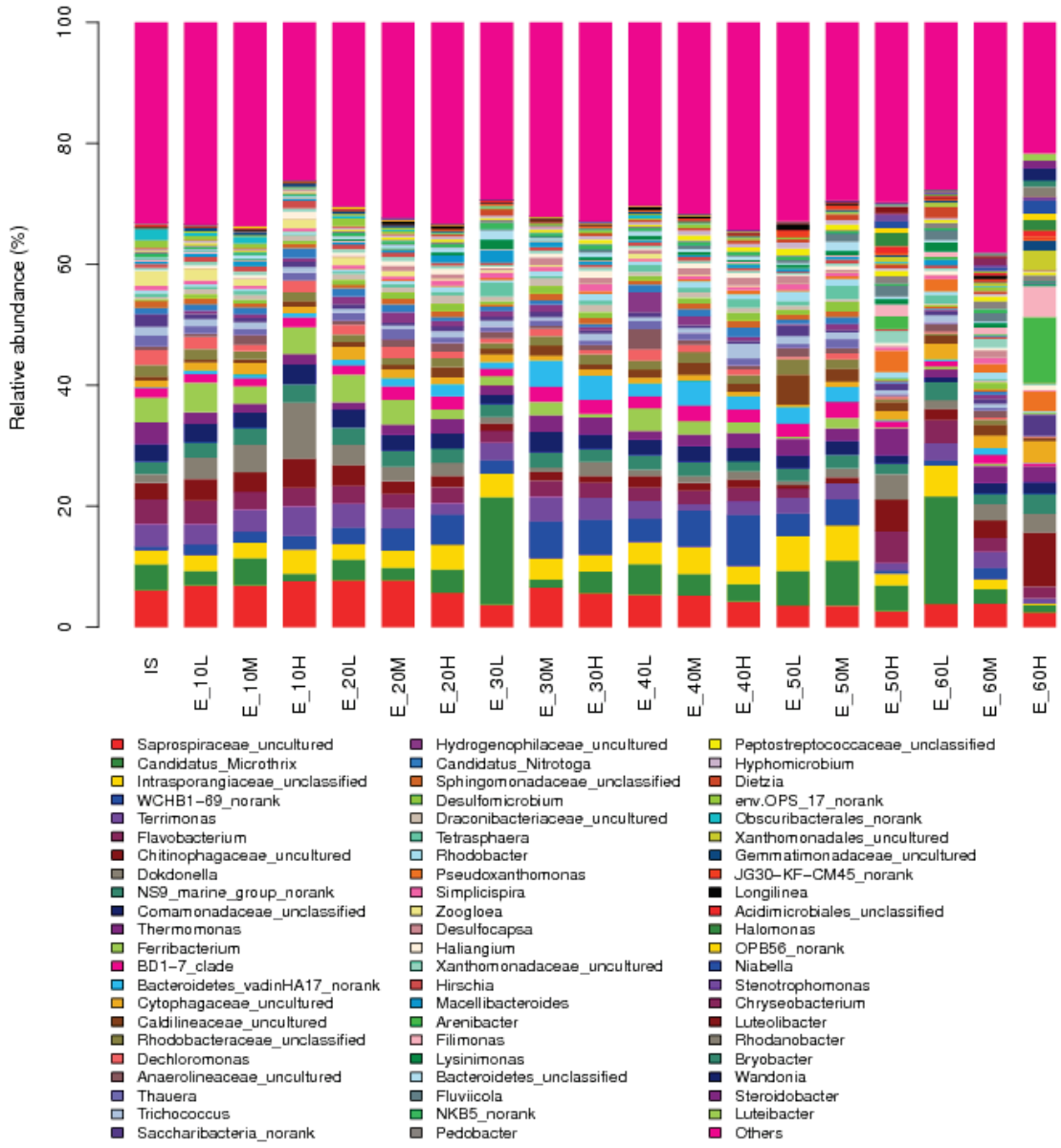

Figure S11 Relative abundance of bacterial composition during vermicomposting of excess sludge at different earthworms' density. IS-initial sludge. L, M and H represent treatments at 15°C, 20°C and 25°C. The number represent sampling time (day). The number at the front of L, M and H represent sampling time (day).

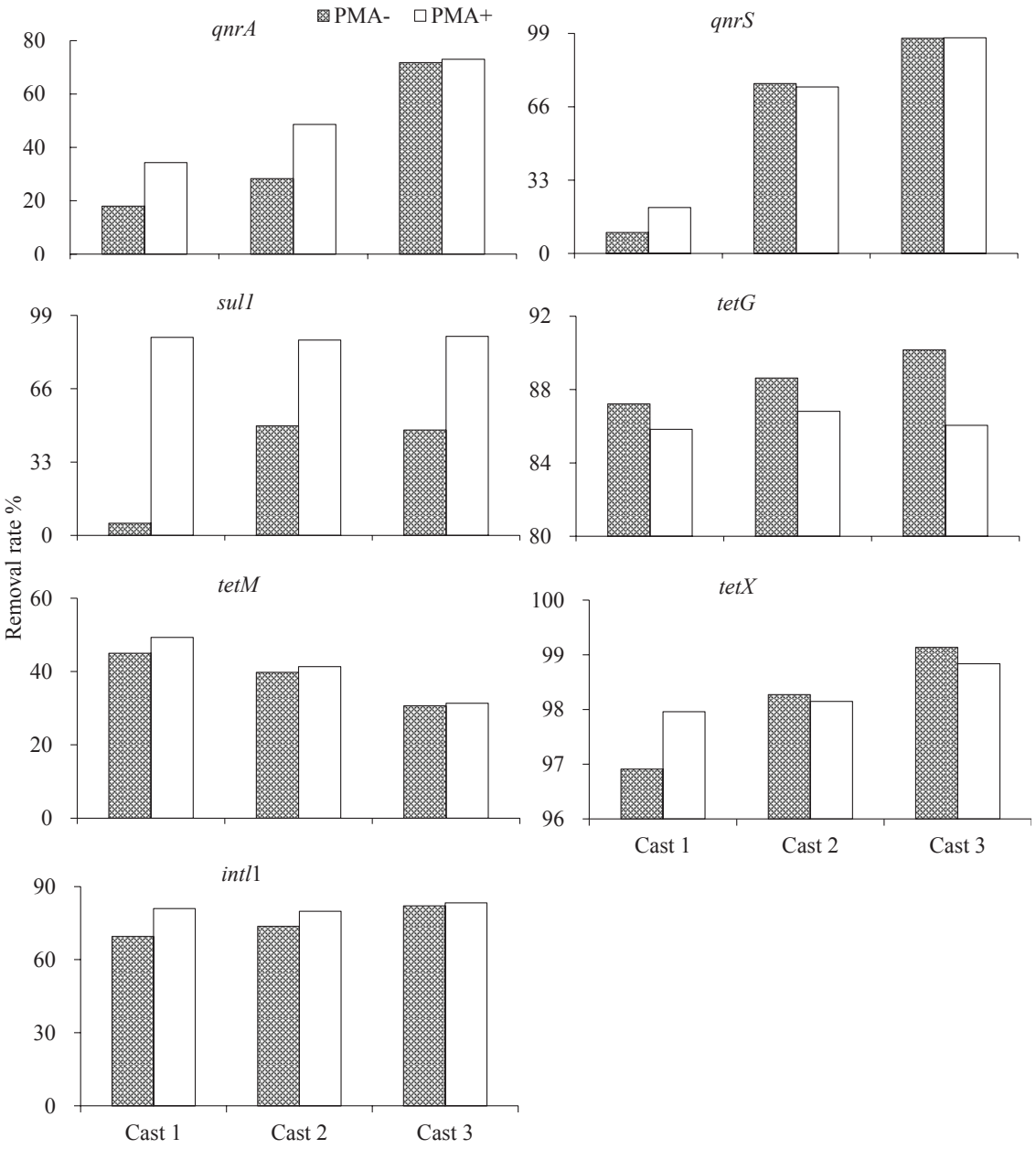

Figure S12 Removal rates of ARGs and *int*1 gene before and after earthworm gut digestion of excess activated sludge. PMA+ and PMA- represent the samples treated without and without PMA, respectively.

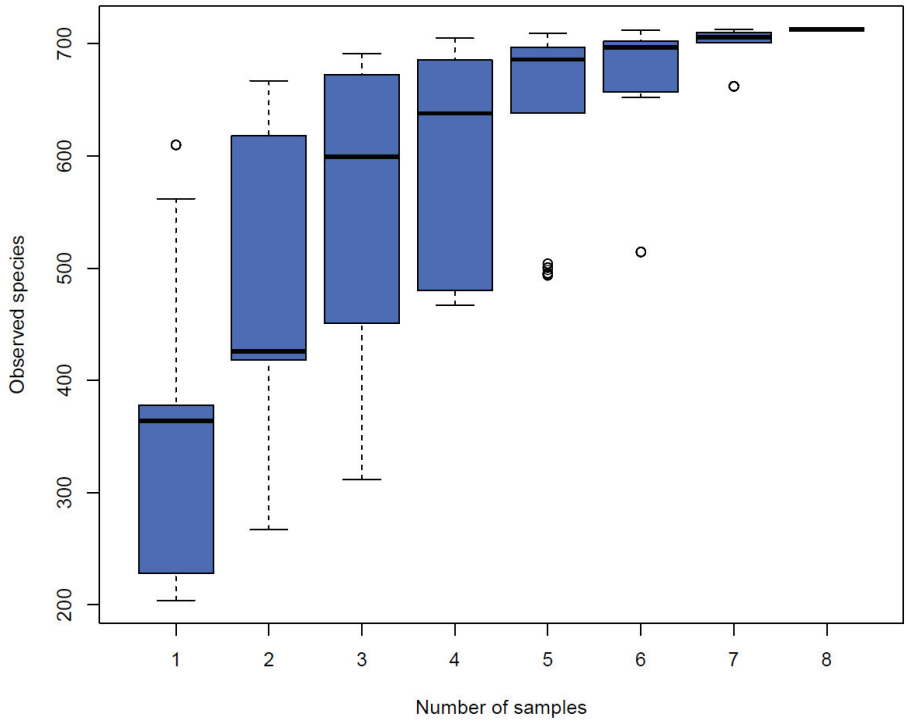

Figure S13 Species accumulation boxplot of samples before and after earthworm gut digestion of activated sludge

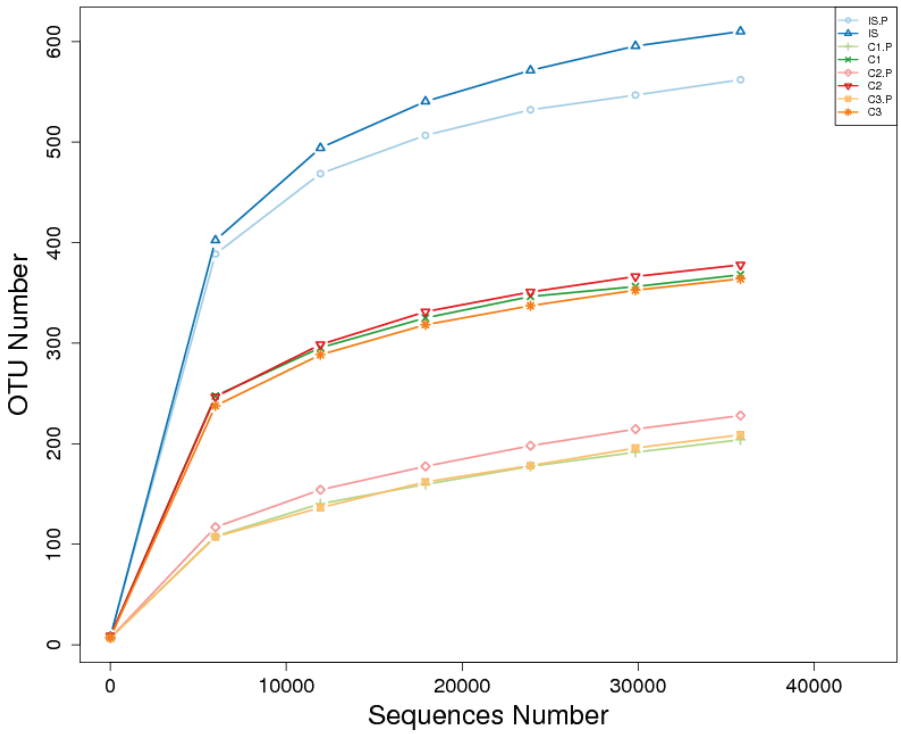

Figure S14 Rarefaction curve of high throughput sequencing for samples before and after earthworm gut digestion of activated sludge. IS, C1, C2 and C3 indicate initial sludge, cast 1, cast 2 and cast 3, respectively. P indicates samples were pretreated with PMA.

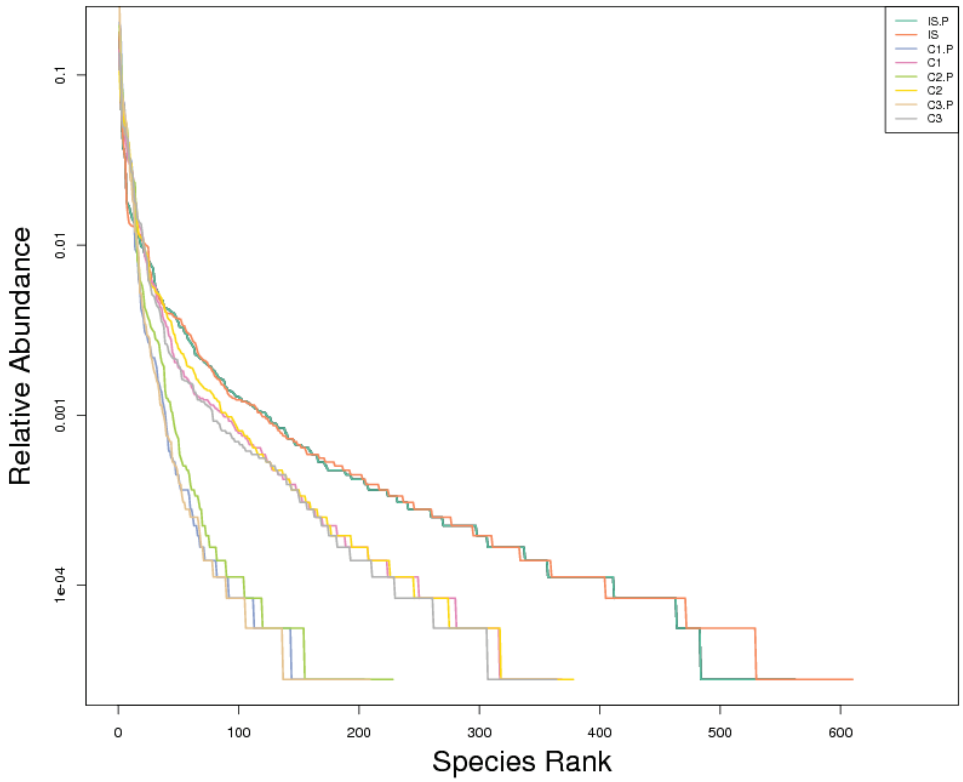

Figure S15 Rank abundance of samples before and after earthworm gut digestion of activated sludge. IS, C1, C2 and C3 indicate initial sludge, cast 1, cast 2 and cast 3, respectively. P indicates samples were pretreated with PMA.

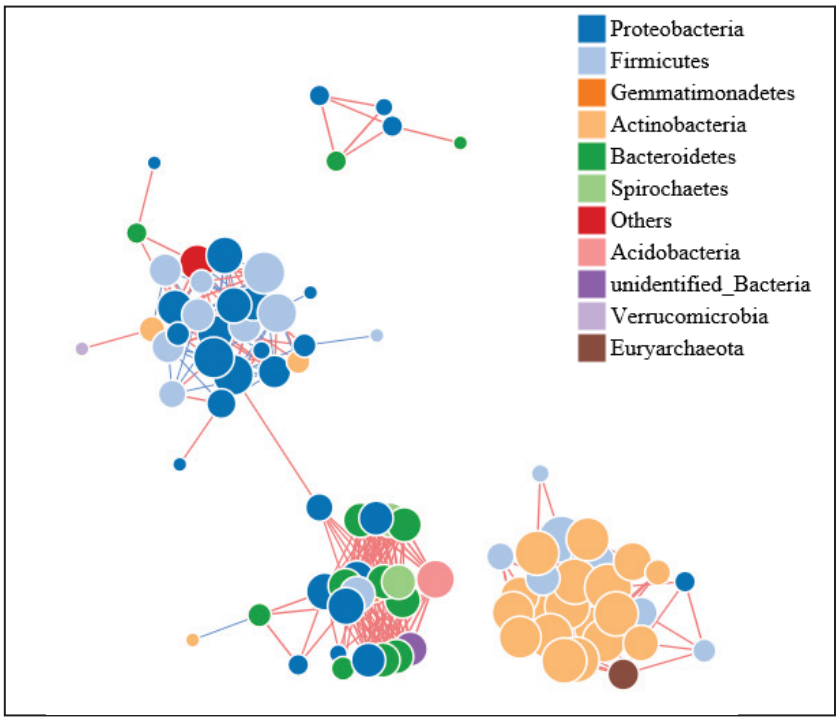

Figure S16 Network analysis of bacterial community (phylum level) during gut digestion of activated sludge

Table S1 Changes of earthworm's biomass before and after the experiment. Values are expressed as average (n=3). EL, EM and EH mean low earthworm density treatment with 40 earthworms/kg wet sludge, medium earthworm density treatment 80 earthworms/kg wet sludge and high earthworm density treatment 120 earthworms/kg wet sludge, respectively. Different letters for the same indictor mean significant difference at the level of $p < 0.05$.

Treatment	Initial earthworm's biomass (g)	Final earthworm's biomass (g)	Growth rate of earthworms (g/day)	Survival rate (%)
EL	0.375	0.684	0.147a	50.0a
EM	0.335	0.778	0.211b	102.5b
EH	0.320	0.740	0.201b	98.3 b