

**Fate and behavior of antibiotic resistance genes
during water and solid waste treatment**

水および固形廃棄物の処理プロセスにおける
抗生物質耐性遺伝子の運命と挙動

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Abstract

Antibiotic resistance genes (ARGs) including extracellular ARGs (eARGs) and intracellular ARGs (iARGs) are concerned worldwide as newly emerging contaminants since their proliferation in environment can bring about serious ecosystem and public health issues. It is estimated that the global deaths caused by antibiotic resistance will be 10 million in 2050 if without any action (World Health Organization, WHO). As the main method for wastewater and solid waste treatment, biological process based on the use of higher concentrations of aerobic and/or anaerobic bacteria possesses the possibility of becoming a new spot for ARGs to spread among different bacterial species, which needs clarification and control. Excess activated sludge (EAS) is a solid waste generated in WWTFs. It has higher recycling value for its rich organic content and nutrients if properly treated. On the other hand, fruit and vegetable waste (FVW) is another category of municipal solid waste that has higher recycle value. Proper treatment for FVW is also necessary due to its larger quantities and the features of easy decaying. Concerning the fate and behavior of ARGs in biological treatment process of EAS and FVW, studies that can contribute to better understanding of the involved mechanisms and the proposal of optimal treatment conditions are necessary. Accordingly, the main objective of this study was to investigate the fate and behavior of ARGs during water and solid waste treatment, hence providing new insights for effective elimination of the proliferation of ARGs in the environment. This study mainly included three parts: the first part was to clarify the fate and behavior of eARGs and iARGs in activated sludge with different settleability; the second part was to design a system for enhancing the biological treatment efficiency of FVW and EAS with the involvement of earthworms

(vermicomposting); and the third one was to clarify the fate and behavior of ARGs from EAS in vermicomposting treatment of FVW and the role of earthworms.

For clarification of the fate and behavior of eARGs and iARGs in sludge with different settleability, sludge samples from the aerobic contact tank of six household WWTFs were fractionated by using a newly designed settling tube into three fractions with different settleability, namely sludge of low settleability, medium settleability and high settleability. The abundances of eARGs and iARGs in the obtained fractions were evaluated by targeting on the widely detected tetracycline resistance genes (*tet G* and *tet M*) and sulfonamide resistance gene (*sul I*) in water environment based on the PMA-*q*PCR method, together with the evaluation for the well-reported mobile genomic element gene (*intl I*) and total bacterial 16S rDNA. The structure of sludge flocs and the distribution of intact and damaged bacterial cells were observed by using a fluorescence microscope. The results indicated that the sludge fraction with lower settleability was mainly consisted of single intact bacterial cells and small sludge flocs formed by more damaged bacterial cells, and possessed higher proportions of eARGs and higher transfer potential judged by the relative abundances of ARGs and *intl I*. For the sludge fraction of higher settleability, flocs with larger sizes were found to contain both intact and damaged bacterial cells, and the relative abundances of ARGs and *intl I* were apparently lower even if the presence percentages of eARGs were comparatively higher. It is thus inferred that sludge fractions of low settleability possesses higher transfer potential for ARGs and that enhancing the settleability of such sludge fractions through optimization of the operation conditions is important for mitigating the proliferation of ARGs in the receiving environment of WWTFs.

To enhance the vermicomposting efficiency of FVW and EAS, a novel vermireactor consisted of a substrate compartment atop a bed compartment was designed and used

to treat different types of FVW (banana peels, cabbage, lettuce, carrot, and potato) with and without the addition of EAS. The higher bacterial density and activity as well as the richer nitrogen and phosphorus of EAS significantly promoted the growth and cocoon production of earthworms in the treatment of FVW. The changes in the dehydrogenase activity of the substrate compartments revealed that the addition of EAS enhanced the microbial activity in all treatments of FVW, except the treatment of carrot. The organic matter content and the total organic carbon showed a significant decrease after adding EAS, proving that the decomposition efficiency of FVW was enhanced through the addition of EAS. The addition of EAS also improved the content of nitrogen and phosphorus in final products accumulated in the bed compartments and significantly lowered the C/N ratio values. The results thus suggested that the addition of EAS could be a feasible approach to enhance the vermicomposting efficiency of FVW using the novel vermireactor.

Following the studies above, vermicomposting treatment of FVW (banana peels, cabbage, lettuce, carrot, and potato) and EAS was conducted for clarification of the fate and behavior of ARGs from EAS added for more effective treatment of FVW and the role of earthworms. The targeted ARGs were *tet G*, *tet M* and *sul I* and the mobile genetic element gene was *intl I*. Significant reductions of ARGs in the substrate compartments were confirmed for the treatments for FVW added with EAS and EAS alone even if the reduction extents differed among the types of FVW. Apparent reductions were not found in all bed compartments where the final products were accumulated, except the bed compartment for the treatment of EAS alone. The result also indicated that, even if abundant ARGs and *intl I* in EAS were added into the vermireactors, the reactors can effectively eliminate their existence, inhibiting their transfer into the final products after vermicomposting. In the fresh cast of earthworms,

the absolute abundances of ARGs and *intl 1* increased; however, the relative abundances reduced significantly as a result of the enrichment of total bacteria in the cast by a magnitude of 2-3 orders. The gut of earthworms played a significant role in selectively reducing the host bacteria harboring ARGs and *intl 1* through activation of non-antibiotic resistance bacteria or digestion of the resistance bacteria. The remaining abundance of ARGs and *intl 1* was confirmed in the final products of all vermireactors, suggesting that a possible risk for the proliferation of ARGs in the environment still exist if applied as fertilizers or soil modifiers. The mobility of ARGs from the final products to the soil environment or the vegetation needs to be clarified through further studies.

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Chapter 1 Introduction

1.1 Background

Antibiotic resistance genes (ARGs) including extracellular ARGs (eARGs) and intracellular ARGs (iARGs), as a newly emerging contaminant, are of great concern worldwide since their proliferation in the environment can bring about serious environment and public health issues. It is estimated that the global deaths caused by antibiotic resistance will be 10 million in 2050 if without any action (World Health Organization, WHO). Wastewater treatment facilities (WWTFs) play a vital role in the collection, treatment and disposal of wastewater that contains antibiotics as well as ARGs from different sources, such as hospitals, households, and agricultural systems. However, WWTFs generally fail to remove ARGs especially eARGs to the expected level. eARGs can remain in wastewater after conventional or even advanced treatment and therefore persist for several months in the receiving water and soil environments of WWTFs. This results in broad dissemination of resistance genes in the total environment and adversary effects on the safety of fisheries and agricultural food products. On the other hand, the quality of treated wastewater associates closely with the sludge settleability which is affected by such factors as the floc size and density, and the proportion of intact/damaged bacterial cells. These factors may also affect the fate and behavior of eARGs and iARGs in sludge fractions with different settleability, a topic of great academic and practical significance requiring clarification.

In addition, WWTFs are not only a point of collection for ARGs but also a hotspot to spread ARGs to related bacteria during biological treatment since they meet the conditions for the growth of bacteria and possess higher selective pressure for the proliferation of ARGs among different bacterial species. These extensively grown bacteria aggregate together to form sludge

flocs and those with higher settleability will settle during the sedimentation process in large quantities. A significant part of the settled sludge flocs, as a byproduct of wastewater treatment process, is named as excess activated sludge (EAS) requiring proper treatment and disposal.

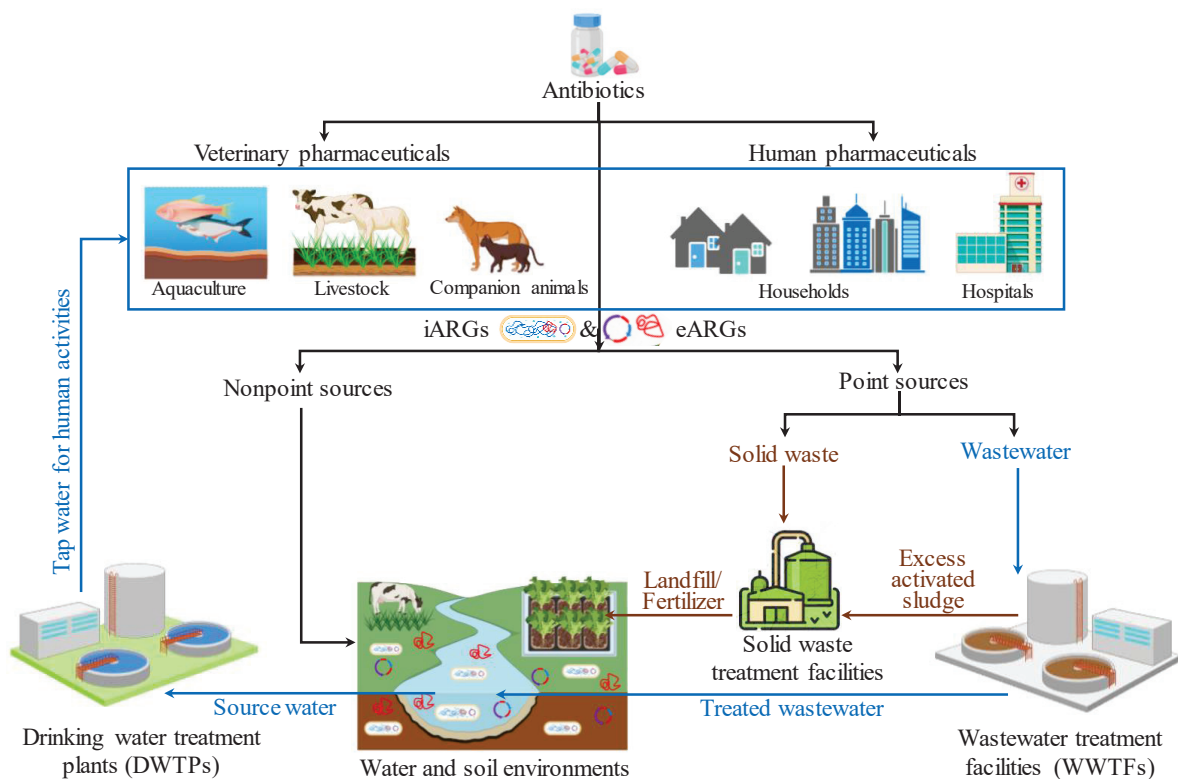


Fig. 1.1 Occurrence and proliferation of extracellular and intracellular antibiotic resistance genes (eARGs and iARGs) in the environment.

EAS is a typical solid waste with higher recycle value for its richer nutrients. However, it is reported also as an important reservoir for ARGs. Biological process based on the use of higher concentrations of aerobic and/or anaerobic bacteria, as the main method for both wastewater and solid waste treatment, possesses the possibility of becoming a new spot for ARGs to spread among different bacterial species, which needs clarification and control.

On the other hand, fruit and vegetable waste (FVW) is another type of solid waste (or liquid-solid waste) generated in large quantities during food production and consumption. Proper

treatment of FVW is a continuous challenge of big urban cities in developed and also most developing countries. Recent studies have reported the existence of ARGs in FVW which include the original ones detected in the waste material and also those newly induced during its biological treatment process. Improper treatment of solid waste including EAS and FVW is one of the likely routes for spreading ARGs into the soil and water environments. Vermicomposting can not only effectively convert FVW or EAS into fertilizers or soil modifiers through the joint action of earthworms and microorganisms but can also attenuate ARGs in the waste materials. Many studies have reported that pretreatment methods like conventional composting are necessary if vermicomposting is applied for treatment of FVW or EAS in order to reduce the risk to earthworms from any harmful substances. Enhancement of the treatment efficiency of FVW and EAS by vermicomposting is necessary for obtaining better final products with higher utilization value. Furthermore, eliminating ARGs during vermicomposting of different compositions of solid waste is necessary, together with deeper understanding of the fate and behavior of ARGs in the waste materials.

1.2 Objective of the study

The overall objective of this study was to investigate the fate and behavior of ARGs (including eARGs and iARGs) during water and solid waste treatment, hence providing new insights for eliminating the proliferation of ARGs in the environment, with following specific aims:

- i. To clarify the fate and behavior of eARGs and iARGs in sludge with different settleability hence proposing a possible pathway to control the spreading of ARGs into the total environment along with the discharge of treated wastewater.
- ii. To enhance the treatment efficiency of FVW and EAS by vermicomposting through

clarifying the effect of EAS on vermicomposting of FVW.

- iii. To clarify the fate and behavior of ARGs in EAS added for more effective vermicomposting of FVW and the role of earthworms on ARGs during vermicomposting.

The technical route of the overall study is shown in **Fig. 1.2**.

1.3 Structure of the dissertation

This dissertation presents the results obtained from the investigations of fate and behavior of ARGs (including eARGs and iARGs) in water and solid waste during their treatments. **Chapter 1** introduces the background and objective of this study. **Chapter 2** is a literature review concerning the eARGs and iARGs in the environment and the conventional treatment methods for water and solid (FVW and EAS). In **Chapter 3**, in order to a better controlling the abundances of ARGs discharged from WWTFs along the treated water, the distribution of eARGs and iARGs in sludge with different settleability are presented and discussed. In **Chapter 4**, a feasible approach for more effective treatment of typical solid waste (FVW and EAS), was suggested and discussed. To treat FVW with the addition of EAS by using a novel vermireactor consists of substrate and bed compartments can result in a more stable final product with higher utilization value as fertilizer. The fate and behavior of ARGs in solid waste (FVW and EAS) and the role of earthworms in the changes of ARGs during vermicomposting were discussed in **Chapter 5**. Finally, the conclusion of this study together with the consideration for future studies, are presented in **Chapter 6**.

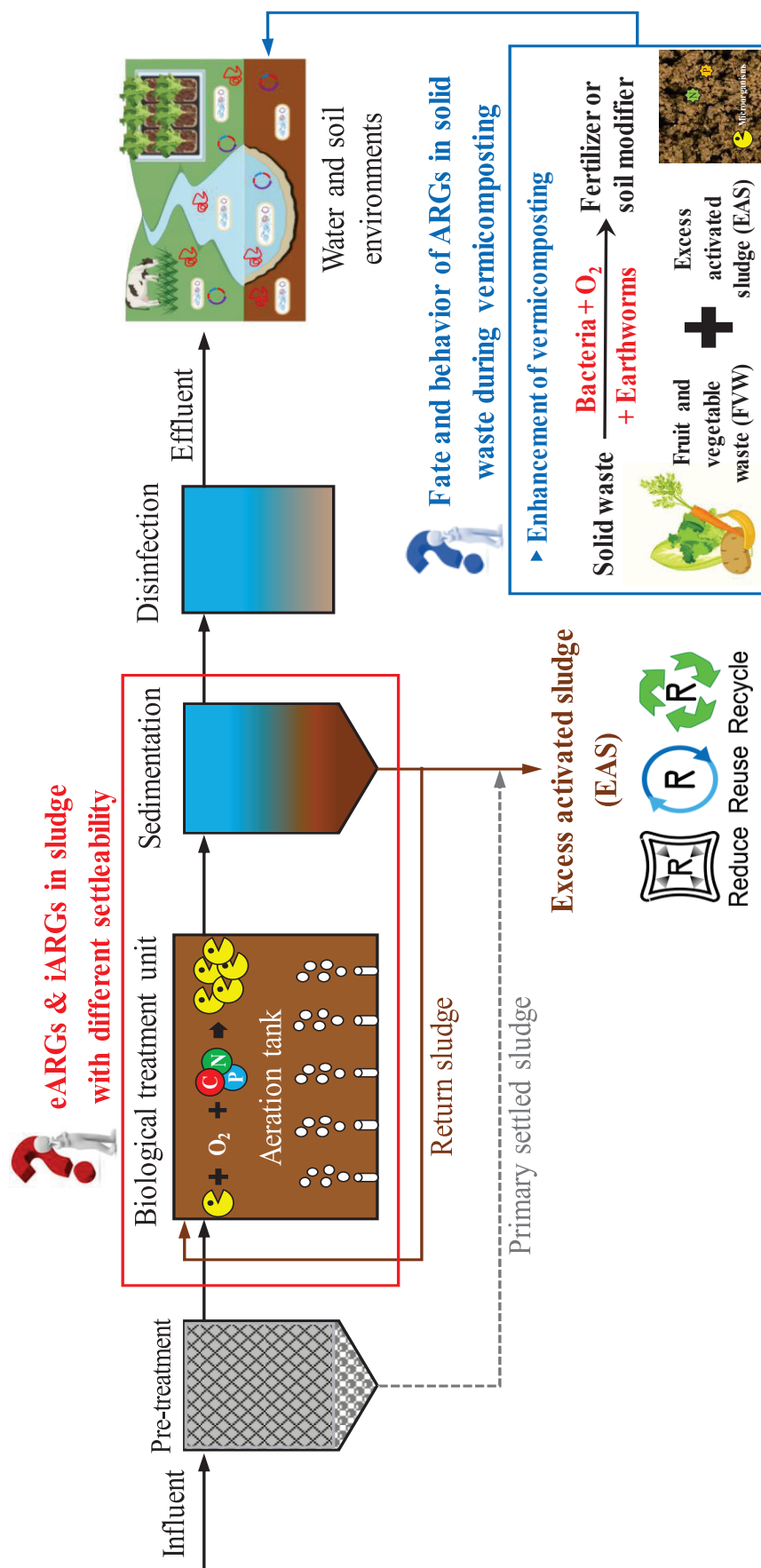


Fig.1.1.2 Technical route of the overall study.

Chapter 2 Review of Literature

2.1 Antibiotic resistance genes (ARGs)

The widespread proliferation of antibiotics resistance genes (ARGs) caused by the overuse of antibiotics has been recognized as a serious environmental and human health issue. The mechanisms for bacteria to be resistant to antibiotics mainly fall into four categories: (1) efflux pump of antibiotics; (2) modification of antibiotic targets; (3) inactivation of antibiotics; and (4) increasement of membrane impermeability (Reygaert, 2018), as shown in **Fig. 2.1**. It is reported that the efflux pump and the target modification are the predominant resistance mechanisms in the natural environment through the detection of ARGs in water and sediment samples (Y. Zhao et al., 2018). ARGs in natural environment and WWTFs exist in two states, the extracellular state (eARGs) and intracellular state (iARGs). eARGs can be derived from the

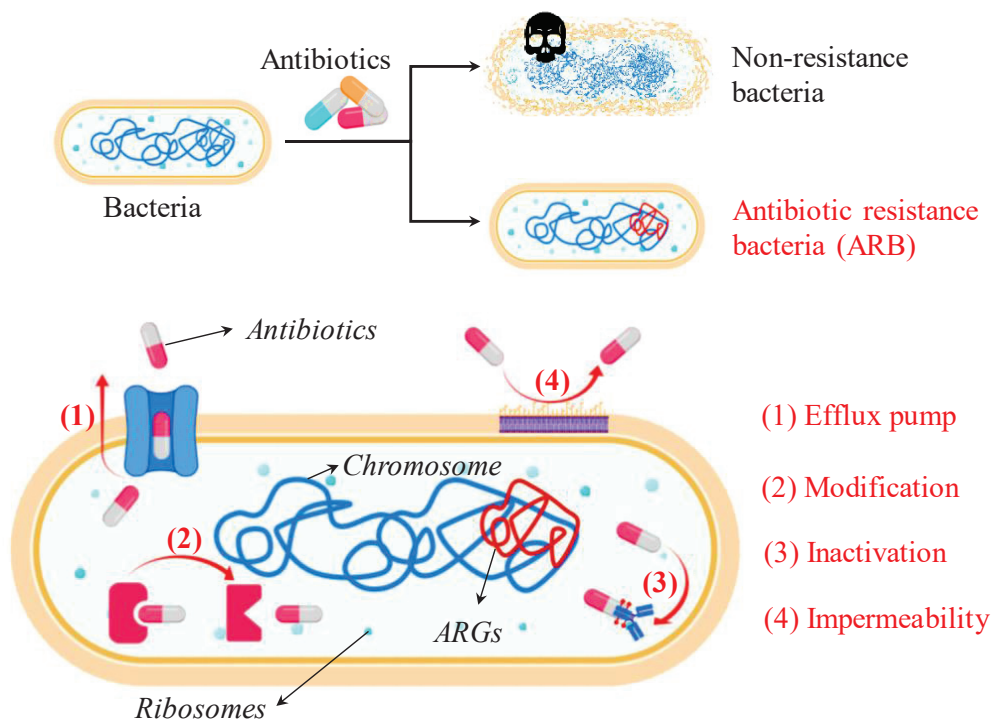


Fig. 2.1 Antibiotic resistance mechanisms of bacteria.

lysis of bacterial cells and the secretion from the live bacteria (Dong et al., 2019). Moreover, the eARGs can infect non-resistant bacteria through natural transformation, hence resulting in further proliferation of antibiotic resistance; while, iARGs in intracellular DNA can pass to the next generation by self-replication (vertical gene transfer) and can also transmit to other bacterial species via conjugation (cell-cell contact) or transduction (infection of phages) (Liu et al., 2018; Zhang et al., 2018; Dong et al., 2019). Transformation, conjugation and transduction are three dominant mechanisms of horizontal gene transfer (HGT) responsible for a larger extent of ARGs proliferation among bacteria (Guo et al., 2018).

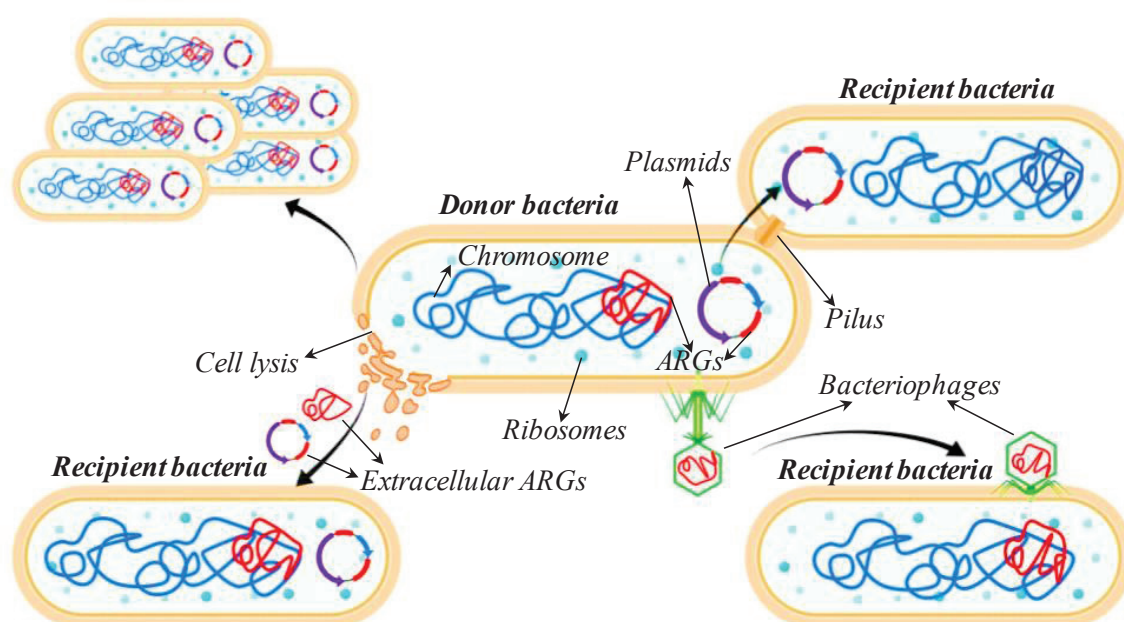


Fig. 2.2 Mechanisms of antibiotic resistance genes transfer.

2.2 ARGs during water treatment process

2.2.1 Abundances of ARGs in water environment

Abundant ARGs have been widely detected in many types of water samples including river, lake, pond, tap water, wastewater, and treated wastewater, as summarized in **Table 2.1**. Hundreds of various ARGs encoding resistance to a broad range of antibiotics have been found

Table 2.1 Abundances of widely detected ARGs in different water samples.

Water samples	Target ARGs	Abundances	Reference
River	<i>tet M, sul I, erm B, qnr S</i>	March: $0 - 5.8 \times 10^{-2}$ copies/16S rDNA; September: $0 - 8.1 \times 10^{-3}$ copies/16S rDNA	(G. Wang et al., 2020)
River	<i>tet C, sul I, sul II</i>	<i>tet C</i> : $8.30 - 13.20 \times 10^{-2}$ copies/16S rDNA; <i>sul I</i> : $(1.41 \pm 1.12) \times 10^{-2}$ copies/16S rDNA; <i>sul II</i> : $(1.58 \pm 1.71) \times 10^{-3}$ copies/16S rDNA	(Qiao et al., 2018)
River	<i>tet a, tet b, tet c,</i>	$2.4 \times 10^{-4} - 2.1 \times 10^{-2}$ copies/16S rDNA	(Z. Wang et al., 2020)
Lake	<i>sul I, sul 2, qnr S</i>	$2.9 \times 10^{-5} - 8.9 \times 10^{-3}$ copies/16S rDNA	
Pond		$1.1 \times 10^{-5} - 1.7 \times 10^{-2}$ copies/16S rDNA	
Tap water	<i>tet A, tet C, tet M, tet X, aad A, bla_{TEM}, sul I, sul 2, cat A1, qnr A, aph(2')-Id, amp C, kat G</i>	eARGs: $0 - 10^5$ copies/L; iARGs: $0 - 10^7$ copies/L	(Hao et al., 2019)
Treated wastewater and its receiving water body (sea)	<i>sul I, sul 2, tet B, tet G, tet X, erm F, erm T, qnr A, qnr B, qnr S</i>	<i>sul I</i> and <i>sul 2</i> : $2.14 \times 10^3 - 2.25 \times 10^6$ copies/ml; <i>qnr A, qnr B</i> and <i>qnr S</i> : $4.6 \times 10^1 - 2.48 \times 10^5$ copies/ml; <i>tet B, tet G, tet X</i> : lower than <i>sul I</i> and <i>sul 2</i> but higher than <i>qnr A, qnr B</i> and <i>qnr S</i> . <i>erm F</i> and <i>erm T</i> : lowest ARGs (data are unknown)	(J. Lu et al., 2020)
Wastewater	<i>tet A, tet O, tet W, sul I, sul II, bla_{CTX-M}</i>	<i>tet A</i> : $10^{4.45} - 10^{5.34}$ copies/ml; <i>tet O</i> : $10^{4.66} - 10^{5.45}$ copies/ml; <i>tet W</i> : $10^{6.42} - 10^{7.00}$ copies/ml; <i>sul I</i> : $10^{5.43} - 10^{5.97}$ copies/ml; <i>sul II</i> : $10^{6.75} - 10^{7.35}$ copies/ml; <i>bla_{CTX-M}</i> : $10^{2.53} - 10^{3.99}$ copies/ml	(Wen et al., 2016)
Treated wastewater	<i>bla_{TEM}, bla_{OXA-48}, bla_{OXA-58}, bla_{CTX-M-15}, bla_{CTX-M-32}, bla_{KPC-3}, sul I, tet M, mcr-I</i>	10^4 /L copies for <i>bla_{TEM}, bla_{OXA-58}, bla_{CTX-M-15}, bla_{CTX-M-32}, sul I, tet M</i> ; <i>bla_{KPC-3}, bla_{OXA-48}</i> and <i>mcr-I</i> were not detected.	(Cacace et al., 2019)

in microorganisms distributed not only in wastewater discharged from hospitals but also in wastewater from households, effluent of WWTFs, surface water, groundwater, and even in tap water (Zhang et al., 2009). It is indicated that the water distribution system (water cycle system) serves as an important reservoir for the dissemination of antibiotic resistance to recipient bacteria, thus greatly affecting the public health and the environment.

2.2.2 Effect of wastewater treatment process on ARGs

Wastewater treatment facilities (WWTFs), which receive wastewater containing not only antibiotics but also ARGs, can become a hotspot to spread ARGs and related bacteria (ARB) since they meet the conditions for the growth of bacteria and possess higher selective pressure for the proliferation of ARGs between different bacterial species (Pazda et al., 2019). So far, many ARGs have been detected from samples associated with WWTFs, such as the influent before treatment (Le et al., 2018), activated sludge (Zhou et al., 2019) and the effluent even after disinfection (Lee et al., 2017a). WWTFs are significant reservoirs of many proliferated and persistent ARGs present in natural water and soil environments (Auerbach et al., 2007; Karkman et al., 2018).

WWTFs generally fail to remove ARGs especially the eARGs (Li et al., 2017). eARGs remaining in wastewater after conventional or even advanced treatment can persist for several months in receiving water bodies and then spread widely in connected fresh water environment and marine water environment, thus affecting the safety of total water environment (Zhou et al., 2019). Zhang et al. (2018) conducted a study on the occurrence of ARGs in coastal areas of Bohai Bay in China and found that eARGs were more abundant than iARGs in both water and sediment samples, implying that eARGs may contribute more to proliferation of ARGs in the water environment than iARGs. In a recent study on the occurrence and distribution of ARGs

in Ba River in China, Jia et al. (2018) found that the rich existence of ARGs in the riverine system (both water and sediment) were mainly attributed to sources such as the effluent of WWTFs. Targeting also on river, the water of which is used as the source for drinking water production, Su et al. (2018) investigated ARGs and bacterial communities in the East, West, and North rivers of the Pearl River Delta region of China. ARGs and ARB were detected from all these three rivers and considered to be the main origin for ARGs detected in the tap water of the region. In addition to the effect on water environment, ARGs in treated wastewater can also bring about large scales of soil environmental pollution when used as the source water for irrigation of agricultural activities (Zhu et al., 2020). Through host bacteria in soil, ARGs can proliferate via horizontal gene transfer and cause pollution of crops and vegetations, hence affecting the safety of soil environment and the food production and supply chains (Gao et al., 2020).

Additionally, WWTFs are not only a point of collection for ARGs but also a hotspot to spread ARGs to related bacteria during biological treatment since they meet the conditions for the growth of bacteria and possess higher selective pressure for the proliferation of ARGs among different bacterial species. These extensively grown bacteria aggregate together to form sludge flocs and those with higher settleability will settle during the sedimentation process in large quantities. A significant part of the settled sludge flocs, as a byproduct of wastewater treatment process, is named as excess activated sludge (EAS) requiring proper treatment and disposal. EAS is a typical solid waste with higher recycling value for its richer nutrients but also an important reservoir for ARGs. Mao et al. (2015) investigated the prevalence and proliferation of ARGs in two municipal WWTFs and found that significant ARGs discharges were occurred through both treated wastewater and EAS. It is also revealed that the antibiotic resistance bacteria in treated wastewater were also relatively resistant to chlorination (Mao et al., 2015).

2.3 ARGs during solid waste treatment process

2.3.1 Fruit and vegetable waste (FVW)

About 1.3 billion tons of food that accounts for 32% of the total food produced for human consumption across the entire food supply chain is lost and wasted every year in the world (Du and Li, 2016). The FVW, accounts for the largest proportion (45%) of the total lost and wasted food (Abubackar et al., 2019a), are also generated in large quantities along with the entire food supply chain, from production, processing to the consumption. According to the statistical data reported by the Food and Agriculture Organization (FAO), approximately 1.8, 6.5, 32 and 15 million tons of FVW are generated each year in India, Philippines, China and the United States of America, respectively (FAO, 2013). From the same information source, it is reported that at least 15% of fruits and 25% of vegetables are wasted at the bottom of the food supply chain worldwide (FAO, 2014). Diverting our attention to the FVW from households, about 10 million tons of FVW are generated every year in Japan alone (*Ministry of agriculture, forestry and fisheries, Japan*, 2012). All the statistical data indicated that the FVW are a very important class of wastes since they are produced in very large amounts in all the wholesale markets and other activities in the world (Scano et al., 2014).

The FVW can be highly variable depending on its source and is strongly dependent on the eating habits of consumers (Cerdeira et al., 2018). In general, the FVW are characterized by high water content and rich biodegradable organic compounds (e.g. carbohydrates, lipids, and organic acids), typically with soil content under 10 %, and 85 % corresponding to organic matter (Edwige et al., 2018; Li et al., 2020). Thi et al. (2015) also reported that the FVW comprises 74 – 90% of water content and have a volatile solid to total solid ratio (VS/ TS) of 80 – 97%, and a C/N ratio of 14.7 – 36.4. Chang Edwige & Frare (2017) have investigated the physical and chemical characteristics of FVW sampled monthly during one year in Brazil, as

summarized in **Table 2.2**. These characteristics indicated that the FVW has a great potential for reuse, recycling and recovery (Plazzotta et al., 2017) and should to be treated with proper methods.

Table 2.2 Physical and chemical characteristics of fruit and vegetable waste.

	Min	Max	Mean
pH	3.9	4.5	4.2
Higher calorific value (MJ/kg)	14.8	21.2	16.5
Total solids (%)	7.2	13.8	9.5
Volatile solids (%-TS)	89.9	93.4	92.0
Proteins (%-vs)	9.6	25.5	15.9
Lipids (%-vs)	1.0	22.3	4.5
Cellulose (%-vs)	13.8	26.9	17.1
Hemicellulose (%-vs)	3.1	15.3	9.4
Lignin (%-vs)	3.0	12.0	6.4
Non-lignocellulosic carbohydrates (%-vs)	20.1	60.1	46.7

TS: total solids; VS: volatile solids.

2.3.2 Excess activated sludge (EAS)

The excess activated sludge (EAS) is a kind of organic solid waste generated from WWTFs. It is reported that the high content of nitrogen in EAS can give rise to a higher ammonia concentration environment thus leading to the death of earthworms (Fu et al., 2015; Li et al., 2020). To solve this problem, a pre-treatment is required to adjust the C/N ratio by mixing some bulking materials with rich carbon content, such as paper mulch (Ndegwa et al., 2000), straw (Contreras-Ramos et al., 2005), and sawdust (C. Zhao et al., 2018). Adding these kinds of bulking materials can not only ameliorate the living environment for earthworms by increasing the C/N ratio, but also cause a longer decomposition process (non-degradable substrate) and lower usage value (lower nitrogen content) of final product as fertilizer (Li et al., 2020a).

Moreover, other pre-treatment methods like air drying, airing, creating pellet, and pre-composting could also increase the whole period of vermicomposting.

2.3.3 Abundances of ARGs in FVW and EAS

Abundant and diverse ARGs have been detected in different solid waste including FVW and EAS, as summarized in **Table 2.3**. Improper treatment and disposal could directly or indirectly

Table 2.3 Abundances of widely detected ARGs in different solid waste.

Solid waste	Target ARGs	Abundances	Reference
Food waste	14 types of ARGs (<i>tet</i> , <i>sul</i> , <i>qnr</i> , <i>erm</i> , <i>bla</i>)	$0 - 4 \times 10^{-4}$ copies/16S rDNA	(Lee et al., 2017b)
Household waste	Vegetable waste: 150 types of ARGs; Meat waste: 128 types of ARGs; Fruit waste: 91 types of ARGs	Vegetable waste: 4.29 copies/cell; Meat waste: 13.45 copies/cell; Fruit waste: 2.65 copies/cell	(Liang et al., 2020)
Dewatered sludge	124 subtypes of ARGs (26 classes)	527.6 ppm	(Huang et al., 2020b)
Sewage sludge	25 types of ARGs	5.1×10^{11} copies/g-dry	(Liao et al., 2018)
Leachate released from municipal solid waste	12 subtypes of ARGs for fluoroquinolones and β -lactams	<i>qnr A</i> : 1.10 copies/16S rDNA; <i>qnr B</i> : 1.13×10^{-5} copies/16S rDNA; <i>qnr D</i> : 4.95×10^{-6} copies/16S rDNA; <i>qnr S</i> : 1.42×10^{-7} copies/16S rDNA; <i>bla_{OXA10}</i> : 3.86×10^{-4} copies/16S rDNA; <i>bla_{CTX-M}</i> : 3.64×10^{-5} copies/16S rDNA; <i>bla_{OXY}</i> : 1.92×10^{-6} copies/16S rDNA; <i>bla_{SHV}</i> : 5.95×10^{-7} copies/16S rDNA; <i>pen A</i> : $1.36 \times 10^{-6} - 1.87 \times 10^{-5}$ copies/16S rDNA; <i>amp C</i> : 7.47×10^{-7} copies/16S rDNA Others: no data	(You et al., 2018)

lead to the dissemination of ARGs in the surrounding soil and water environment, hence endangering the food chain and public health.

2.3.4 Conventional treatment methods for FVW and EAS

With the rapid economic development, the lack of resources and energy, and environmental issues have become more serious. The concept of recovery, recycling, and reuse form waste resources has been widely accepted for the sustainable treatment of wastes in large quantities and attracted the attention of many researchers. The fruit and vegetable waste (FVW), as one of the major components of wastes, is generated in large quantity during production, processing and consumption. The FVW is characterized by high water content and rich biodegradable organic compounds, typically with soil content under 10%, and 85% corresponding to organic matters (Edwiges et al., 2018). These characteristics indicated that the FVW may contribute to negative environmental issues (e.g. leachate and greenhouse gas) and provide a great potential for reuse, recycling and recovery (Hartmann and Ahring, 2006; Plazzotta et al., 2017). Therefore, it is important to find a sustainable method for the treatment and recycling of FVW. Several conventional methods are used for treatment and recycling of FVW, such as incineration, landfill, anaerobic digestion, and composting. Compared to these conventional methods, vermicomposting has the advantage of effective stabilization of the organic wastes through the joint function of earthworms and microorganisms in decomposition of easily decaying organic constituents, and of high utilization value for its final product as fertilizer. On the other hand, excess activated sludge (EAS), as the main byproduct of sewage treatment process, is an organic waste mainly consist of microorganisms which can be treated by vermicomposting.

The FVW are distinguished as liquid and/or semi-liquid wastes due to high water content.

These kinds of wastes are conventionally disposed by non-scientific methods such as transported to landfill sites or incineration plants with other municipal solid wastes without proper treatment and recycling (Liu et al., 2012). In most industrialized countries, incineration is a widely used method to treat burnable municipal solid waste including FVW (Du and Li, 2017). The exhaust gas and ash produced in incineration plant during combustion process has serious harmful effects to the human health and the environment (Cangialosi et al., 2008). Moreover, the incineration plant is also accounted as a pollution source of heavy metals for the surrounding area. In many developing countries, however, landfill and direct dumping are still prevailing. The landfill and dumping can worsen soil and water environments. Direct dumping to small urban rivers and garbage collection stations near residential areas is blamed for foul smells and leachates that affect the living environment of humans in most developing countries (Du and Li, 2017). Therefore, it is imperative to overcome the relevant defects of landfill or incineration method for recycling FVW, simultaneously develop an environment friendly method which can convert FVW to high value products.

Currently, anaerobic digestion and composting are recognized as two efficient and environmentally friendly methods to recycle/recover available resources from organic wastes and are used extensively worldwide (Cerdeira et al., 2018). Diverting organic wastes like FVW from landfill or incineration to anaerobic digestion or composting has many environmental benefits, such as reduction of greenhouse gas emission and improvement of soil properties through compost application (Bernstad Saraiva Schott et al., 2016). Anaerobic digestion is a biological degradation process, in which stabilization of organic wastes is achieved by microbial consortia, in the absence of oxygen. The main products of this process are biogas, a mixture of CH₄, H₂ and other gases in traces, and digested sludge as the main byproduct (Moukazis et al., 2018). The biogas produced can be used to generate heat and/or electricity or

can be scrubbed to natural gas quality for use as transport fuel or injection into the natural gas grid (Singh et al., 2010). However, it is reported that the required range of pH, carbon to nitrogen (C/N) ratio and moisture for anaerobic digestion are around 7.0 (Khalid et al., 2011), 20 – 30, and 70 – 80 % (Hernández-Berriel et al., 2008), respectively. Therefore, it is necessary to introduce a pretreatment process for FVW before the anaerobic digestion. Composting, as one of the most sustainable options for the treatment of organic wastes including the FVW, can not only cut down the volume of organic wastes but also produce a useful product as fertilizer (Lou and Nair, 2009; Cerda et al., 2018). Since the composting is an aerobic biochemical process via the function of thermophilic microorganisms, it is important to maintain the requirements for the growth of microorganisms. Some environmental factors that may influence the microbial activity like temperature, oxygen concentration, pH, water content, C/N ratio and particle size need to be adjusted by a pre-treatment process before composting (Domínguez et al., 2009). Besides these two methods, some other sustainable treatment methods are being developed (e.g. fermentation, microbial fuel cell). **Table 2.4** summarized some published investigations on the treatment of solid waste by using different treatment methods.

2.3.5 Vermicomposting of FVW and EAS

Vermicomposting is attracting researchers' attention in recent years owing to the reason that it can degrade organic wastes like FVW and can recycle and convert the valuable nutrients into organic fertilizer (Li et al., 2020a). Compared to composting, vermicomposting has more effective functions of biodegradation and stabilization of the organic wastes through the combined action of earthworms and microorganisms (Domínguez, 2004). In general, there are two operation systems are widely used for vermicomposting, namely mixed system and separated system. The substrate and bed material are mixed together in the mixed system, while

the substrate and bed material are simply separated into two layers or separated by using a mesh with holes in the separated system (Li et al., 2020a). Many researchers investigated the vermicomposting of FVW with different operation systems and conditions are summarized in **Table 2.5**. It is worth to note that these previous studies used dry FVW or with the addition of other bulking materials for earthworms. The pre-treatment for drying the fresh FVW normally takes one to three weeks before conducting the vermicomposting process (Li et al., 2020a). This kind of pre-treatment not only increases the whole time needed for vermicomposting but also leads to the loss of considerable amounts of nutrients via leachate (Huang et al., 2012). Moreover, limited literatures related to the vermicomposting of fresh FVW reported that the earthworms cannot survive in the fresh FVW due to the high water content and high electrical conductivity of the leachate generated from the fresh FVW (Gunadi and Edwards, 2003; Huang et al., 2012; Li et al., 2020). Therefore, it is necessary to enhance the efficiency of the treatment of FVW by vermicomposting urgently owing to that the sustainable treatment methods are regarded as one of the key approaches to achieve the urban sustainability via recycling resources and recovering energy (D. Wang et al., 2020).

Table 2.4 Different methods for treating solid waste and their final products.

Treatment method	Types of solid waste	Pre-treatment	Treatment volume	Treatment time	Final products	References
Anaerobic digestion	FVW from municipal central supply	Drying at 60 °C; Ground to a diameter smaller than 2 mm	ND	32 days	Biogas (CH ₄ : 377±67 L/kg-vs)	(Edwiges and Frare, 2017)
Anaerobic digestion	FVW from municipal market	Without any pre-treatment for FVW, while the digester was initially loaded with cow rumen Mixed with hyacinth/garden prune/sawdust;	108 kg/day (wet basis)	365 days	Biogas (CH ₄ : 0.11 – 0.15 m ³ /kg-vs)	(Martí-Herrero et al., 2019)
Composting	Vegetable waste	Inoculation of cow dung; Adjusted C/N ratio; Chopped to a size of 1–2 cm	100 kg	30 days	Fertilizer (N increased by 14.83 %)	(Rich et al., 2018)
Fermentation (mesophilic condition)	Mixture of different fruits and vegetables	Ground or cut into small pieces of size < 5 cm; Autoclaved at 120 °C for 20 min;	ND	8 - 14 days	Biogas (H ₂ : 10.77 – 23.53 Nml/g-vs)	(Abubackar et al., 2019a)
Fermentation (thermophilic condition)		Inoculation of sludge from biogas plant	6.5 kg (wet basis)	9 – 10 days	Biogas (H ₂ : 20.81 - 27.19 Nml/g-vs)	(Abubackar et al., 2019b)
Microbial fuel cell (two chambers)	Potato	Boiled and cut into small cubes (length of 5mm); Inoculation of anaerobic bacterial consortia	2.5 g (wet basis)	52 days	Electricity (Highest current density: 160.1 – 253.9 mA/m ²)	(Du and Li, 2016)

FVW: fruit and vegetable waste; ND: No data.

Table 2.5 Operation condition and final product value of vermicomposting for solid waste.

Types of solid waste	Pre-treatment	Earthworm species and density	Operation system	Temperature and time	Initial content of N and P	Final content of N and P	References
Vegetable-market solid waste	Dried at 60 °C in hot air oven; Chopped and sieved (<2 mm); Mixed with cow dung/biogas slurry/wheat straw; Thermal stabilization for 3 weeks	<i>Eisenia fetida</i> ; 26.7 worms/kg-dry (258 - 278 mg/worm)	Mixed system	26.9 ± 0.36 °C; 105 days	5.82 – 17.1 g-N/kg; 2.73 – 5.74 g-P/kg	8.24 – 30.6 g-N/kg; 3.92 – 8.9 g-P/kg	(Suthar, 2009a)
Vegetable greenhouse waste	Over-dried at 25 °C and chopped; Mixed with cow dung or straw; Aerated for a week	<i>Eisenia andrei</i> ; 100 worms/kg-dry (0.17 - 0.31 g/worm)	Mixed system	24 °C; 12 weeks	15.0 – 22.8 g-N/kg; 3.9 – 5.8 g-P/kg	14.1 – 23.0 g-N/kg; 6.9 – 8.4 g-P/kg	(Fernández-Gómez et al., 2010b)
Tomato-fruit wastes	Pre-composting of bed material for 15 days	<i>Eisenia fetida</i> ; 50 g/kg-wet	Separated system; Sheep manure was used as bed material	25 °C; 210 days	Sheep manure: 9.6 g-N/kg; 2.6 g-P/kg	12.9 g-N/kg; 2.04 g-P/kg	(Fernández-Gómez et al., 2010a)
Banana peels; Cabbage; Lettuce; Potato; Watermelon peels	Chopped and wetted by tap water	<i>Eisenia fetida</i> ; 200 worms/kg-wet (Juvenile earthworms)	Separated system; FVW and vermicompost as a bed layer was separated by a plastic mesh with holes	25 ± 2 °C; 28 days	6.6 – 21.6 g-N/kg; 2.7 – 5.5 g-P/kg	5.8 – 7.7 g-N/kg; 3.8 – 5.2 g-P/kg	(Huang et al., 2012)

Apple pomace	Mixed with chopped wheat straw (soaked in water for 1 month); Pre-composting at 25 °C for 14 days under aerobic condition	<i>Eisenia</i> ; 150 worms/L	Mixed system	4 months	13 – 19 g-N/kg; 5.53 – 6.43 g-P/kg	N increased by 58%; 10.18 – 17.79 g-P/kg	(Hanc and Chadimova, 2014)
Vegetable waste	Mixed with cattle manure; Pre-composting for 20 days	<i>Eisenia fetida</i> ; 65 worms/kg (Adult earthworms)	Separated system; Chopped hay, banana pulp, and tree leaves were used as bed material	25 °C; 45 days	13 – 19 g-N/kg; 5.31 – 6.30 g-P/kg	29 – 31 g-N/kg; 7.95 – 9.33 g-P/kg	(Varma et al., 2015)
Vegetable market waste	Chopped into pieces (1 cm × 1 cm); Mixed with rice straw;	<i>Eisenia fetida</i> , and <i>Perionyx excavates</i> ; 10 worms/kg-wet	Mixed system	2 months	100.8 – 129.7 mg-N/kg; 35.5 – 56.6 mg-P/kg	103.3 – 784.0 mg-N/kg; 88.1 – 227.5 mg-P/kg	(Hussain et al., 2016)
Kitchen vegetable waste	Mixed with paddy straw; Pre-composting for 5 days;	<i>Eisenia fetida</i> , <i>Eudrilus eugeniae</i> , and <i>Perionyx excavates</i> ; 10 worms/kg-wet (Juvenile earthworms)	Separated system; Cow dung was used as bed material	27 – 30 °C; 120 days	ND	N increased by 5.86 – 6.6 folds; P increased by 5 folds in maximum	(Hussain et al., 2018)
Kitchen waste	Dried at 65 °C; Smashed and passed 10 mesh sieves; Mixed with rice straw;	<i>Eisenia fetida</i> ; 5 worms/kg-wet (300 mg/worm)	Mixed system	20 – 25 °C; 45 days	19.2 – 22.2 g-N/kg; 1.36 – 1.75 g-P/kg	Both N and P decreased	(Song et al., 2019)

FVW: fruit and vegetable waste; N: nitrogen; P: phosphorus.

2.4 Summary

The large amounts of FVW generated during the whole food supply chain should be treated, recycled, and recovered properly. The vermicomposting is considered as one of the most sustainable methods for the treatment of FVW. EAS is another typical solid waste generated from WWTFs in large quantities, which can be also treated by vermicomposting. However, the vermicomposting of fresh FVW and EAS without any pre-treatment still have some problems. The treatment efficiency of FVW and EAS by vermicomposting should be enhanced for better quality of final products. Furthermore, various ARGs have been detected in different environmental samples related to water and solid waste, indicating a serious risk of the proliferation for ARGs in the total environment. It is necessary and important to have deeper investigations on the fate and behavior of ARGs during the water and solid waste treatment.

Chapter 3 Extracellular and intracellular antibiotic resistance genes in sludge of different settleability

3.1 Background and objective

The widespread proliferation of antibiotics resistance genes (ARGs) caused by the overuse of antibiotics has been recognized as a serious environmental and human health issue. Wastewater treatment facilities (WWTFs), which receive wastewater containing not only antibiotics but also ARGs, can become a hotspot to spread ARGs and related bacteria (ARB) since they meet the conditions for the growth of bacteria and possess higher selective pressure for the proliferation of ARGs between different bacterial species (Pazda et al., 2019). So far, many ARGs have been detected from samples associated with WWTFs, such as the influent before treatment (Le et al., 2018), activated sludge (Zhou et al., 2019) and the effluent even after disinfection (Lee et al., 2017a). WWTFs are significant reservoirs of many proliferated and persistent ARGs present in natural water and soil environments (Auerbach et al., 2007; Karkman et al., 2018).

ARGs in natural environment and WWTFs exist in two states, the extracellular state (eARGs) and intracellular state (iARGs) (Dong et al., 2019). WWTFs generally fail to remove ARGs especially the eARGs (Li et al., 2017). eARGs remaining in wastewater after conventional or even advanced treatment can persist for several months in receiving water bodies and then spread widely in connected fresh water environment and marine water environment, thus affecting the safety of total water environment (Zhou et al., 2019). Zhang et al. (2018) conducted a study on the occurrence of ARGs in coastal areas of Bohai Bay in China and found that eARGs were more abundant than iARGs in both water and sediment samples, implying that eARGs may contribute more to proliferation of

ARGs in the water environment than iARGs. In a recent study on the occurrence and distribution of ARGs in Ba River in China, Jia et al. (2018) found that the rich existence of ARGs in the riverine system (both water and sediment) were mainly attributed to sources such as the effluent of WWTFs. Targeting also on river, the water of which is used as the source for drinking water production, Su et al. (2018) investigated ARGs and bacterial communities in the East, West, and North rivers of the Pearl River Delta region of China. ARGs and ARB were detected from all these three rivers and considered to be the main origin for ARGs detected in the tap water of the region. In addition to the effect on water environment, ARGs in treated wastewater can also bring about large scales of soil environmental pollution when used as the source water for irrigation of agricultural activities (Zhu et al., 2020). Through host bacteria in soil, ARGs can proliferate via horizontal gene transfer and cause pollution of crops and vegetations, hence affecting the safety of soil environment and the food production and supply chains (Gao et al., 2020). Therefore, as pointed out also by Yuan et al. (2019b), quantitative investigation of the abundances of eARGs and iARGs is of great significance for better understanding the occurrence, fate and behavior of ARGs in environment and for effective control and elimination of their detrimental impacts to humans and ecosystems. However, regarding the presence of eARGs and iARGs in WWTFs, particularly the distribution of both the genes in activated sludge, the key component that determines the performance of biological wastewater treatment process and hence the quality of water to be discharged to environment after treatment, available literature for reference is very limited.

The performance of WWTFs is closely related to the solid-liquid separation efficiency for flocs of suspended solids in the settling stage (Wilén et al., 2008a; Li and Pagilla, 2017). In general, flocs with higher settleability are effectively separated from water through gravity settling (Y. Cui et al., 2019). Many factors affect the property and settling

velocity of the flocs, such as the size and density, bacterial activity and community contained in the flocs, and the content of extracellular polymeric substances (EPS) (Chen et al., 2019). Flocs of lower settleability can remain in the effluent after treatment and bring about detrimental effect to the quality of receiving water bodies via small sludge flocs and/or free bacterial species (Li and Pagilla, 2017). A previous study reported that sludge flocs of lower settleability had lower bacterial activity due probably to their composition or structure containing more damaged cells caused by lysis or decay; while, the sludge flocs of higher settleability contained more intact cells and therefore revealed relatively higher bacterial activity (Chen et al., 2019). The bacterial community structure is also found to be a factor affecting the settleability of sludge flocs ((Wilén et al., 2008b). These factors may also affect the existence and distribution state of ARGs in sludge, since it is very likely that the release of ARGs from cells to enable them to exist as extracellular ones, i.e., eARGs, may change with the conditions of cells, and the extent of existence as eARGs may also change with the properties of sludge cells according to the content of EPS that contribute greatly to the adhesion due to their higher viscosity feature, and hence increase the risk of ARGs proliferation via HGT. The possible pathways for the release, diffusion and transfer of eARGs in sludge can probably be very complex; since the structure of the sludge itself is complex, which contains free water, bound water and intracellular water (Dai et al., 2018), and cells inside the sludge flocs comprise both intact and damaged ones (Y. Lu et al., 2020). As depicted in **Fig. 3.1**, a large proportion of eARGs in sludge may come from iARGs of intact cells through secretion (1) or lysis (Nagler et al., 2018) and can be further separated into two types: adsorbed eARGs on the bacterial surface, colloids or larger molecular organic compounds (2, 3) and free eARGs existing in the free water (4) (Q. Bin Yuan et al., 2019). The activity of bacteria in sludge, which varies with the presence proportions of intact and damaged bacterial cells, may

also affect the secretion of EPS (1) and the release of eARGs (2, 3, 4). The released eARGs may find their way to become either adsorbed ones (2, 3, 5) or free ones (4, 6, 7), depending greatly on the property of sludge flocs (size, surface charge, the content of EPS, etc.). Therefore, it is reasonable to presume that the distribution of eARGs and iARGs in sludge may vary among its constituting fractions having different settleability, a topic of great significance that requiring evidence of new experimental data to support.

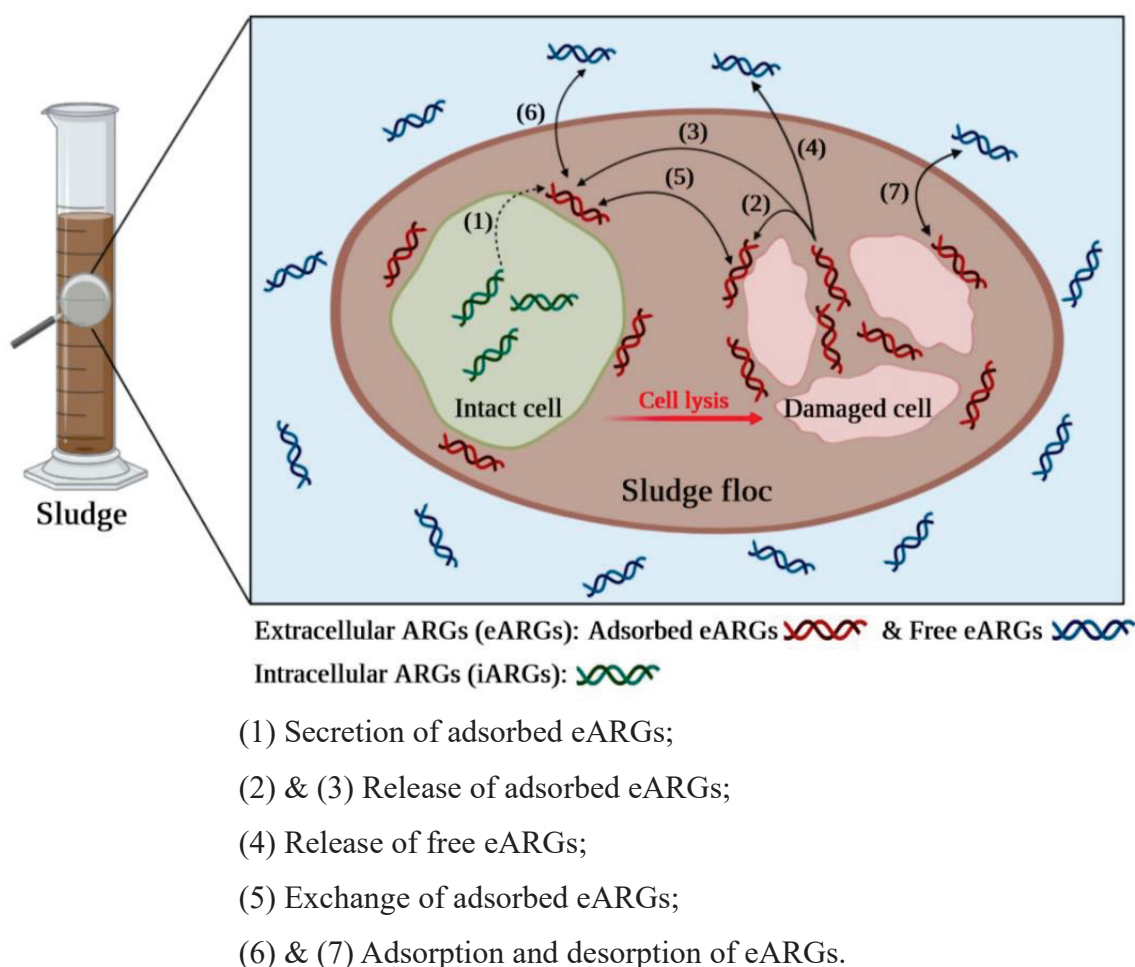


Fig. 3.1 Existence and transfer of extracellular and intracellular ARGs in sludge flocs.

Accordingly, the main objective of this study was to clarify the association of ARGs including eARGs and iARGs with the sludge settleability, which included two specific aims: 1) to investigate the distribution of eARGs and iARGs in sludge fractionated in

terms of settleability; and 2) to evaluate the transfer potential of ARGs in sludge fractions with different settling velocities based on the relative abundances of ARGs and mobile genomic element. To our best knowledge, this study was the first one that hypothesized the distribution of eARGs and iARGs may differ with the sludge properties reflected by settleability. Better understanding of the distribution of eARGs and iARGs in sludge of different settleability can assist to the enhanced reduction of ARGs, especially eARGs through WWTFs in practice, thus mitigating their proliferation in the environment.

3.2 Materials and methods

3.2.1 Fractionation of sludge samples in terms of settleability

Six sludge samples collected from the sediment tank of six household WWTFs in Gifu, Japan, were used as raw sludge samples for this study. These WWTFs treat individual household wastewater and are consisted of an anaerobic tank, aerobic contact tank, sedimentation tank and a contact disinfection chamber. Based on their treatment efficiency for BOD and SS by these WWTFs, the sludge samples were divided into three groups: A1 and A2 (higher treatment efficiency), B1 and B2 (average efficiency performance), and C1 and C2 (lower treatment efficiency), as could be seen in **Fig. 3.2**. The detailed information on the treatment efficiency of the six WWTFs are given in **Table 3.1**. For each sludge sample, using a newly designed settling apparatus, which has a length equivalent to the depth of the settling tank of the WWTFs and therefore enables tracing the whole settling process of suspended solids from the water surface of the tank, the suspended solids in the sludge was fractionated into three fractions in terms of settleability: the fraction of low settleability (LS: $v \leq 6$ m/h), intermediate settleability (MS: $6 \leq v \leq 12$ m/h) and high settleability (HS: $v \geq 12$ m/h). The fractionation process for

each sludge was made in triplicate. The diagram on the settling apparatus is shown in **Fig.**

3.3.

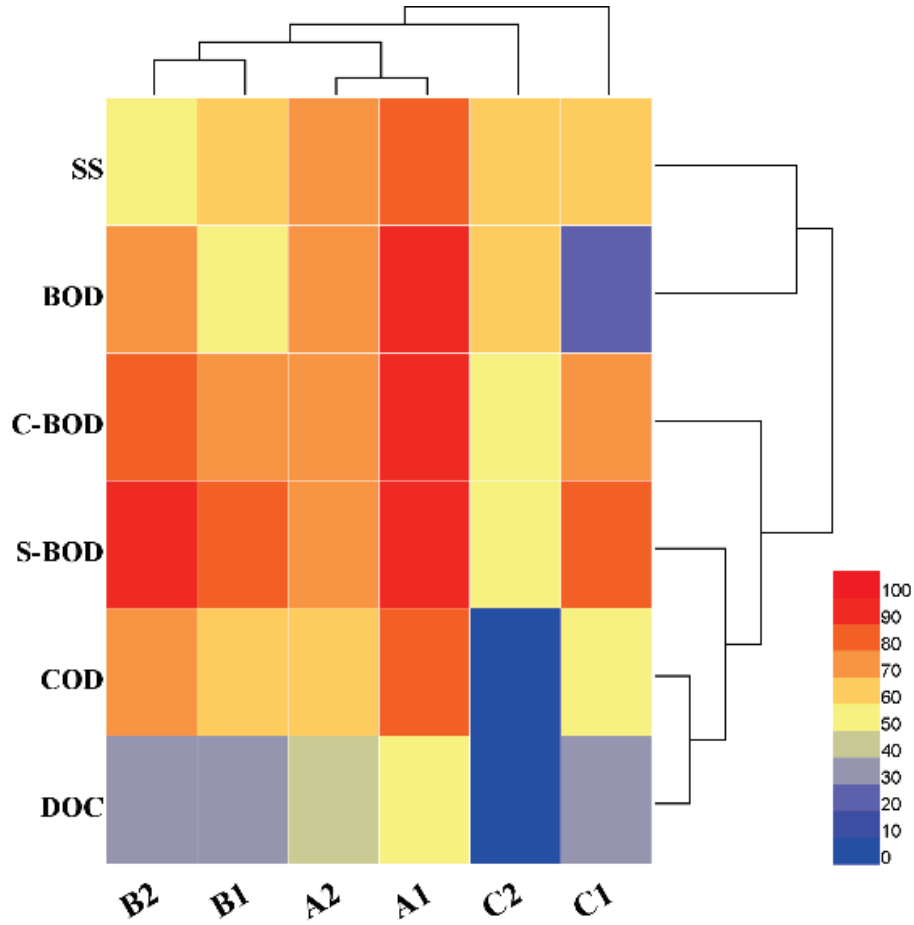


Fig. 3.2 Classification of six WWTFs used in this study based on treatment efficiency.

3.2.2 PMA pretreatment and DNA extraction

Intracellular DNA was quantified by following the documented method of PMA-*q*PCR, a method combining a pretreatment process by propidium monoazide (PMA) and quantitative PCR (*q*PCR) and has been used frequently for detection of intracellular DNA in environmental samples (Li et al., 2014). PMA can enter the compromised membranes of damaged bacterial cells and be subsequently bound to DNA in extracellular part of cell or DNA inside the damaged cells under light condition. Since DNA bound with PMA cannot be amplified during PCR, therefore, this pretreatment enables qualification of

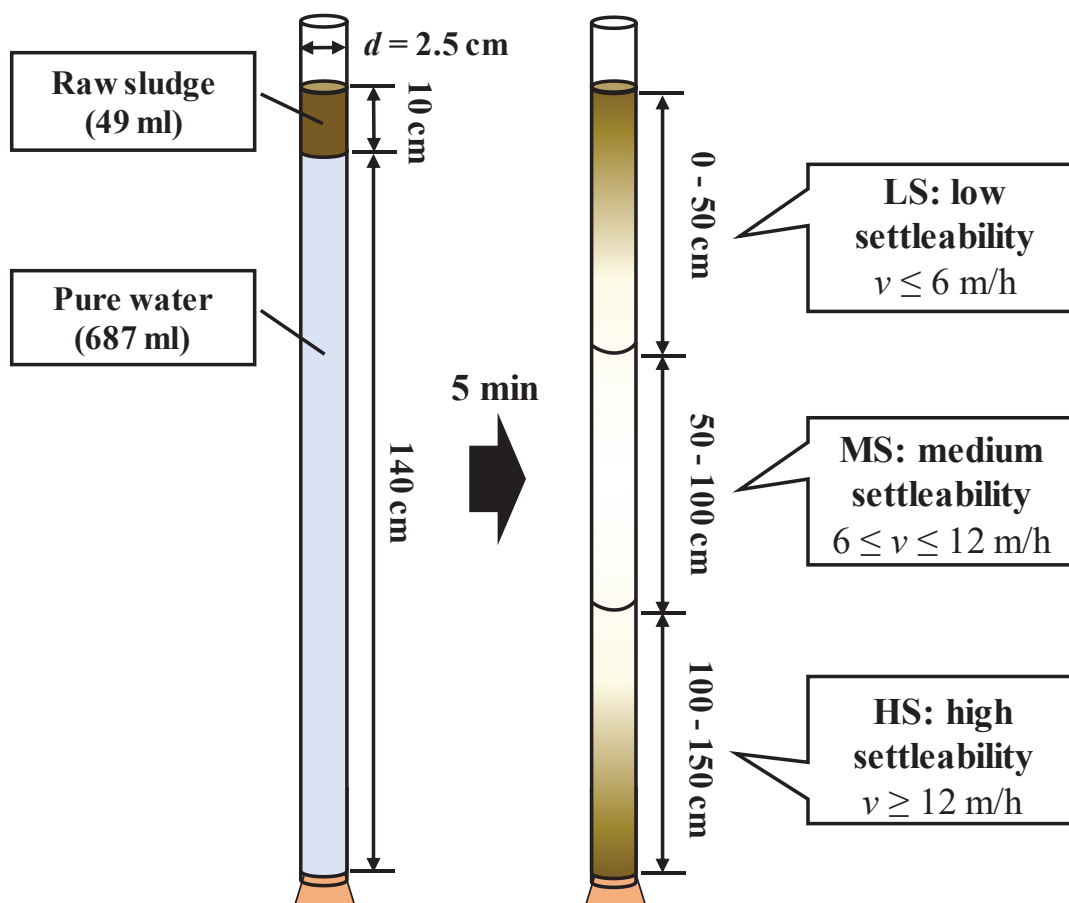


Fig. 3.3 Images of settling tube and settling depths for sludge fractionation based on settleability.

DNA protected by complete membranes of intact bacterial cells by *q*PCR (Li et al., 2014). The fractionated sludge samples were pretreated before DNA extraction by PMA following the process described (Li et al., 2014; G. Cui et al., 2019). Briefly, for each sludge sample, 2 mL was taken and was divided uniformly into two parts (each with 1 mL) inside two 2 mL centrifuge tubes, respectively. One tube was mixed with 10 μL of PMA solution (25 μM) and was used for *q*PCR of intracellular DNA; while, the other tube without the addition of PMA was used for *q*PCR of total DNA. Both tubes were shaken and placed under dark condition for 5 min and were then photolyzed by a PMA-Lite™ LED Photolysis Device (Biotium, USA) for 15 min. DNA extraction for all

samples after the photolyzing treatment was conducted using Power soil DNA Extraction Kit (MOBIO, USA) based on manufacturer's manual. The extracted DNA was stored at -25 °C prior to determination for target genes by *q*PCR.

3.2.3 Determination of ARGs, *intl 1*, and 16S rDNA

The tetracycline resistance genes (*tet G*, *tet M*), sulfonamide resistance gene (*sul I*), the mobile genetic element (MGE) associated integrase class 1 gene (*intl 1*), and 16S rDNA gene were quantified by *q*PCR with the utilization of SYBR® Premix Ex Taq™ (TaKaRa, Japan) based on the manufacturers' protocol. The reason for selecting these three types of ARGs for study is because tetracycline and sulfonamide are the most commonly used antibiotics (Luo et al., 2010). The *intl 1* gene plays a significant role in the proliferation of ARGs owing to its capability to capture and spread gene cassettes containing ARGs (Gaze et al., 2011). The absolute abundance of each target gene was quantified in total (both extracellular and intracellular) and in intracellular depending on if the sample underwent the pretreatment with PMA or not. The absolute abundance of the extracellular genes for each sample was evaluated as the difference between the quantified total abundance and intracellular abundance. The relative abundance of ARGs and MGE was defined by dividing the absolute abundance with the total 16S rDNA gene copy numbers. The primer information and *q*PCR conditions are given in **Table 3.2** (G. Cui et al., 2019).

3.2.4 Observation of intact/damaged bacteria and sludge flocs

The distribution of intact/damaged bacteria and the morphological features of sludge flocs in fractionated sludge samples were observed by using fluorescence microscope. Double-staining and observation were conducted following the report of Corno et al. (2019) and Xia et al. (2019). The double-staining procedure was used to assess the distribution of

intact/damaged bacterial cells through simultaneous staining by membrane permeable nucleic acid dye (SYBR Green I) and the membrane impermeable dye (Propidium iodide: PI). All sludge samples were diluted 100 times by sterilized phosphate-buffered saline before staining. Incubation was conducted under dark condition at 4 °C for 15 min after adding SYBR Green I and PI with a final concentration of 10 µg/mL for each. After incubation, 1 mL of each sample was filtered through gray polycarbonate filter with a pore size of 0.2 µm. The filter paper was transferred to a glass slide and covered by a glass cover slip, and then immediately observed with a fluorescence microscope (DS-Ri1, Nikon Co., Japan). Intact bacterial cells showed green color under blue excitation, which differ from the damaged bacterial cells red-marked.

Table 3.2 Information of primers for target genes and the quantitative PCR conditions.

Genes	Primer sequence (5'- 3')	Annealing temperature	Amplicon size (bp)
16S rDNA	Com1: CAG CAG CCG CGG TAA TAC	50 °C	407
	Com2: CCG TCA ATT CCT TTG AGT TT		
<i>intl 1</i>	F: GGC TTC GTG ATG CCT GCT T	57 °C	148
	R: CAT TCC TGG CCG TGG TTC T		
<i>tet G</i>	F: TTA TCG CCG CCG CCC TTC T	55 °C	133
	R: TCA TCC AGC CGT AAC AGA AC		
<i>tet M</i>	F: ACA GAA AGC TTA TTA TAT AAC	45 °C	171
	R: TGG CGT GTC TAT GAT GTT CAC		
<i>sul 1</i>	F: CAC CGG AAA CAT CGC TGC A	55 °C	158
	R: AAG TTC CGC CGC AAG GCT		

※ F: forward; R: reverse

3.2.5 Statistical analysis

For better understanding the classification of the fractionated sludge in terms of settleability from the viewpoint of AGRs, heatmap and cluster figures were drawn by using the software of HemI (Heatmap Illustrator, version 1.0) as used by others (Xia et al., 2019).

3.3 Results and discussion

3.3.1 Intact/damaged bacteria and sludge features

The observation results on intact/damaged bacteria and the morphological features of sludge flocs fractionated based on settleability are shown in **Table 3.1**. Three typical types of flocs with different structures were observed: primary flocs (single bacterial cells), secondary flocs formed by bacterial colonies (single cells bound together), and tertiary flocs formed by bacterial colonies (Wilén et al., 2008). The sludge flocs in the LS fractions of all raw sludge samples were generally smaller than those in the MS and HS fractions (except sludge C2). It means, in other words, that more single bacterial cells and smaller bacterial colonies existed in the LS fractions with low settleability. It is also noticeable that, most LS fractions contained a larger proportion of damaged cells marked in red by PI (e.g. sludge A2); even if there were exceptions where some LS fractions (e.g. sludge A1) contained more single intact bacterial cells marked in green by SYBR Green I. The exact proportion of intact and damaged bacterial cells will be further discussed later.

Table 3.1 Distribution of intact/damaged bacteria and sludge flocs in sludge fractionated in terms of settleability (Scale bar: 200 μm). The intact bacteria are shown in green stained by SYBR Green I and the damaged bacteria are shown in red stained by propidium iodide (PI). LS: sludge of low settleability; MS: sludge of medium settleability; HS: sludge of high settleability.

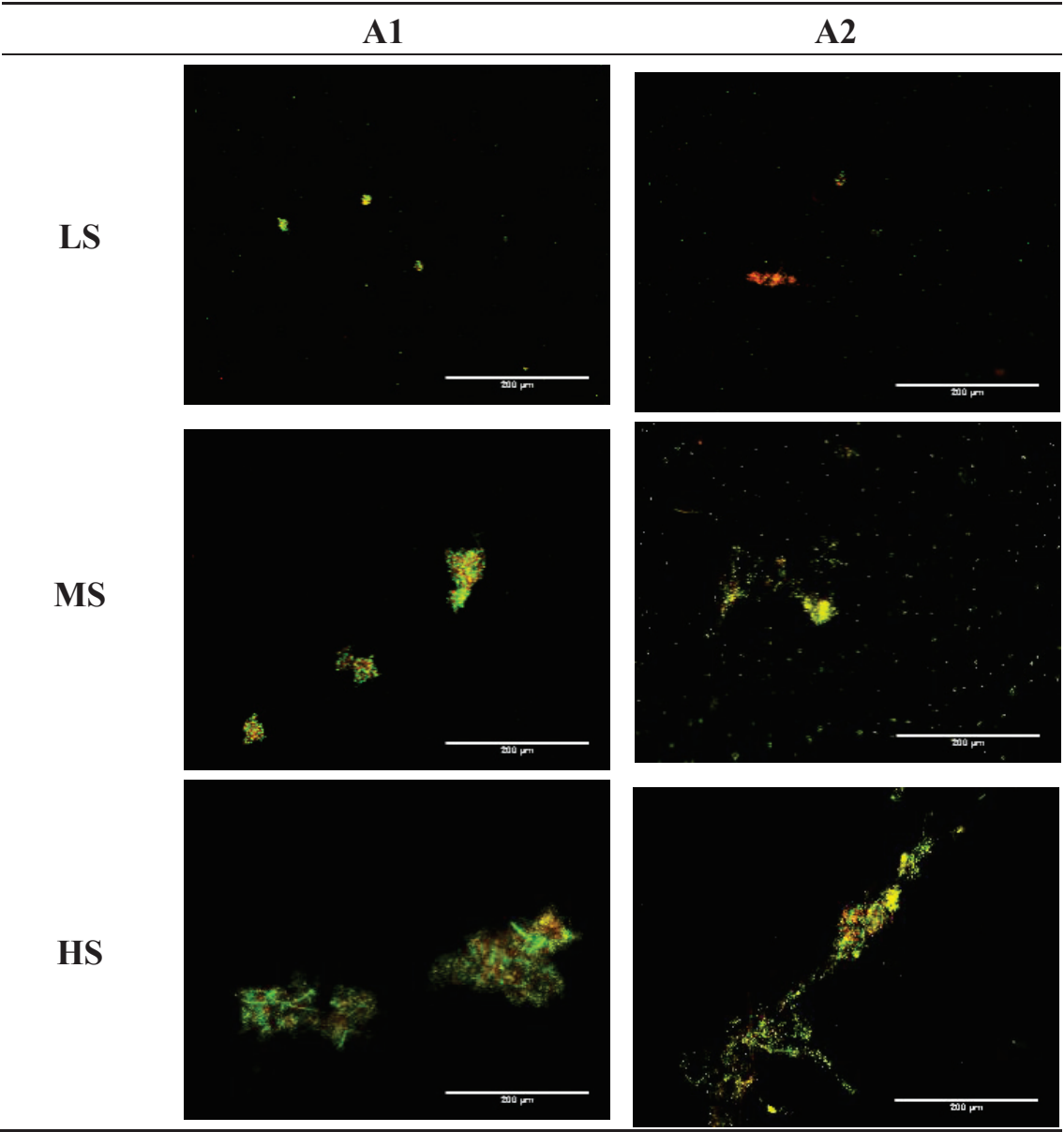


Table 3.1 Distribution of intact/damaged bacteria and sludge flocs in sludge fractionated in terms of settleability (Scale bar: 200 μm). The intact bacteria are shown in green stained by SYBR Green I and the damaged bacteria are shown in red stained by propidium iodide (PI). LS: sludge of low settleability; MS: sludge of medium settleability; HS: sludge of high settleability. **(Continued)**

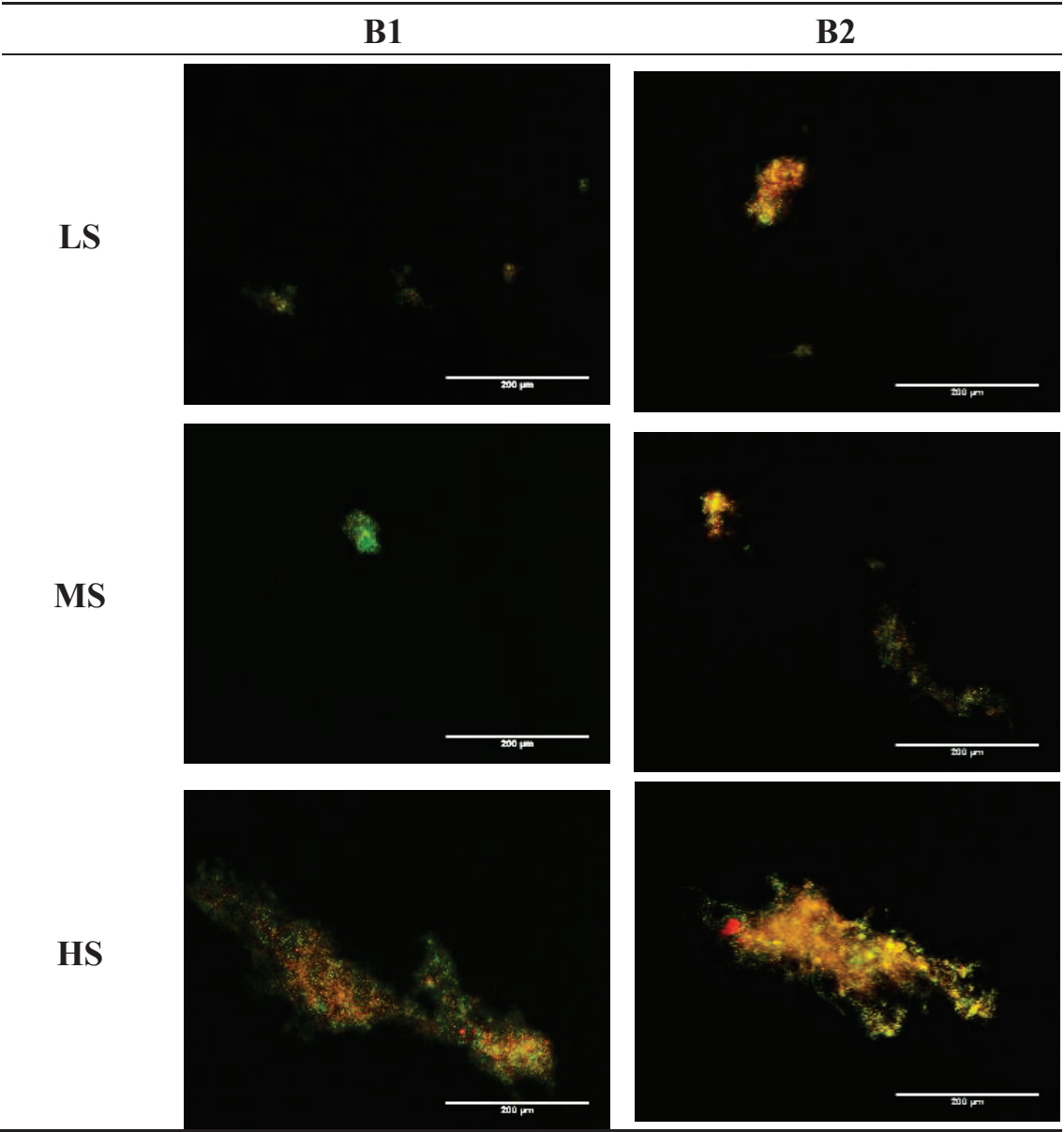
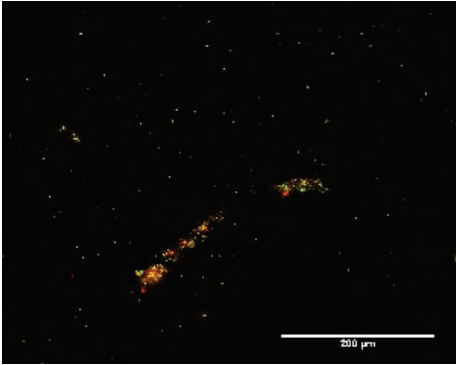
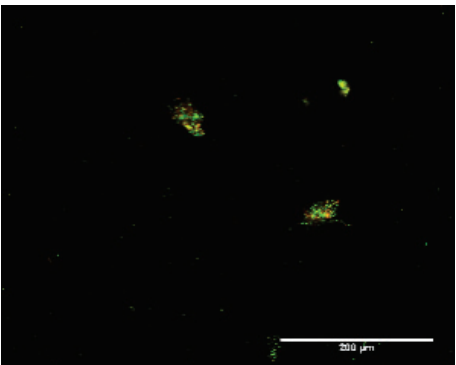
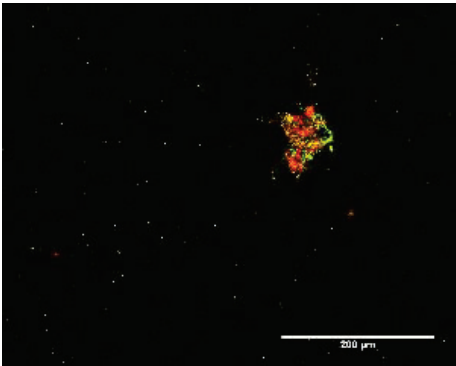
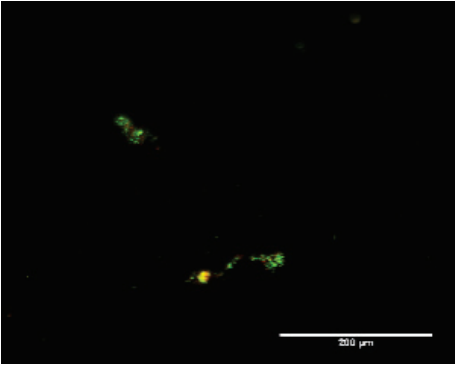
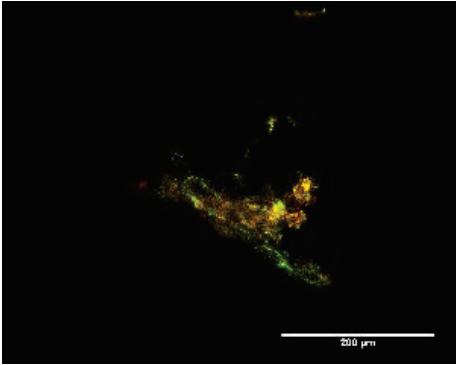
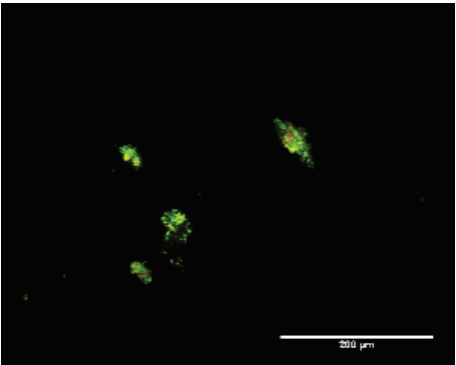


Table 3.1 Distribution of intact/damaged bacteria and sludge flocs in sludge fractionated in terms of settleability (Scale bar: 200 μm). The intact bacteria are shown in green stained by SYBR Green I and the damaged bacteria are shown in red stained by propidium iodide (PI). LS: sludge of low settleability; MS: sludge of medium settleability; HS: sludge of high settleability. **(Continued)**

	C1	C2
LS		
MS		
HS		

For sludge flocs with high settleability (in the HS fractions), their sizes were about two or three times larger than flocs with intermediate settling velocities (in the MS fractions), except the sludge sample of C2. For C2, the floc sizes of its three fractions showed no significant differences, implying that aggregation of cells to larger flocs seen in other sludge samples did not occur or occurred less distinctly in this sludge that was associated with more suspended particles in the effluent of the facility, although the proportion of intact bacterial cells seemed to be higher. Moreover, in most HS fractions of the sludge samples, filamentous bacteria, a species reported to be able to promote cells aggregation and thus contributing to their separation from water (Burger et al., 2017), were observed. Effective separation of sludge cells in sedimentation is crucial and smaller and less dense cell flocs are always blamed for their remaining in the final effluent of WWTFs (Wilén et al., 2008b). Interestingly, larger flocs formed by filamentous bacteria were also observed in the LS fraction of sludge B2. However, since a larger proportion of the bacteria in the flocs were damaged or dead ones stained in red by PI, the lower settleability of the sludge flocs of this fraction was probably associated with their property of being less dense for settling.

3.3.2 Absolute abundances of eARGs and iARGs

The absolute abundances of *tet G*, *tet M*, and *sul I* in both the extracellular (eARGs) and intracellular (iARGs) parts of the fractionated sludge samples are shown in **Fig. 3.3**. The absolute abundances of the targeted genes in the sludge of A1, C1 and C2 were smaller by 1 - 2 orders of magnitude as compared to those in the sludge of A2, B1, and B2. This supports our previous finding in vermicomposting that the presence extent of the ARGs in excess activated sludge changes with the sludge types and the utilization frequency for antibiotics (G. Cui et al., 2019). Even for a given raw sludge type, the

concentrations of eARGs and iARGs differed greatly among the fractions divided in terms of settleability. This difference probably reflected the likely differences of the contained bacteria in the fractions regarding their structure and activity, which had probably affected the aggregation capability of the contained bacteria to form colonies or flocs with different settling velocities. As reported before, for instance, *Betaproteobacteria* can grow in colonies with sizes of 10-100 μm ; while, *Alphaproteobacteria* can exist as single cells or in colonies of 5-20 μm (Wilén et al., 2008b).

For *tet G*, the absolute abundances in the extracellular part of the sludges A1, B2 and C2 were extremely higher than those in the intracellular part, with the largest abundance values being found for the fractions of low settleability (the LS fractions). The antibiotic resistance mechanism of *tet G* is efflux pump (Roberts, 2005), which is known to reduce the accumulation of antibiotics inside the bacterial cells and slows the function process of antibiotics to provide sufficient time for bacteria to adapt to the antibiotics, thus becoming resistant through mutation or alteration of antibiotic targets (Li and Nikaido, 2011). Unlike *tet G*, the antibiotic resistance mechanism of *tet M* is ribosome protection through the allosteric disruption of antibiotic binding sites to ribosomes, thereby leading to the release of antibiotic molecules from the ribosome (Roberts, 2005). The absolute abundances of *tet M* in both extracellular and intracellular parts showed similar results with those of *tet G* in the fractionated sludge of all raw sludge types, except the extracellular *tet M* in sludge C2. On average, the absolute abundance of *tet M* in all fractionated sludge was about one order lower than *tet G*. As a plasmid-borne gene, *sul I* is reported to be generally linked to other resistance genes in integron for proliferation (Sköld, 2000). This means, no matter the extracellular or the intracellular *sul I* is concerned, both may give rise to the high risk of HGT. The sludge fraction of low

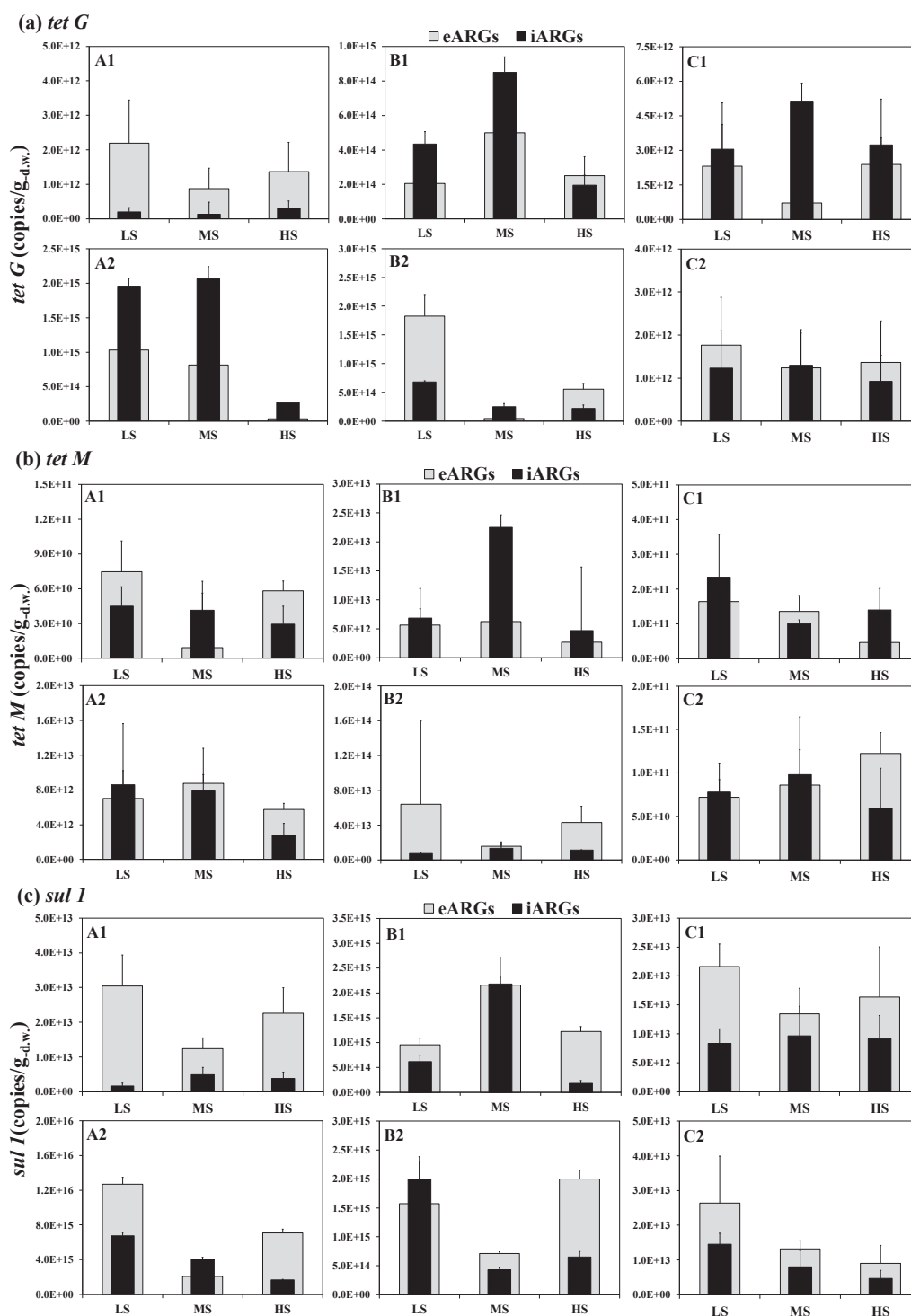


Fig. 3.3 Absolute abundances of extracellular and intracellular ARGs, eARGs and iARGs, in sludge fractionated in terms of settleability (against dry weight of sludge): (a) *tet G*, (b) *tet M*, and (c) *sul I*. Data are presented as mean and standard deviation, $n = 3$. LS: sludge of low settleability; MS: sludge of medium settleability; HS: sludge of high settleability.

settleability (the LS fractions) had the highest abundance level for *sul 1* in the extracellular part. This was found in all used raw sludge types excluding B1 and B2. For the sludge B1, the abundance levels of both the extracellular and intracellular *sul 1* were markedly higher in the MS fraction.

Since eARGs and iARGs are presented in extracellular and intracellular parts of bacteria, their abundances may closely associate with the respective abundances of 16S rDNA (eDNA and iDNA). As shown in **Fig. 3.4**, the total copy number of 16S rDNA in the fractions of the sludges A2, B1 and B2 reached 10^{17} - 10^{18} copies/g, which was about two orders higher the sludges A1, C1 and C2 detected for 10^{15} - 10^{16} copies/g, clearly revealing the difference of the investigated sludges in their composition regarding the cell density. In similarity with the distribution of eARGs and iARGs, the distribution of eDNA and iDNA in the fractions of sludge fractionated in terms of settleability was also distinct among the raw sludges. For sludges A1, A2 and C2, the abundances of eDNA in the LS fractions were relatively higher than those in the MS and HS fractions. For sludges B2 and C1, however, the abundances of eDNA in the corresponding HS fractions of high settleability were also higher. In regard of the abundance of iDNA, the MS fractions with intermediate settling velocities showed relatively higher levels than the LS and HS fractions with low and high settleability, respectively for the sludges of B1, A2, C1 and A1. The highest abundance levels of iDNA were found with the LS fractions of the sludges of B2 and C2. The above results revealed clear that the presence and distribution of eARGs and iARGs in sludges were in close association with the abundance of the respective total DNA, i.e., eDNA and iDNA. This can thus serve as an indirect evidence to support a recent indication by Wang et al. (2020) after investigation of full-scale membrane bioreactors for wastewater treatment that a strong correlation may exist between the total 16S rDNA and the number of ARB or the related genes (ARGs).

The presence of the extracellular genes in the total genes of the targeted *tet G*, *tet M*, *sul 1*, *intl 1* and 16S rDNA among all sludge fractions of different settleability are shown **Table 3.2**. For all raw sludges, a general trend of higher presence percentages of eARGs in the fractionated sludge based on settleability seemed to be existent, excluding only a few exceptions that showed lower presence percentages in the LS fractions for the gene of *tet M* in the sludges A2 and C1 and the gene of *sul 1* in the sludge of B2. Similar to eARGs, eDNA also showed larger percentages in the LS and HS fractions than in MS fraction except for the sludge B1. Chen et al. (2019) indicated in their study that the apoptotic-like decaying cells, late apoptotic cells and intact cells were significantly different in the gravity-separated sludge fractions of different settling velocities. This may imply that the state of bacterial cells may affect the settleability and the distribution of

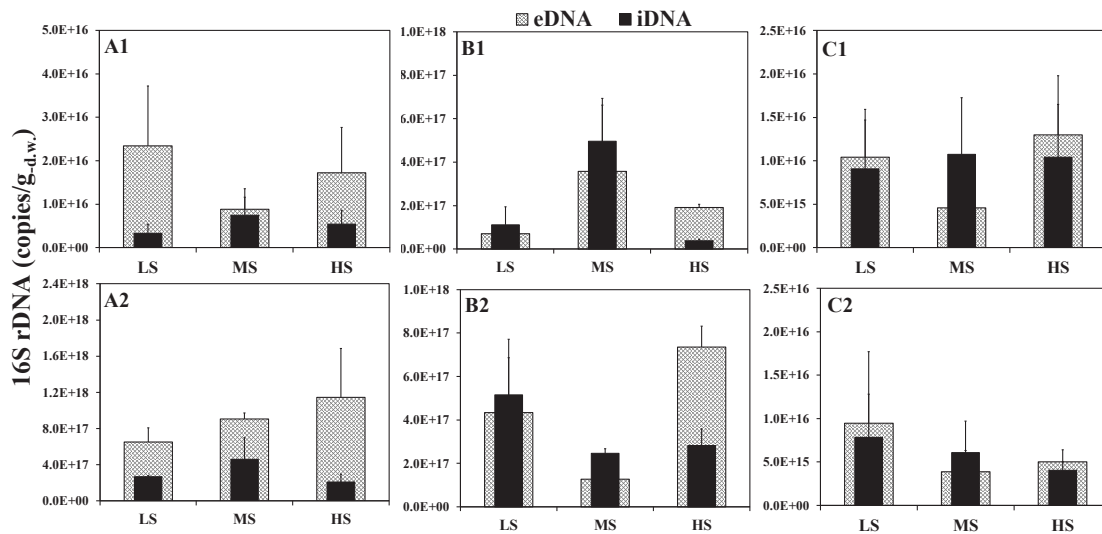


Fig. 3.4 Distribution of extracellular 16S rDNA (eDNA) and intracellular 16S rDNA (iDNA) in sludge fractionated in terms of settleability (against dry weight of sludge). Data are presented as mean and standard deviation, $n = 3$. LS: sludge of low settleability; MS: sludge of medium settleability; HS: sludge of high settleability.

eDNA and iDNA, linking to the distribution differences of eARGs and iARGs. It is highly considerable that eARGs and eDNA in the LS fractions were mainly contributed to the lysis or decay of bacteria, as suggested by the observed images that, in most cases, the sludge fractions of low settleability contained more damaged bacteria (**Table 3.1**). By effectively reducing the concentration of low settling sludge flocs remaining in the wastewater after treatment, the transfer and spreading potential of eARGs in receiving water bodies and the total water environment can be probably reduced. This will minimize the extend of the adversary effects on soil environment and food production and supply chains as a result of controlling of the pathways of ARGs through the treated wastewater reuse and discharge (Manaia et al., 2018; Corno et al., 2019; Gao et al., 2020). For the detected higher presence percentages of eARGs in the HS fraction, however, other reasons beyond the damaged cells probably existed since the proportion of damaged cells was very low as shown earlier (**Table 3.1**). There are two ways related to the occurrence of eARGs or eDNA: the lysis or decay of damaged bacterial cells and the secretion of intact bacterial cells (Yuan et al., 2019b). The distinct presence of eARGs and eDNA in the HS fractions may be mainly caused by the secretion of intact cells. The higher concentration of EPS secreted by bacterial cells in the HS fractions might have contributed to the formation of the bigger sludge flocs with a tightly compact structure. EPS can not only increase the hydrophobicity and the negative surface charge of sludge flocs, thus resulting in enhanced sorption capacity for DNA (Basuvaraj et al., 2015), but can also contain abundant DNA in itself. The higher concentration of sludge flocs in the HS fractions may also be a possible reason for the higher presence percentages of eARGs, since more particles and extracellular organic matter molecules are reported to be capable of alleviating the susceptibility of genes or DNA to nuclease attack (Mao et al., 2014; Dong et al., 2019).

Table 3.3 The presence of the extracellular genes in the total genes of *tet G*, *tet M*, *sul I*, *intl 1* and 16S rDNA. A1-C2 represent raw sludge from 6 wastewater treatment facilities. LS, MS and HS represents three sludge fractions with low, medium and high settleability, respectively. Data are presented as mean, n =3.

Extracellular <i>tet G</i> /Total <i>tet G</i> (%)						
	A1	A2	B1	B2	C1	C2
LS	91.7	34.5	32.2	72.9	43.2	58.9
MS	86.6	28.2	37.0	15.7	12.2	48.8
HS	81.6	10.7	56.3	71.5	42.4	59.7
Extracellular <i>tet M</i> /Total <i>tet M</i> (%)						
	A1	A2	B1	B2	C1	C2
LS	62.5	45.0	45.2	89.6	41.2	48.1
MS	18.1	52.6	21.7	54.3	57.4	46.7
HS	66.5	67.4	36.4	79.0	25.1	67.3
Extracellular <i>sul I</i> /Total <i>sul I</i> (%)						
	A1	A2	B1	B2	C1	C2
LS	94.8	65.3	60.8	44.1	72.2	64.5
MS	71.6	34.0	49.7	62.4	58.3	62.2
HS	85.4	81.0	87.4	75.4	64.0	65.9
Extracellular <i>intl 1</i> /Total <i>intl 1</i> (%)						
	A1	A2	B1	B2	C1	C2
LS	91.9	70.0	71.2	21.6	85.4	67.6
MS	73.7	16.5	78.6	19.5	49.9	57.7
HS	94.2	75.9	83.7	70.9	65.5	59.8
Extracellular 16S rDNA/Total 16S rDNA (%)						
	A1	A2	B1	B2	C1	C2
LS	87.6	70.9	38.8	45.7	53.4	54.7
MS	54.4	66.4	41.9	33.9	29.9	38.9
HS	76.2	84.7	83.6	72.3	55.5	55.5

Scale (%)
100
80
60
40
20
0

IS: low settleability; MS: medium settleability; HS: high settleability.

3.3.3 Relative abundances of eARGs and iARGs

The relative abundances of three target ARGs (*tet G*, *tet M*, and *sul I*) in both extracellular (eARGs) and intracellular (iARGs) parts of the fractionated sludge samples are shown in **Fig. 3.5**. For each raw sludge, the abundance levels of either eARGs or iARGs differed significantly among the fractions of different settleability. For sludge B2, the highest abundance level of *tet G* appeared in the LS fraction, while the highest abundance levels for *tet M* and *sul I* were recorded in its MS fraction. For sludge B1 and C2, the highest relative abundance levels for all eARGs and the total ARGs were found also in their LS fractions. The results may thus imply that small sludge flocs or damaged bacterial cells in the sludge fractions of lower settleability may possess higher transfer potential for ARGs after entering the natural water environment due to their higher distribution in the extracellular part of the cells, i.e., the observed higher relative abundances of eARGs. The higher relative abundances of eARGs are probably attributed to their slower degradation rate and higher persistence, as could be inferred from the report of Mao et al. (2014) that plasmid eDNA hosting the ARGs was more persistent than 16S rDNA genes in chromosome. The persistence and transforming ability of eARGs have also been reported by Dong et al. (2019) who emphasized the important roles of the extracellular genes in ARGs proliferation. The relative abundance of ARGs higher than 10^{-4} gene copies/16S rDNA copies is considered as a highly contaminated reception (Graham et al., 2011). Based on this, the sludge samples A1, C1 and C2 used in this study can be classified as highly contaminated by ARGs.

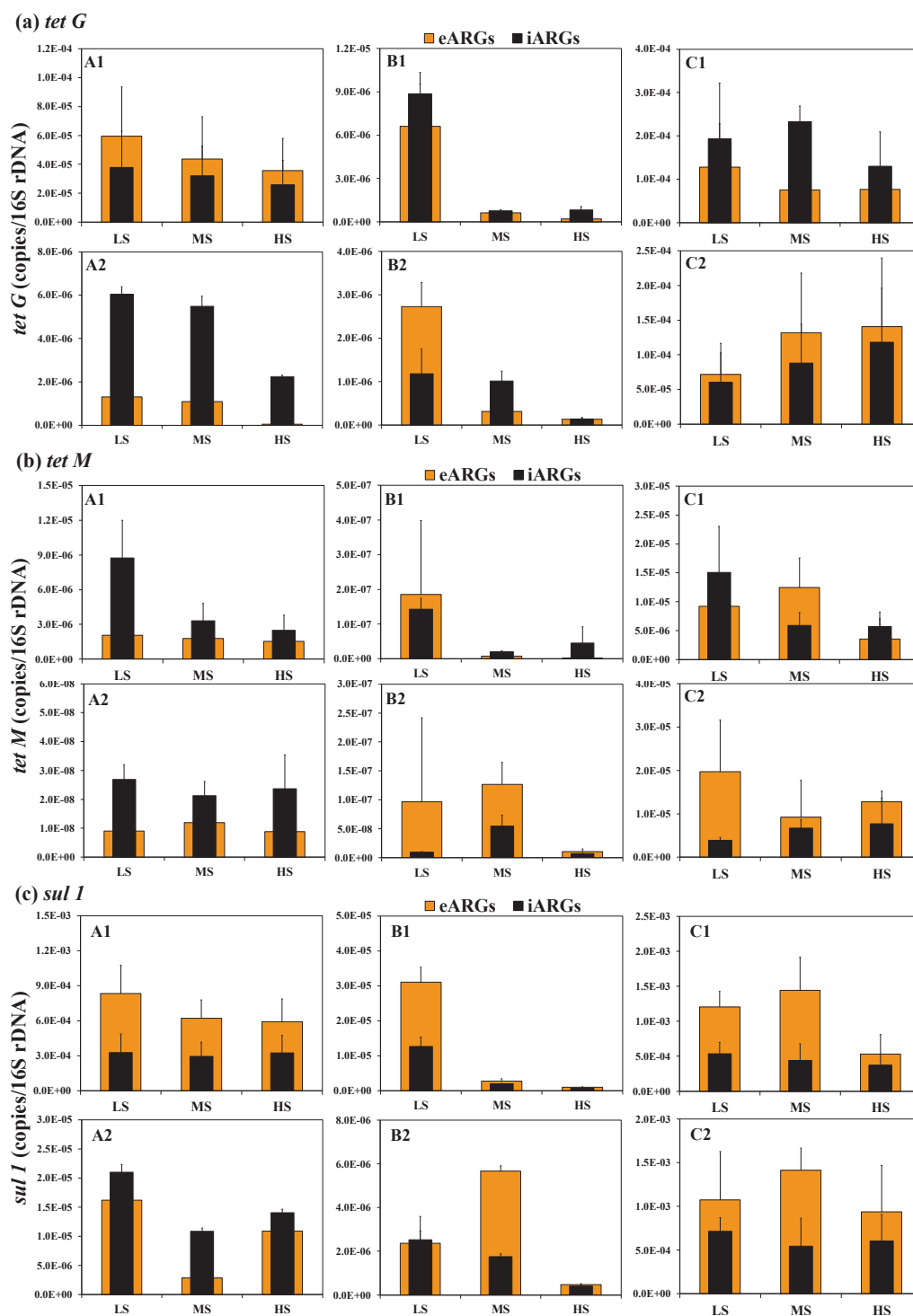


Fig. 3.4 Relative abundances of extracellular and intracellular ARGs, eARGs and iARGs, in sludge fractionated in terms of settleability (against total 16S rDNA): (a) *tet G*, (b) *tet M*, and (c) *sul I*. Data are presented as mean and standard deviation, $n = 3$. LS: sludge of low settleability; MS: sludge of medium settleability; HS: sludge of high settleability.

3.3.4 Absolute and relative abundances of eMGE and iMGE

The absolute and relative abundances of *intl 1*, the well targeted MGE, in both the extracellular and intracellular parts of fractionated sludges (eMGE and iMGE) are shown in Fig. 3.6. Similar with 16S rDNA, the MGE gene in the sludges A2, B1 and B2 was

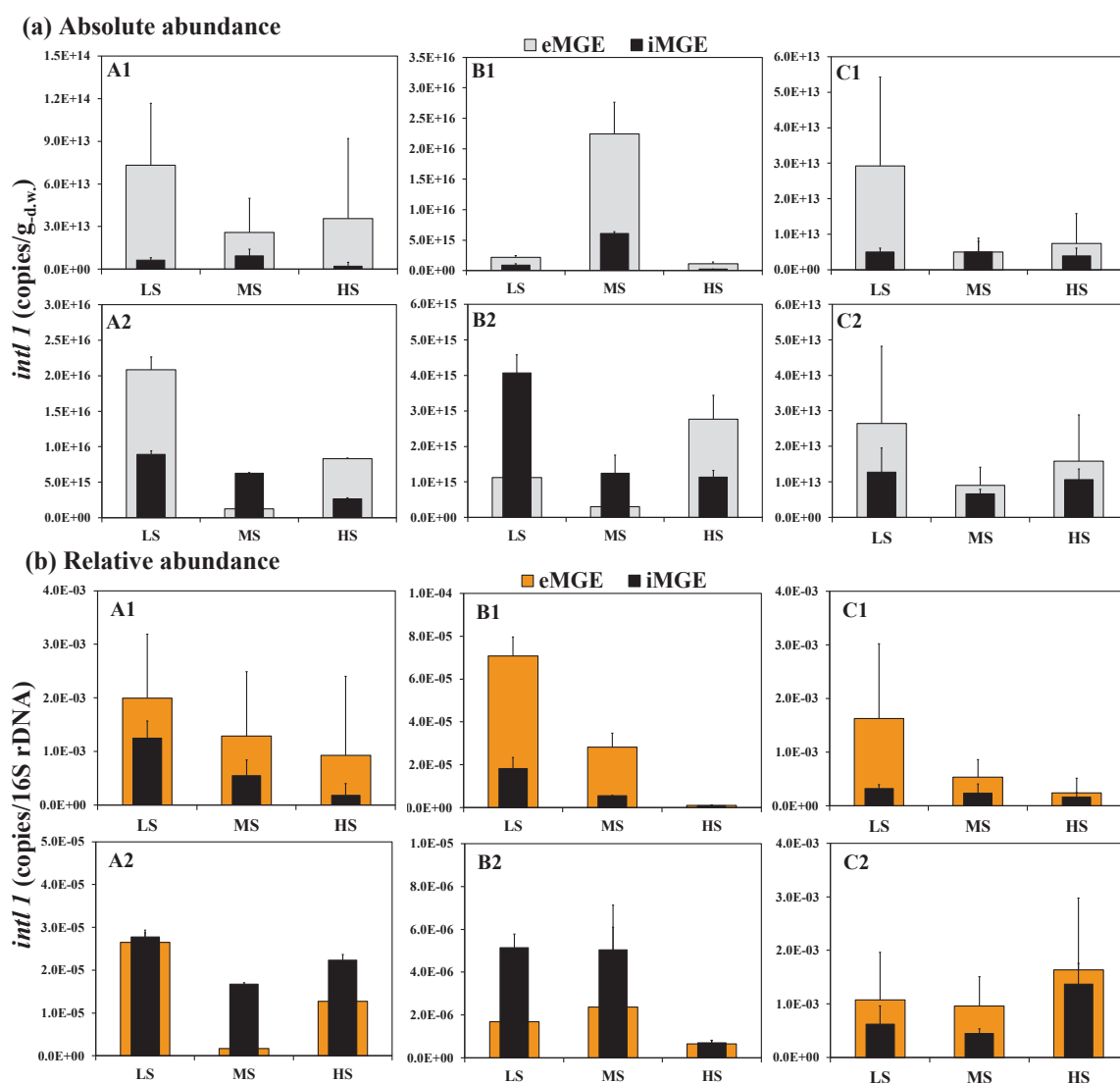


Fig. 3.5 Absolute (a) and relative (b) abundances of the mobile genomic element gene *intl 1* in extracellular part (eMGE) and intracellular part (iMGE) of the sludge fractionated in terms of settleability (d. w.: dry basis). Data are presented as mean and standard deviation, $n = 3$. LS: sludge of low settleability; MS: sludge of medium settleability; HS: sludge of high settleability.

generally 1-2 orders higher than the sludges A1, C1 and C2 in its absolute abundances. A general trend of higher absolute abundances for the total *intl 1* was found for the LS fractions of all raw sludges, except B1 which showed higher abundance level in its MS fraction. It is thus conceivable that the sludge with lower settleability may have higher ARGs transfer potential. Sui et al. (2019) pointed out that the ARGs could locate on MGE and present as extracellular and intracellular DNA. Although some MGEs (such as plasmid) are incompatible, they can still promote HGT of ARGs by extracellular DNA which could be uptaken by competent cells through transformation. On the other hand, for the relative abundance of *intl 1*, the highest levels were also recorded in the LS fractions of all raw sludge samples except A2 and C2. For the sludge C2, significant differences in the relative abundance of *intl 1* was not observed among its three fractions of different settleability, revealing a close link with the similar features of the sludge flocs observed from their fluorescent microscopic images (**Table 2**).

3.3.5 Classification of fractionated sludge samples

The results of the heatmap and cluster analysis conducted based on both absolute and relative abundances of ARGs and MGE in the LS, MS and HS fractions of six all raw sludges are shown in **Fig. 3.7**. It could be seen from **Fig. 3.7a** that, for all sludges except A1, the HS fraction was separated with the LS and MS fractions. The genes of *tet G* and *tet M* were clustered together and were separated from the cluster of *sul 1* and MGE. This may imply that the responses of bacteria regarding their resistance to tetracyclines and sulfonamide were distinctly different. A close relation was found between *sul 1* and MGE, serving probably as a new evidence supporting the previous report that *sul 1* was a plasmid-borne ARG proliferated greatly through HGT (Sköld, 2000). eARGs and iARGs distributed in different clusters, hinting that they may have different transfer potential and

may transfer through different pathways (Y. Zhang et al., 2018). Similar results are also displayed by clustering based on the relative abundances of ARGs and MGE, as could be seen from **Fig. 3.7b**. For the sludge A1, its HS fraction was not separated from its LS fraction (**Fig. 3.7a**). This could be explained as the reason that the absolute abundances of eARGs and eMGE in the HS fraction were as higher as the LS fraction, thereby resulting in similar distribution of eARGs or eMGE.

The observed results implied that the sludge with different settleability may contribute differently to the proliferation of ARGs regarding its potential and/or pathways. For sludge with lower settleability (LS fractions, or MS fractions in few cases), which, in most cases, contains smaller flocs or more damaged bacterial cells, the presence of ARGs, MGE and 16S rDNA was found more abundant in the extracellular part. This sludge fraction can thus become a main contributor for further proliferation of ARGs in the receiving water environment if discharged through the effluent from WWTFs. Enhancing its settleability, therefore to reduce its remaining concentration in the effluent, is a way to control the dissemination of ARGs in the environment, an important indication, which is also hinted in a recent research of Dong et al. (2019) that emphasized the need to focus more on eARGs in relevant studies.

On the other hand, for sludge with higher settleability (the HS fraction), which is consisted of more bigger flocs or more intact bacterial cells, the higher absolute abundances of 16S rDNA, ARGs, and MGE detected in both extracellular and intracellular parts may suggest that this fraction is also a significant repository of ARGs, even if the relative abundances are generally lower than the LS and MS fractions. Sludge with higher settleability is considered as a stable colloid system, in which water is wrapped around the sludge particles by organic matter and cannot be easily released (Dai et al., 2018). However, eARGs loosely attached on the surface of sludge flocs are easy to

enter water through the dewatering process of the sludge. To reduce the eARGs in wastewater from dewatering process, some pretreatment is necessary from the viewpoint of ARGs, for instance pre-coagulation investigated by Li et al. (2019) for removal of ARGs, especially eARGs. For the dewatered sludge, biological methods, such as vermicomposting (G. Cui et al., 2019), are possible approaches to reduce ARGs. To eliminate the number of ARGs in sources where the selection of antibiotic resistance is likely to occur is a way to inhibit the expansion of antibiotic resistance in the environment (Bengtsson-Palme et al., 2016). It thus reasonable to infer that increasing sludge settleability through optimization of its properties (e.g. floc size, bacterial activity) is probably an effective way to mitigate the proliferation of ARGs in environment through WWTFs.

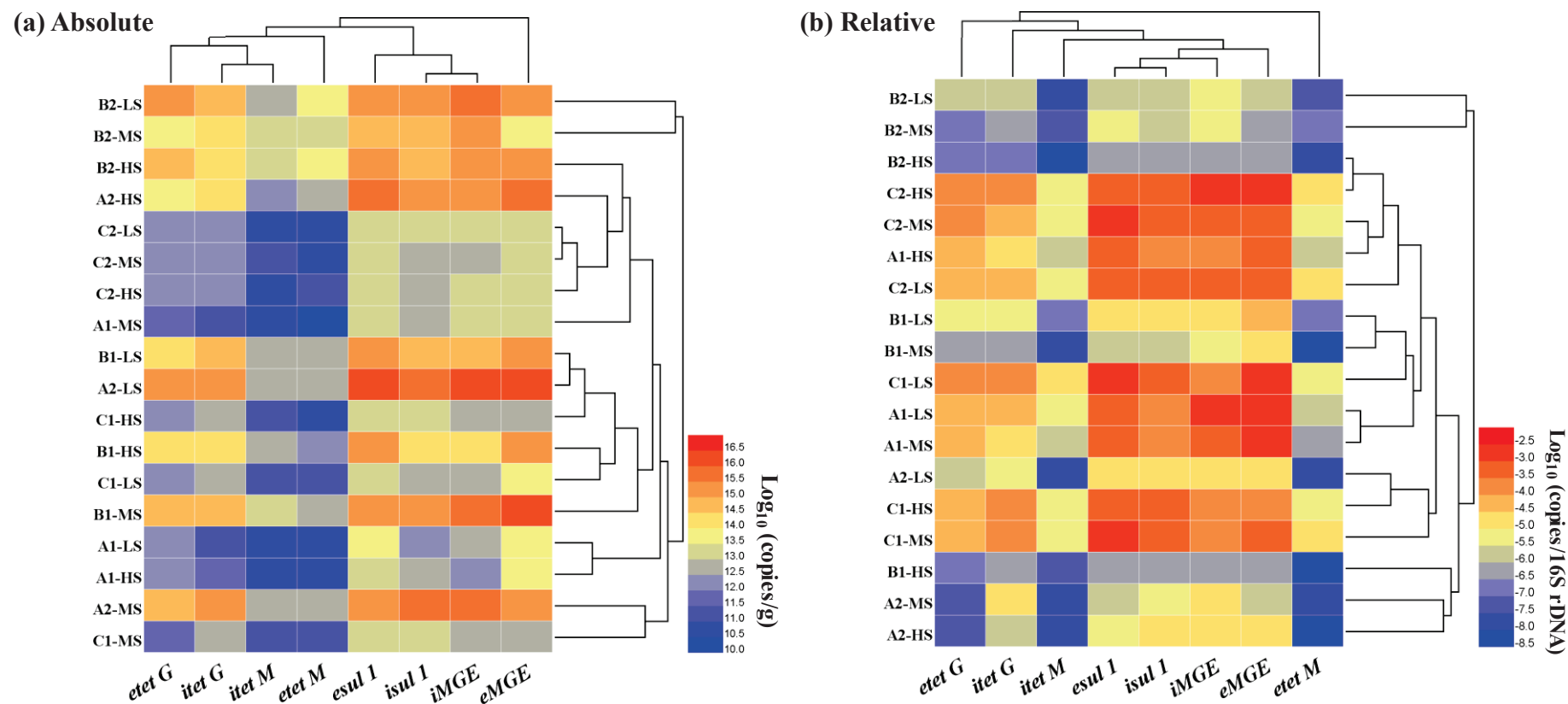


Fig. 3.7 Classification results of heatmap and cluster analysis based on the absolute (a) and relative (b) abundances of extracellular and intracellular ARGs and MGE of all sludge samples fractionated in terms of settleability. *etet G*, *etet M* and *esul 1*: extracellular *tet G*, *tet M* and *sul 1*; *itet G*, *itet M* and *isul 1*: intracellular *tet G*, *tet M* and *sul 1*; *eMGE* and *iMGE*: extracellular and intracellular *intl 1*; LS: sludge of low settleability; MS: sludge of medium settleability; HS: sludge of high settleability.

3.4 Summary

The present study is the first one that explored the association of ARGs in sludge with its settleability. It is also the first one that identified the absolute abundances of eARGs and iARGs in sludge fractionated in terms of settleability. The main findings of this study can be summarized as follows:

1) The sludge fraction with lower settleability (the LS fraction) was mainly consisted of single intact bacterial cells and small sludge flocs formed by more damaged bacterial cells, and possessed higher proportions of eARGs and higher transfer potential judged by the detected higher relative abundances of ARGs and rich MGE;

2) The sludge fraction with higher settleability (the HS fraction) generally contained flocs with larger sizes formed by both intact and damaged bacterial cells. The relative abundances of ARGs and MGE in this sludge fraction were apparently lower, even if the presence percentages of eARGs were comparatively higher.

The findings above are considered significant as they can serve as new evidence for establishing more effective operation and management strategies to minimize the discharge of ARGs from WWTFs through improving the settleability of sludge. This will greatly inhibit the transfer and spread of ARGs through both eARGs and iARGs in the total water and soil environments, and at the same time, minimizing the risk to the food production and supply chains. The results obtained through this study can also benefit future studies needed for further exploring the mechanisms associated with the fate and behavior of ARGs in biological wastewater treatment process.

Chapter 4 Vermicomposting of excess activated sludge and fruit and vegetable waste

4.1 Background and objective

Fruit and vegetable waste (FVW) are generated in large quantities along with the entire food supply chain, from production, processing to the consumption. According to the statistical data reported by the Food and Agriculture Organization (FAO), approximately 1.8, 6.5, 32 and 15 million tons of FVW are generated each year in India, Philippines, China and the United States of America, respectively (FAO, 2013). For the FVW from households, about 10 million tons of FVW are generated each year in Japan alone (*Ministry of agriculture, forestry and fisheries, Japan, 2012*). Normally, FVW is characterized by high water content and rich biodegradable organic compounds (e.g. carbohydrates, lipids, and organic acids). These characteristics may contribute to negative environmental issues in traditional solid waste management systems (e.g. landfill and incineration), such as the discharge of leachate and the emission of greenhouse gases (Hartmann and Ahring, 2006). The FVW has a great potential for reuse, recycling and recovery (Plazzotta et al., 2017), and instead of landfill or incineration, it can be recycled with more environmentally friendly methods.

Composting and vermicomposting are attracting researchers' attention in recent years owing to the reason that these two methods can degrade organic wastes like FVW and can recycle and convert the valuable nutrients into organic fertilizers. Compared to composting, vermicomposting has more effective functions of biodegradation and stabilization of the organic wastes through the combined action of earthworms and microorganisms (Domínguez, 2004). In vermicomposting, microorganisms are mainly

responsible for the degradation of organic wastes, while the earthworms are important drivers for conditioning the substrate and altering the biological activity (Suthar, 2009a). Many researchers investigated the vermicomposting of FVW, including tomato (Fernández-Gómez et al., 2010a), mixed vegetables (Fernández-Gómez et al., 2010b), and food waste (Othman et al., 2012). These previous studies on vermicomposting used dry FVW with the addition of other bulking materials for earthworms. The pretreatment for drying the fresh FVW normally takes one to three weeks before conducting the vermicomposting process. This not only increases the whole time needed for vermicomposting but also lead to the loss of considerable amounts of nutrients via leachate (Huang et al., 2012). For treating fresh FVW by vermicomposting, limited literatures demonstrated that the earthworms were unable to survive in the fresh FVW possibly because of the high water content and high electrical conductivity of fresh FVW (Gunadi and Edwards, 2003).

On the other hand, excess activated sludge (EAS), as the main byproduct of sewage treatment process, can be treated by vermicomposting. However, several scientific literatures revealed that the rich nitrogen in EAS can give rise to a high ammonia concentration environment where the earthworms cannot survive. To solve this problem, some carbon-rich bulking materials like paper mulch (Ndegwa and Thompson, 2000), straw (Contreras-Ramos et al., 2005), and sugarcane trash (Suthar, 2009b), were added into the vermicomposting system for treating EAS. Although these carbon-rich bulking materials can adjust C/N ratio and ameliorate the live environment for earthworms, they can also bring some substances that are difficult to be degraded, thus leading to a longer decomposition process and lower nitrogen content in final products. However, the nitrogen rich EAS, may have some positive effects on the vermicomposting of FVW. It may promote the growth of earthworms and specific bacterial species, thus improving the

decomposition efficiency and could improve the nutritional content of the final products as fertilizer. Moreover, the EAS consists of microorganisms with great population and diversity, for example, various nitrogen fixing bacteria and the phosphate-accumulating bacteria like *Gemmatimonas* (Shchegolkova et al., 2016). It is well known that the complex microbial communities are reported to play a key role in the vermicomposting (Chen et al., 2018), the inoculation with suitable microbial strains could enhance the vermicomposting process and the nutrient enrichment in final products (Lukashe et al., 2019). Earlier studies have showed that the inoculation of nitrogen fixing bacteria (*Azotobacter chroococcum* and *Azospirillum lipoferum*) and phosphate-solubilizing bacteria (*Pseudomonas striata*) beneficially effected the vermicomposting process (Kumar and Singh, 2001). Another study observed that some organic waste containing microorganisms could also act as an inoculum for the decomposition of vegetable waste and help in the degradation of organic matters (Kalamdhad et al., 2008). Therefore, it is reasonable to consider that the wide variety of microorganisms contained in EAS may enhance the microbial activity and positively contribute to the decomposition of FVW, thus accelerating the nitrification and mineralization process, and leads to higher nitrogen and phosphorus contents in the final product.

Another fact worth to be mentioned is that the operation system of vermicomposting is deemed to be an important factor for the growth of earthworms (Glenn Munroe, 2007). In general, vermicomposting is conducted by using the mixed system, in which the substrate and bed material are mixed together (Singh and Suthar, 2012; Suthar et al., 2012; Garg and Gupta, 2011). On the other hand, few studies preferred using the separated system for treating organic wastes with high water content like FVW, in which the substrate and bed material are simply separated into two layers or separated by using a mesh with holes (Fernández-Gómez et al., 2010a; Huang et al., 2014; Huang et al., 2016).

Although the bed material in separated system can play as a supporting layer for the growth of earthworms, the earthworms still cannot survive if excessive volume of leachate is generated from the fresh FVW (Huang et al., 2016). Therefore, it is necessary to establish a novel vermireactor to separate the substrate and bed material very well, thus avoiding the risk of the leachate generated by FVW to earthworms. Furthermore, many researchers suggested that the bed material closely responded to the decomposition of substrate, thus affecting the final products of the vermicomposting (Domínguez et al., 2000). What seems to be lacking is the discussion about the substrate or bed material individually during vermicomposting. To investigate the effect of EAS on the vermicomposting of FVW, especially the effect on the decomposition of FVW, the changes of both substrate and bed material should be monitored individually.

Accordingly, the overall objective of this study was to investigate the effect of EAS on vermicomposting of FVW by using a novel vermireactor consists of substrate and bed compartments, with following specific aims:

- 1) to clarify the effect of EAS on earthworms by investigating weight and cocoon production of earthworms;
- 2) to evaluate the effect of EAS on the decomposition efficiency of FVW by quantitatively analyzing the contents of organic matter and total carbon, and the microbial activity;
- 3) to clarify the effect of EAS on the final product as organic fertilizer by investigating the content of total nitrogen and total phosphorus.

4.2 Materials and methods

4.2.1 Materials preparation

The *epigeic* earthworm species *Eisenia fetida* was chosen for this experiment due to its wide tolerance against environmental variables, such as pH, moisture, and temperature (Suthar, 2009a, Fernández-Gómez et al., 2010a). Five types FVW, namely banana peels, cabbage, lettuce, carrot and potato, were obtained from a supermarket in Gifu, Japan. The reason for the selection of these five types of FVW is that they are consumed in considerable amounts throughout the whole year. Besides, only the flesh or pulp of them are consumed commonly, as a result, significant amounts of nutrients are present in their peels and other components are wasted. The FVW were cut into pieces with a width of about 1 cm (and a thickness of 2 mm for carrot and potato) before the experiment. In order to avoid the threat to earthworms from leachate generation during vermicomposting, a supporting bed was designed. The bed material was a mixture of soil and the final product from previous vermicomposting of FVW. For EAS, dewatered sludge from a slaughterhouse wastewater treatment plant in Gifu, Japan, was used. The initial properties of FVW, EAS and bed material used in this study are shown in **Table 4.1**.

4.2.2 Experimental set up

4.2.2.1 Design of novel vermireactor

Vermicomposting was performed in a laboratory scale by using eleven plastic containers as vermireactors. Each reactor was formed with two compartments, namely a substrate compartment and a bed compartment. The two compartments were separated by a plastic plate opened with eight holes (1 cm× 1 cm) for earthworms to move freely between the two compartments. To provide a constant moisture and aerobic condition, all reactors were covered with a plastic cap with eight venting holes. In addition, a box was placed below the reactor for collecting the leachate. Compared with the vermicomposting reactors used in other studies, this novel reactor not only simply separated the substrate

and bed material into two compartments but is also easy to sample from two compartments and avoids the effect of the bed material on the substrates. The diagram of the novel vermireactor is displayed in **Fig. 4.1**.

4.2.2.2 Vermicomposting process

For the bed compartments, 100 g (wet basis) bed material was added and then 10 adult earthworms with an individual weight of 350-500 mg were inoculated. For the substrate compartments of six reactors, 100 g (wet basis) of banana peels, cabbage, lettuce, carrot, potato, and EAS were added respectively. For the remaining five substrate compartments,

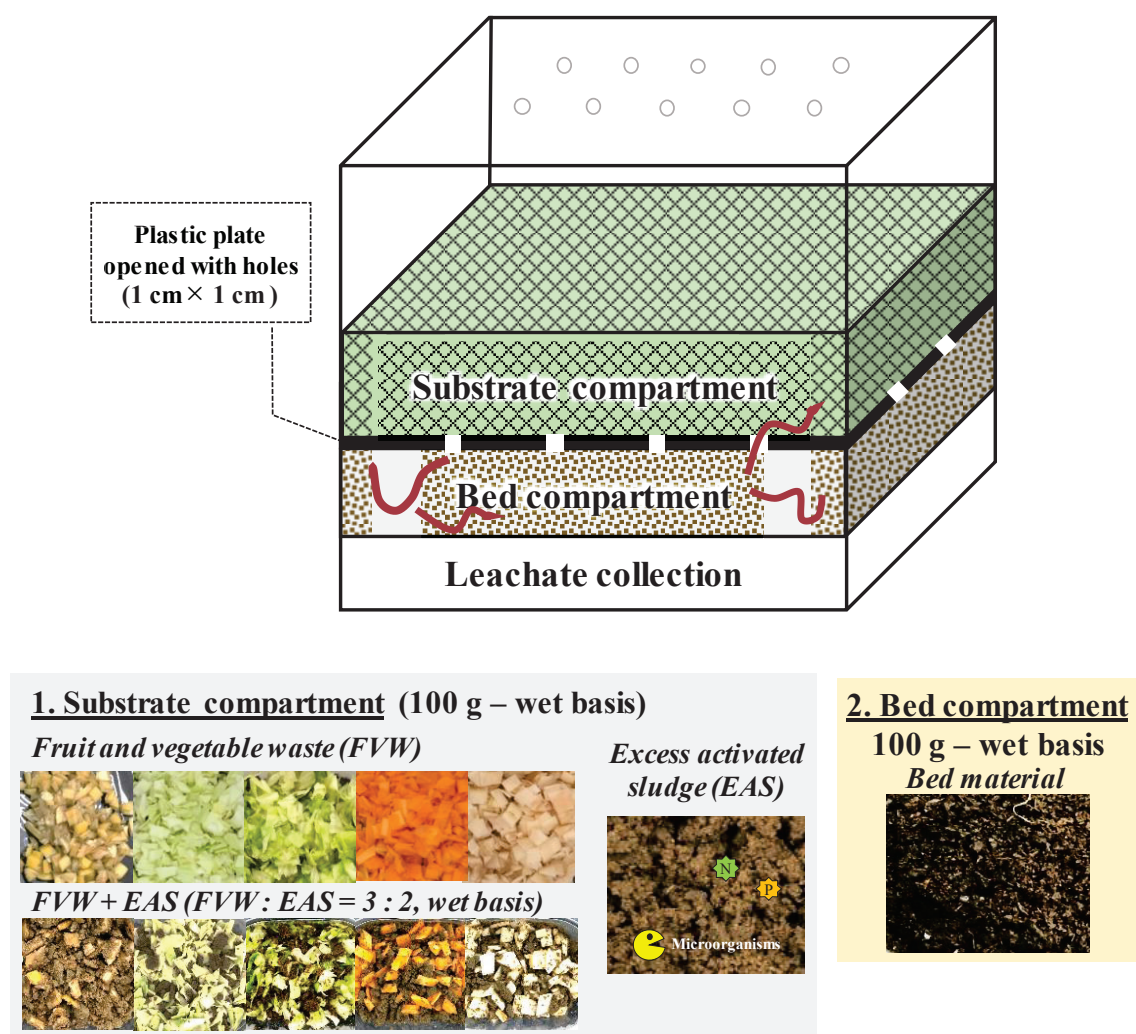


Fig. 4.1 Diagram of the novel vermireactor used in this study.

Table 4.1 Initial properties of FVW, EAS and the bed material used for vermicomposting. Except for pH and electrical conductivity, results are shown as means \pm SD, n = 3. EAS: excess activated sludge.

	pH	Electrical conductivity (mS/m)	Water content (%)	Organic matter content (%)	Total carbon (mg/g)
Banana peels	5.79	589	88.2 \pm 0.3	71.2 \pm 0.7	413.5 \pm 1.3
Banana peels + EAS	5.51	455	86.6 \pm 0.3	84.1 \pm 1.4	424.2 \pm 1.1
Cabbage	5.93	347	93.7 \pm 0.1	89.8 \pm 0.1	386.6 \pm 0.5
Cabbage + EAS	5.69	308	89.7 \pm 0.3	90.7 \pm 1.1	405.8 \pm 0.1
Lettuce	5.90	258	96.7 \pm 0.0	79.4 \pm 1.3	376.7 \pm 6.9
Lettuce + EAS	5.70	244	92.6 \pm 0.3	89.7 \pm 0.9	405.4 \pm 0.4
Carrot	6.07	420	91.6 \pm 0.1	78.1 \pm 3.0	384.5 \pm 1.1
Carrot + EAS	5.85	311	88.5 \pm 0.4	90.6 \pm 0.4	404.7 \pm 0.5
Potato	6.05	529	90.1 \pm 0.5	90.8 \pm 1.2	417.0 \pm 1.5
Potato + EAS	5.95	387	80.5 \pm 3.0	93.2 \pm 0.3	418.2 \pm 0.1
EAS	5.80	197	84.3 \pm 0.1	88.1 \pm 0.1	422.1 \pm 2.7
Bed material	5.76	72.6	76.1 \pm 1.0	71.1 \pm 2.2	333.0 \pm 2.6
	Total nitrogen (mg/g)	C/N ratio	Total phosphorus (mg/g)	16S rDNA ($\times 10^9$ copies/g)	Dehydrogenase activity [μ g-TF/(g \cdot h)]
Banana peels	10.5 \pm 0.1	39.6 \pm 0.3	1.3 \pm 0.0	1.3 \pm 0.1	0.0 \pm 0.0
Banana peels + EAS	33.8 \pm 0.7	12.6 \pm 0.2	6.0 \pm 0.4	22.5 \pm 3.6	1568.8 \pm 113.3
Cabbage	28.8 \pm 0.0	13.4 \pm 0.0	3.0 \pm 0.1	9.8 \pm 2.2	31.5 \pm 7.4
Cabbage + EAS	52.7 \pm 0.3	7.7 \pm 0.0	6.3 \pm 0.9	11.1 \pm 0.2	1036.5 \pm 35.5
Lettuce	33.9 \pm 0.4	11.1 \pm 0.3	4.5 \pm 0.1	18.6 \pm 0.2	73.1 \pm 7.4
Lettuce + EAS	58.5 \pm 0.3	6.9 \pm 0.0	9.7 \pm 0.1	25.6 \pm 0.3	1447.1 \pm 195.6
Carrot	15.0 \pm 0.3	25.7 \pm 0.4	2.3 \pm 0.1	17.2 \pm 1.2	0.0 \pm 0.0
Carrot + EAS	46.1 \pm 0.5	8.8 \pm 0.1	8.0 \pm 0.0	32.7 \pm 0.05	1567.1 \pm 11.0
Potato	10.0 \pm 0.2	41.9 \pm 0.6	1.3 \pm 0.0	15.1 \pm 0.1	0.0 \pm 0.0
Potato + EAS	28.2 \pm 1.0	14.9 \pm 0.5	4.5 \pm 0.4	21.3 \pm 0.1	861.4 \pm 55.7
EAS	70.7 \pm 0.8	6.0 \pm 0.1	12.9 \pm 0.3	24.4 \pm 2.2	3642.8 \pm 213.1
Bed material	40.9 \pm 0.6	8.1 \pm 0.1	7.0 \pm 0.2	27.6 \pm 2.9	35.5 \pm 16.8

100 g (wet basis) of the mixed substrates of each type of FVW with the EAS (FVW:EAS = 3:2, wet basis) were added respectively. All reactors were kept in dark and conducted under the constant temperature of 25 °C. The information of each reactor in detail is shown in **Table 4.2**. At designated time during vermicomposting, samples from both substrate compartment and bed compartment of each reactor were homogenized and collected separately. Collected samples were separated into two parts, one part was used for the measurement of water content, organic matter content, and the dehydrogenase activity (DHA) immediately, and the remaining part was dried by using a freeze-vacuum dryer. The dried samples were pulverized and stored at -25 °C for further analysis of physicochemical and microbial parameters.

4.2.3 Analysis methods

4.2.3.1 *Earthworms' growth parameters*

Earthworms were gently taken out from each reactor by hand and washed with pure water for removing the adhering materials from their body every three days. Subsequently, earthworms were placed to a wet adsorption paper towel and then weighted on a live weight basis. The cocoon number in each reactor was counted at the end of vermicomposting. The growth rate of the earthworms was calculated as given by Huang et al. (2016).

$$\text{Growth rate} = \frac{\text{Final mean weight of earthworms} - \text{Initial mean weight of earthworms}}{\text{Experimental days}} \text{ (mg/worm/day)}$$

4.2.3.2 *Physicochemical parameters*

Water content was determined by drying the samples to a constant weight at 105 °C (at least 5 hours) in an oven. The organic matter content (loss on ignition) was measured by

combusting the dried samples in a muffle furnace (Yamato, Japan) at 600 °C for 2 hours. The pH and electrical conductivity (EC) were measured by using the aqueous solution of pulverized dry sample with deionized water (w/v = 1/10) after shaken for 2 hours (Fernández-Gómez et al., 2010b; Cui et al., 2018). Total carbon and total nitrogen were measured by using a nitrogen and carbon analyzer (Huang et al., 2012). The result of C/N ratio was obtained through dividing total carbon by total nitrogen. Total phosphorus was measured by using the molybdenum blue-absorption method with a spectrophotometer at the designated wavelength of 880 nm (Huang et al., 2012). For better evaluation on the decomposition efficiency of the substrates, the mass reduction rate of substrates was calculated based on the changes in their dry weight, as following equation:

Mass reduction rate =

$$\frac{\text{Initial dry weight of substrate} - \text{Final dry weight of substrate} + \text{Sampled dry weight of substrate}}{\text{Experimental days}} \quad (\text{g/day})$$

4.2.3.3 Microbial parameters

Dehydrogenase activity (DHA), a parameter reflecting total microbial activities (Suthar, 2008a), was determined by triphenyl tetrazolium chloride (TTC) method (Cui et al., 2018). Briefly, the fresh samples with the addition of TTC solution were kept in a water bath shaker at 37 °C for 30 min under the dark condition to ensure the reaction. After that, acetone was added to dissolve the reaction product triphenyl formazan (TF). Finally, the resulting acetone solution after centrifuged at 3500 rpm for 5 min was measured with a spectrophotometer at the designated wavelength of 485 nm. The genomic DNA was extracted from dried samples by using the DNA extraction kit (MOBIO, USA) based on manufacturer's protocol. Two primers, Com1 (5-CAGCAGCCGCGGTAATAC-3) and Com2 (5-CCGTCAATTCCTTTGAGTTT-3), were used to amplify the 16S rDNA gene

of bacteria. The quantitative PCR program consisted of initial denaturation at 95 °C for 5 min followed by 40 three-step cycles of 95 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s (Huang et al., 2013).

Table 4.2 Details of the vermicomposting reactors used in this study. FVW: fruit and vegetable wastes; EAS: excess activated sludge.

Substrate compartment			Bed compartment	Earthworms	
Composition	Mixing ratio (FVW:EAS)	Weight (g-wet basis)	Weight (g-wet basis)	Individual weight (mg)	Numbers (worms)
1 Banana peels		100	100	350 - 500	10
2 Banana peels + EAS	3:2	100	100	350 - 500	10
3 Cabbage		100	100	350 - 500	10
4 Cabbage + EAS	3:2	100	100	350 - 500	10
5 Lettuce		100	100	350 - 500	10
6 Lettuce + EAS	3:2	100	100	350 - 500	10
7 Carrot		100	100	350 - 500	10
8 Carrot + EAS	3:2	100	100	350 - 500	10
9 Potato		100	100	350 - 500	10
10 Potato + EAS	3:2	100	100	350 - 500	10
11 EAS		100	100	350 - 500	10

4.2.4 Statistical analysis

The significant differences between the mean values of several parameters (organic matter content, total carbon, C/N ratio, total nitrogen and total phosphorus) of the treatment for FVW alone and the treatment for FVW with the addition of EAS were analyzed by *t*-test with a 95% confidence level using Statistics 21 software.

4.3 Results and discussion

4.3.1 Changes in appearance of substrates

The changes in appearance of substrates during the vermicomposting are shown in **Table 4.3**. It is explicated that all reactors performed a markedly mass reduction of the substrates no matter with or without the addition of EAS. Previous researches also proved that the vermicomposting has a great mass reduction capacity over a shorter processing time due to the joint action of earthworms and microorganisms (Suthar, 2008; Fernández-Gómez et al., 2010b). Such kinds of mass reduction on the substrates are likely related to the conversion of substrates into earthworms cast/excreta (Rodrigues et al., 2017). For better understanding, the mass reduction rate of the substrates in each reactor was calculated according to their changes in dry weight (**Fig. 4.1**). A significantly higher mass reduction rate was recorded in the treatment with the addition of EAS. It indicated that the addition of EAS accelerated the entire vermicomposting process.

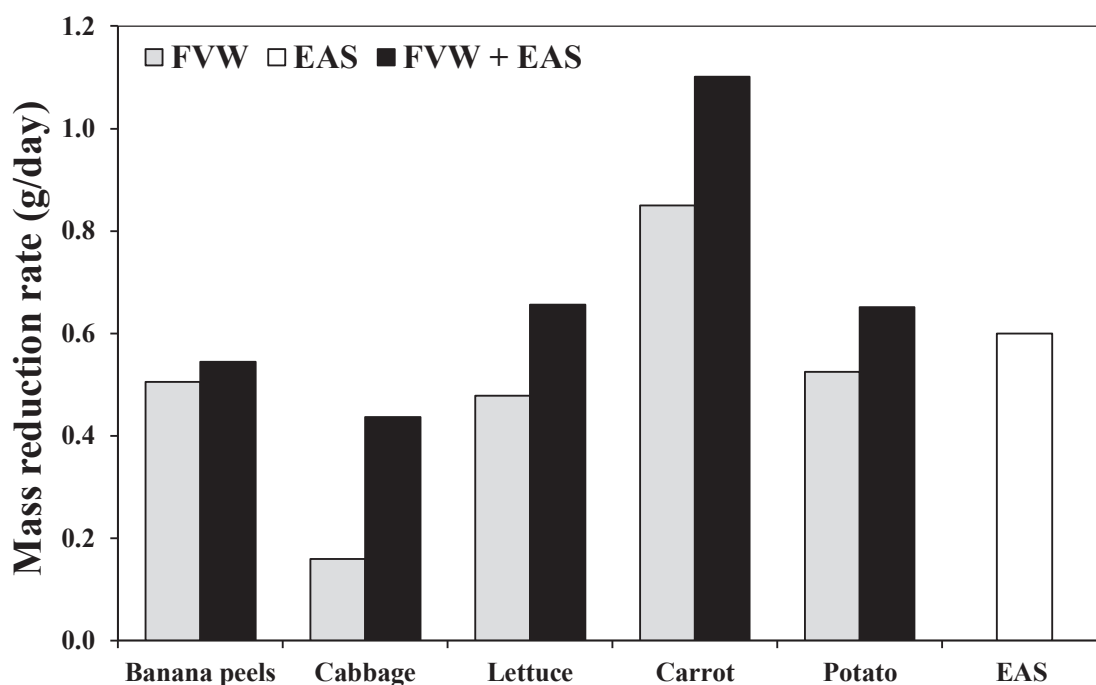
























Fig. 4.1 Mass reduction rate of substrates used for vermicomposting. FVW: fruit and vegetable wastes; EAS: excess activated sludge.

Table 4.3 Changes in appearance of substrates during vermicomposting. FVW: fruit and vegetable wastes; EAS: excess activated sludge.

	FVW or EAS		FVW + EAS	
	Day 0	Day 6	Day 0	Day 6
Banana peels				
Cabbage				
Lettuce				
Carrot				
Potato				
EAS				

4.3.2 Effect of EAS on the growth of earthworms

Healthy growth status of earthworms directly reflects a successful vermicomposting process owing to that earthworms are considered as crucial drivers during vermicomposting (C. Zhao et al., 2018). Earthworm weight and cocoon production are two critical indicators for the growth of earthworms. Body weight changes of the earthworms over vermicomposting are displayed in **Fig. 4.2**. The body weight of earthworms increased in the initial period and then declined gradually at the end of vermicomposting for most of treatments. Such a decline was corroborated by few earlier studies and can be explained as the aging of substrate materials (Gómez-Brandón et al., 2011b); the exhaustion of food (Gupta and Garg, 2008); the reduction of bioavailable nutrients (Gunadi and Edwards, 2003); and the conversion of most of the used substrate to final products (Suthar et al., 2012). Overall, earthworms in the treatments for FVW with the addition of EAS performed excellent trends of weight gain compared to the earthworms in the treatments for FVW alone. The better weight gain of earthworms could be related to the enhancement of the biochemical quality of substrates by the addition of EAS.

The growth rate of earthworms is considered as an appropriate indicator to assess the earthworm growth in different substrates. As shown in **Fig. 4.3**, the growth rate of earthworms differed with the types of FVW during the vermicomposting. There was a negative growth rate occurred in the treatment for banana peels, cabbage, potato and its mixture with EAS, while a positive growth rate was observed in other treatments. The maximum growth rate was found in the treatment for lettuce with the addition of EAS (27.7 mg/worm/day), which is about twice the value of the treatment for lettuce alone (14.3 mg/worm/day). It is suggested that the growth rate of earthworms depends on the microbial populations and the nutrient pool availability in substrates as the feeds (Suthar,

2009a). Furthermore, the addition of EAS also promoted the cocoon production in treatment for banana peels, cabbage, carrot and potato as the number of cocoons in the reactors treating banana peels, cabbage, carrot and potato mixed with EAS (2, 2, 3, and

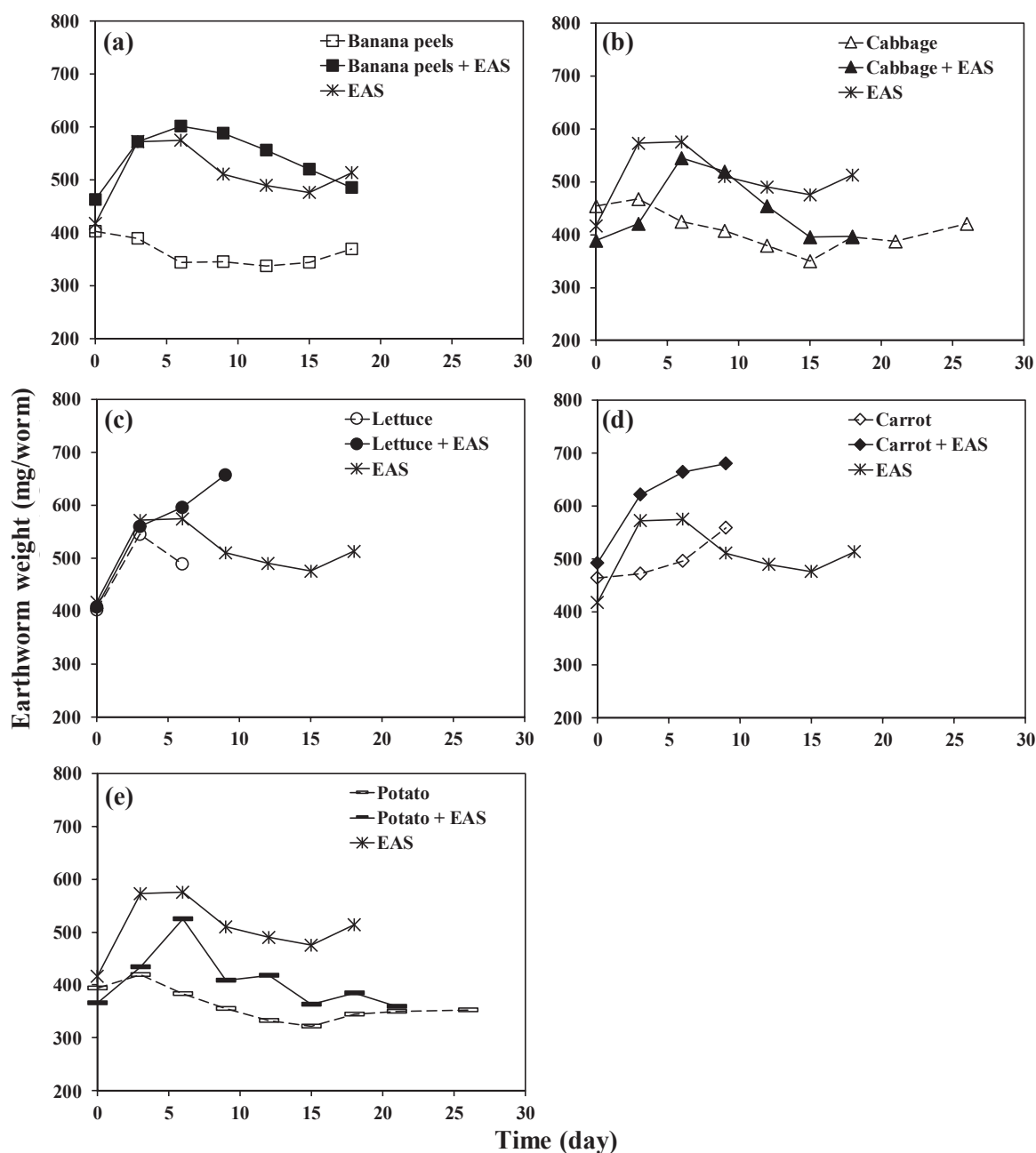


Fig. 4.2 Body weight changes of earthworm during vermicomposting of five types of FVW including (a) banana peels, (b) cabbage, (c) lettuce, (d) carrot, and (e) potato with and without the addition of EAS. FVW: fruit and vegetable waste; EAS: excess activated sludge.

3, respectively) was larger than that treating respective FVW alone (0, 0, 0, and 2, respectively), as displayed in **Fig. 4.3**. It is concluded that the cocoon production must be related to the nutrient profile especially nitrogen or C:N ratio of substrate materials for earthworms (Edwards et al., 1998) (Suthar, 2008). The results of the present study revealed that adding EAS into the vermicomposting for treating FVW can provide better growth medium and nourishment for earthworms.

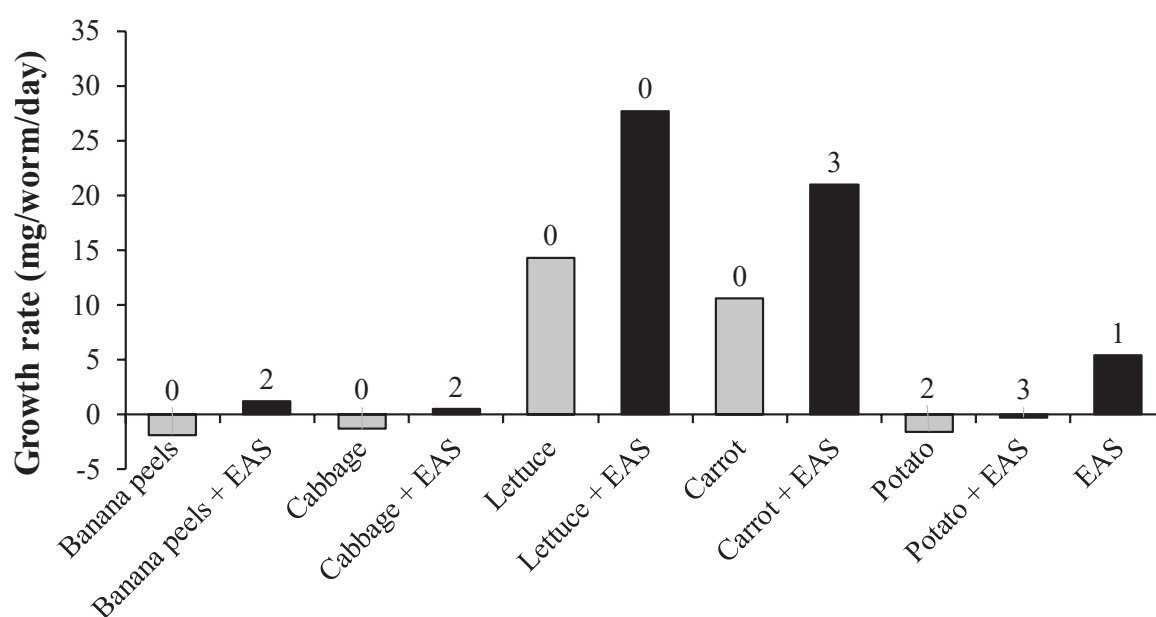


Fig. 4.3 Growth rate of earthworms during vermicomposting. Values showed the cocoon numbers produced by earthworms after vermicomposting in each reactor. EAS: excess activated sludge.

4.3.3 Effect of EAS on chemical properties

All the substrates used in this study were converted into more stable materials by vermicomposting, meanwhile, the chemical properties (e.g. pH, electrical conductivity) of them were also changed drastically. As summarized in **Table 4.3**, the pH value in the substrate compartments increased from the initial range of 5.51 - 6.07 to the final range

of 6.13 - 8.65 at the end of vermicomposting. For the bed compartments, where the initial pH was 5.76, a variation of pH in the range of 5.41 - 7.80 was recorded. The variation of pH is probably in the reflection of the chemical qualities of substrates that lead to different changes in the processes of organic acid production and mineralization during vermicomposting (Sharma and Garg, 2018). It is suggested by earlier studies that different substrates may lead to different levels of the production for dissimilar intermediate species, thus results different behaviors in pH shift (Garg et al., 2006). In general, the pH value in the bed compartment of treatment with the addition of EAS was relatively lower than the treatment for FVW alone. The lower pH is reported to be associated with the increases in the mineral nitrogen content, the changes in the ammonium-nitrate equilibrium, and the accumulation of organic acids from microbial metabolism or from the production of fulvic and humic acids during the decomposition (Dominguez and Edwards, 2004).

The changes in the electrical conductivity (EC) before and after vermicomposting are showed in **Table 4.3**. EC decreased substantially in the substrate compartments of all reactors after vermicomposting. The maximum decreasing rate was measured in the treatment for lettuce and potato as 76.1%, followed by the treatment for carrot as 75.5%. The minimum decreasing rate was found in the treatment for EAS alone as 36.7 %. The decrease of EC during vermicomposting could be explained by the reason that the decomposition of organic substrates can lead to a great loss of dissolved ions (Huang et al., 2017). In contrast, the EC in all bed compartments increased from the initial level of 72.6 mS/m to 77.2 - 173.0 mS/m after vermicomposting. Such an increase was possibly related to the release of different mineral salts in available forms such as ammonium, phosphate, and potassium through the decomposition of the organic substrates. Similar results were also reported regarding the vermicomposting of FVW (Huang et al., 2012).

It is worthy of special notice that the increasing percentage of EC in the treatment for FVW with the addition of EAS was higher than that in the treatment for FVW alone except for cabbage. For the treatments of lettuce and carrot with or without the addition of EAS, the increasing percentage was a little bit lower compared to other treatments. The lower increasing percentage may be in association with the higher growth rate of earthworms in these two treatments (27.7 and 21.0 mg/worm/day). A larger amount of leachate was released from the treatments for lettuce and carrot due to their higher water content and was transferred to the bed compartment where some ions in the leachate were consumed by earthworms for their body growth.

4.3.4 Effect of EAS on the decomposition efficiency of FVW

The reduction in organic matter content and total carbon during the vermicomposting process denotes the decomposition efficiency of the organic wastes. The changes of organic matter content in both substrate and bed compartments after vermicomposting are summarized in **Table 4.4**. The organic matter content in the substrate compartment of all treatments decreased from the initial levels, except for lettuce. For the bed compartment, reductions of the organic matter content were also revealed in all treatments except for carrot and its mixture with EAS, and EAS alone. The largest drop of organic matter content in the substrate compartment was found in the treatment for EAS (23.4 %), and the largest drop in the bed compartment was in the treatment for cabbage with the addition of EAS (11.8 %). Similarly, the total carbon in the substrate compartment of the treatment with addition of EAS was lower than that of the treatment for FVW along except for cabbage (**Fig. 4.4**). The total carbon was lost as CO₂ during vermicomposting owing to the consumption of the available carbon as a source of energy for earthworms and microorganisms (Hait and Tare, 2011). The lower total carbon may be caused by the

Table 4.3 Changes of pH value and electrical conductivity (EC) in all substrate compartments and bed compartments after vermicomposting. The positive values mean increasing percentages, and the negative values mean decreasing percentages. EAS: excess activated sludge.

pH	Substrate compartment			Bed compartment		
	Initial	Final	Changes (%)	Initial	Final	Changes (%)
Banana peels	5.79	8.65	49.4	5.76	6.83	18.6
Banana peels + EAS	5.51	7.75	40.7	5.76	6.02	4.5
Cabbage	5.93	6.13	3.4	5.76	5.68	-1.4
Cabbage + EAS	5.69	6.23	9.5	5.76	5.55	-3.6
Lettuce	5.90	6.62	12.2	5.76	7.80	35.4
Lettuce + EAS	5.70	7.26	27.4	5.76	6.27	8.9
Carrot	6.07	6.79	11.9	5.76	7.42	28.8
Carrot + EAS	5.85	8.34	42.6	5.76	6.04	4.9
Potato	6.05	6.74	11.4	5.76	5.80	0.7
Potato + EAS	5.95	6.27	5.4	5.76	5.49	-4.7
EAS	5.80	6.27	8.1	5.76	5.41	-6.1
Electrical conductivity (mS/m)	Substrate compartment			Bed compartment		
	Initial	Final	Changes (%)	Initial	Final	Changes (%)
Banana peels	455	146.8	-67.7	72.6	159.4	119.6
Banana peels + EAS	589	161.2	-72.6	72.6	162.4	123.7
Cabbage	347	86.1	-75.2	72.6	162.2	123.4
Cabbage + EAS	308	134.9	-56.2	72.6	162.3	123.6
Lettuce	258	61.6	-76.1	72.6	77.2	6.3
Lettuce + EAS	244	67.9	-72.2	72.6	124.0	70.8
Carrot	420	102.9	-75.5	72.6	114.5	57.7
Carrot + EAS	311	89.4	-71.3	72.6	127.2	75.2
Potato	529	126.6	-76.1	72.6	159.6	119.8
Potato + EAS	387	131.8	-65.9	72.6	173.0	138.3
EAS	197	124.7	-36.7	72.6	157.8	117.4

better growth of earthworms and higher microbial activity in the treatment with the addition of EAS. For the bed compartment, however, no significant changes in the total carbon was exhibited except for the treatment of cabbage.

Table 4.4 Changes of organic matter content in all substrate compartments and bed compartments after vermicomposting. Data are shown as means \pm SD, $n = 3$. The positive values mean increasing percentages, and the negative values mean decreasing percentages. The asterisk (*) denotes the difference between initial value and final value is statistically significant at 0.05 level. EAS: excess activated sludge; SD: standard deviation.

	Organic matter content (%)					
	Substrate compartment			Bed compartment		
	Initial	Final	Changes (%)	Initial	Final	Changes (%)
Banana peels	71.2 ± 0.7	55.0 ± 2.3 *	-22.8	71.1 ± 2.2	67.7 ± 0.8	-4.8
Banana peels + EAS	84.1 ± 1.4	77.4 ± 1.2 *	-8.0	71.1 ± 2.2	64.8 ± 3.0 *	-8.9
Cabbage	89.8 ± 0.1	84.0 ± 2.4 *	-6.5	71.1 ± 2.2	63.4 ± 8.8	-10.8
Cabbage + EAS	90.7 ± 1.1	85.3 ± 2.8 *	-6.0	71.1 ± 2.2	62.7 ± 2.8	-11.8
Lettuce	79.4 ± 1.3	87.7 ± 0.2 *	10.5	71.1 ± 2.2	66.9 ± 1.4	-5.9
Lettuce + EAS	89.7 ± 0.9	85.7 ± 0.7 *	-4.5	71.1 ± 2.2	68.6 ± 0.8	-3.5
Carrot	78.1 ± 3.0	69.5 ± 2.9 *	-11.0	71.1 ± 2.2	71.5 ± 0.6	0.6
Carrot + EAS	90.6 ± 0.4	80.5 ± 2.7 *	-11.1	71.1 ± 2.2	71.3 ± 0.7	0.3
Potato	90.8 ± 1.2	85.2 ± 2.7 *	-6.2	71.1 ± 2.2	69.2 ± 7.2	-2.7
Potato + EAS	93.2 ± 0.3	83.5 ± 2.6 *	-10.4	71.1 ± 2.2	65.2 ± 6.2	-8.3
EAS	88.1 ± 0.1	67.5 ± 2.3 *	-23.4	71.1 ± 2.2	73.5 ± 1.2	3.4

The C/N ratio is commonly used to indicate the degree of decomposition of organic wastes, as carbon is lost as CO₂, whereas nitrogen is lost at a lower rate, and therefore the more decomposed organic wastes, the lower the C/N ratio (Lazcano et al., 2008). As shown in **Fig. 4.5**, the C/N ratio was significantly lower in the treatment for FVW with the addition of EAS in both substrate and bed compartments, except for the bed

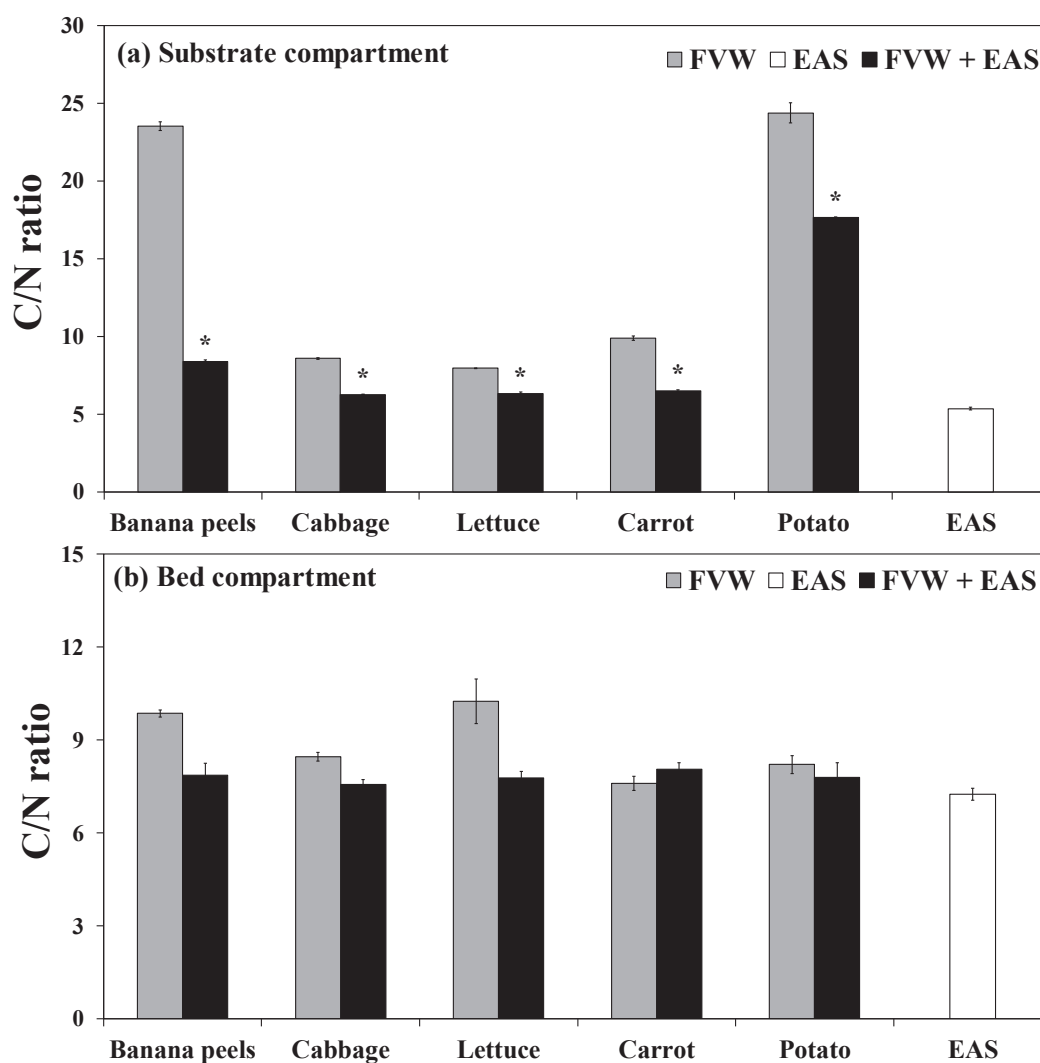


Fig. 4.5 C/N ratio in the (a) substrate compartment and the (b) bed compartment after vermicomposting. Data are presented as mean and standard deviation, n = 3. EAS: excess activated sludge. The asterisk (*) denotes the difference between vermicomposting of FVW and FVW + EAS is statistically significant at 0.05 level.

compartment of the treatment for carrot. Researchers have also found that the correlation between the C/N ratio and the consumption of substrates as food for earthworms is negative. A high C/N ratio indicates low food quality and in consequence, provokes a lower consumption rate of food (Flegel and Schrader, 2000). From the result of mass reduction rate, the lower consumption rate can also be found in the treatments for FVW

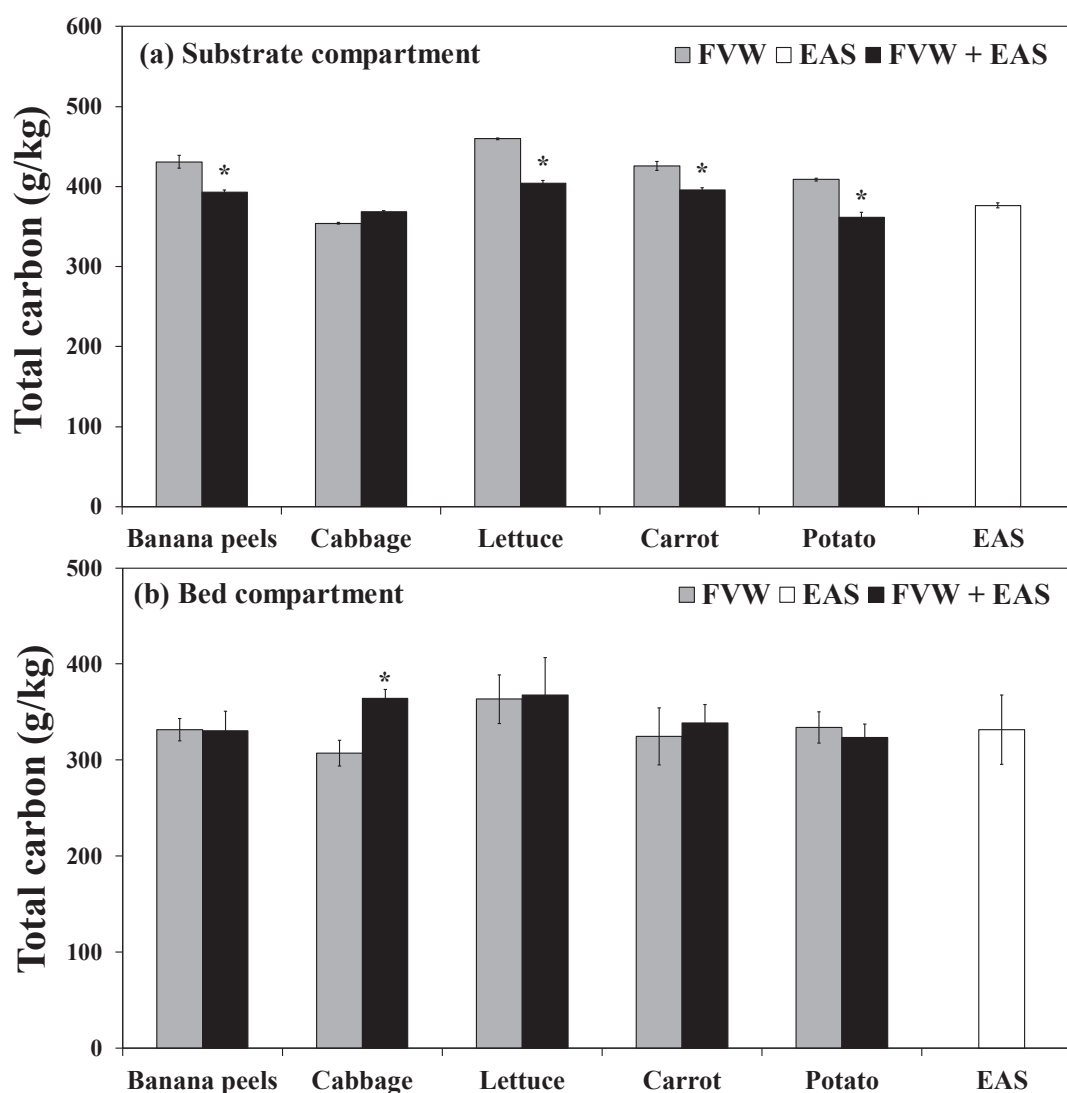


Fig. 4.4 Total carbon in the (a) substrate compartment and the (b) bed compartment after vermicomposting. Data are presented as mean and standard deviation, $n = 3$. EAS: excess activated sludge. The asterisk (*) denotes the difference between vermicomposting of FVW and FVW + EAS is statistically significant at 0.05 level.

alone. These findings suggest that a higher degree of decomposition of substrates used for vermicomposting was achieved by the addition of EAS. Furthermore, the C/N ratio is also widely used as an index for the maturity and stability of the final products obtained after vermicomposting. The value of C/N less than 20 indicated the acceptable maturity of final products, and the value lower than 15 is thought to be preferable for the agronomic value of final products as fertilizer (Huang et al., 2012). In this study, the C/N ratio of all

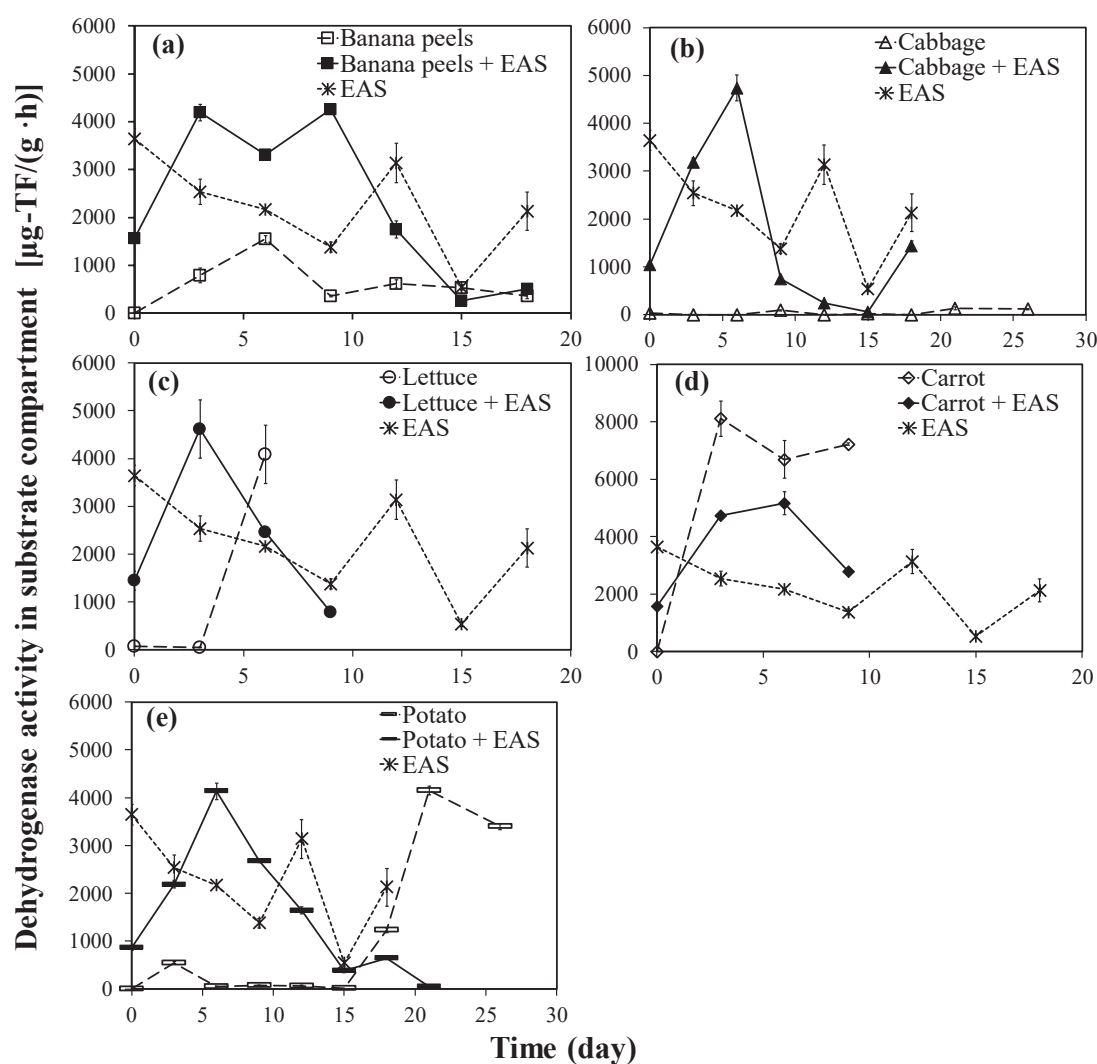


Fig. 4.6 Changes of dehydrogenase activity (DHA) in the substrate compartment of all reactors during vermicomposting. Data are presented as mean and standard deviation, $n = 3$. EAS: excess activated sludge.

substrates decreased markedly from the initial value to a value less than 20 (except for banana peels and potato), and the C/N ratio of all bed compartments were lower than 15.

Dehydrogenase activity (DHA) is often used for monitoring the microbial activity and assessing the degree of biological stability during the decomposition process of organic wastes (Huang et al., 2014). **Fig. 4.6** showed the dynamic changes of DHA in the substrate compartments, an increasing trend in the beginning followed by a decreasing trend at the

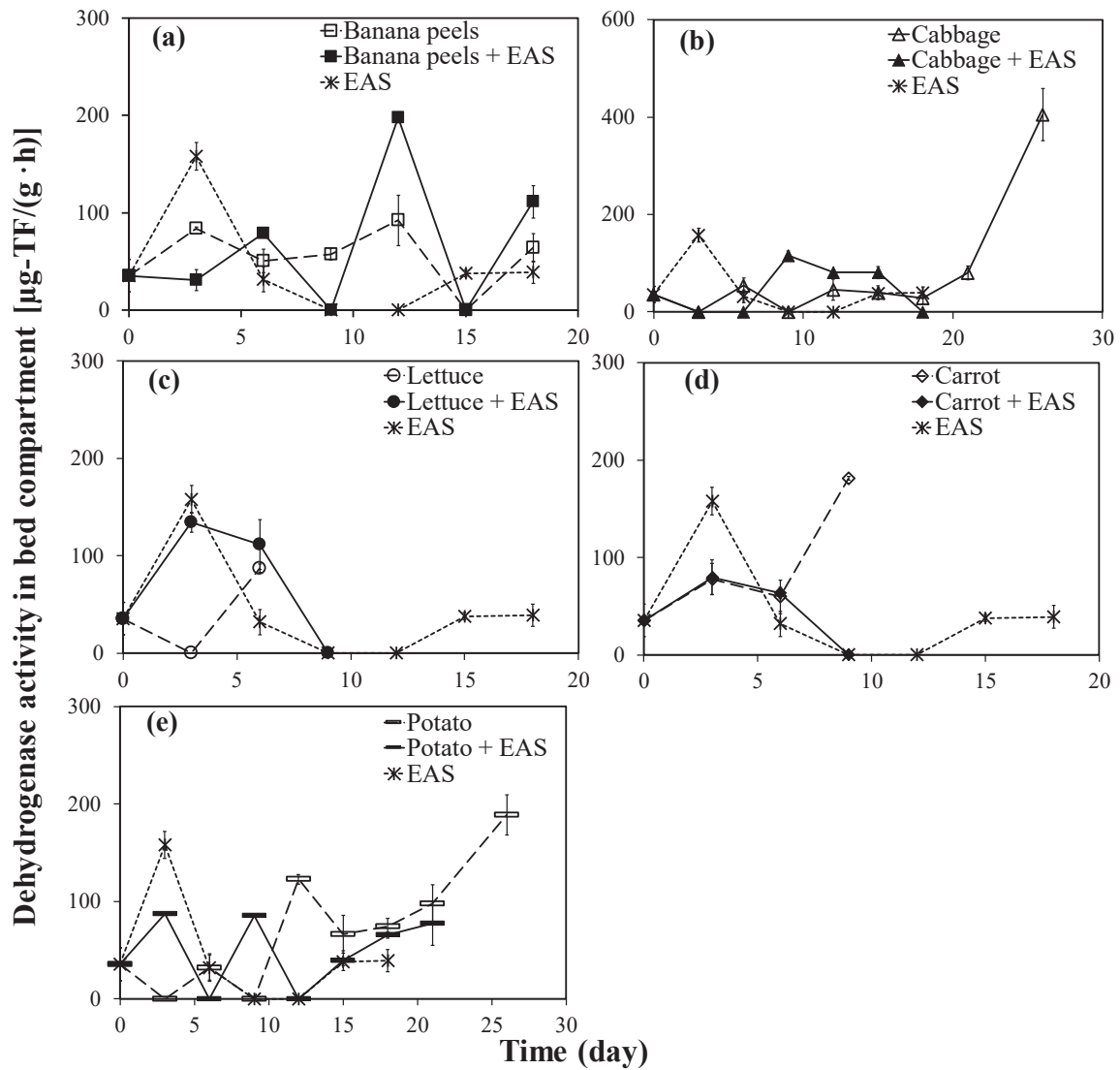


Fig. 4.7 Changes of dehydrogenase activity (DHA) in the bed compartment of all reactors during vermicomposting. Data are presented as mean and standard deviation, $n = 3$. EAS: excess activated sludge.

end of vermicomposting was observed for the treatment with the addition of EAS. While the DHA in the substrate compartment of the treatment for different types of FVW showed different trends. The DHA suddenly increased in the initial stage in the substrate compartment of the treatment for lettuce and carrot. For the treatment of cabbage and potato, a minor fluctuation was recorded in the substrate compartment during the whole vermicomposting period. Different types of FVW probably related to different decomposition efficiencies of organic matters during vermicomposting, thus causing the different dynamic changes of DHA. It is reported that the DHA are highly influenced by the different types of food sources (Flegel and Schrader, 2000).

In general, a lower DHA was observed in the substrate compartment of the treatment for FVW except for carrot, but a higher DHA in the substrate compartment of the treatment for FVW with the addition of EAS, as display in **Fig. 4.6**. Moreover, it is worth to notice that the occurrence time for the DHA reaching its maximum value was much earlier in the substrate compartment for the treatment with the addition of EAS. These findings may suggest that the enrichment of microbial population and activity by adding EAS could lead to a rapid turnover of microorganisms and the nitrogenous substrates obtained by adding EAS or induced by earthworms (e.g. mucus or casts) could also accelerate the priming effects, thus enhancing the decomposition and mineralization efficiency of FVW (Kuzyakov, 2010; Bernard et al., 2012; Bityutskii et al., 2012). In contrast, an extremely higher DHA was found in the treatment for carrot but a lowered DHA was found in treatment for its mixture with EAS. The excellent body weight gain (27.7 mg/worm/day) recorded in the treatment for carrot with the addition of EAS could be explained as one of the possible reasons for the lower DHA owing to the collaboration and the competition relationship between earthworms and microorganisms. Past literatures suggested that the decomposition of organic wastes by earthworms may

negatively influence the microbial biomass caused by the depletion of the food resources for the microorganisms (Gómez-Brandón et al., 2011b). The obtained results indicated that the EAS enhanced the decomposition efficiency of FVW by improving the microbial activities of FVW (except for carrot) or by providing higher nutrients for earthworms.

On the other hand, the DHA in the bed compartment of all treatments fluctuated within a narrow range during the vermicomposting. Compared to the DHA values recorded in the substrate compartment, the DHA values in the bed compartment were extremely lower (shown as supplementary information). Since the DHA can provide a clear indication of the dynamics of organic matter decomposition which is useful in characterizing the status of the final products (Fernández-Gómez et al., 2010b). The lower values of DHA further prove that the final products of vermicomposting are very stable (Fu et al., 2015).

4.3.5 Effect of EAS on nitrogen and phosphorus in final products

As displayed in **Fig. 4.8**, a significantly higher total nitrogen was observed in both the substrate and bed compartments of the treatment for FVW with the addition of EAS after vermicomposting, except the bed compartment of the treatment for carrot. It may imply that adding EAS into FVW as the substrate provided with a favored condition for earthworms and microorganisms thus enhancing the nitrification and/or nitrogen mineralization. This result is largely consistent with several earlier studies, the higher total nitrogen after vermicomposting is contingent upon the initial total nitrogen in the substrates and the extent of decomposition due to the easily degradable organic matters (Garg and Gupta, 2011). Moreover, the mineralization of the proteins in substrates, reduction in dry mass of the substrates, and the loss of organic carbon etc. could be the major causes for the increase of nitrogen during the vermicomposting. Same finding has been reported that the loss of dry mass (organic carbon) as CO₂ and water by evaporation

during mineralization of organic matters might have determined the relative increase in nitrogen content of different vermicomposting treatments (Khatua et al., 2018). Furthermore, the increased microbial activity and the increased nitrogen-fixing bacteria number brought about by the addition of EAS as a consequence of the increasing earthworm activity were likely another important reason for the enhanced nitrogen content in the end products (Suthar, 2010; Huang et al., 2012). However, the lower

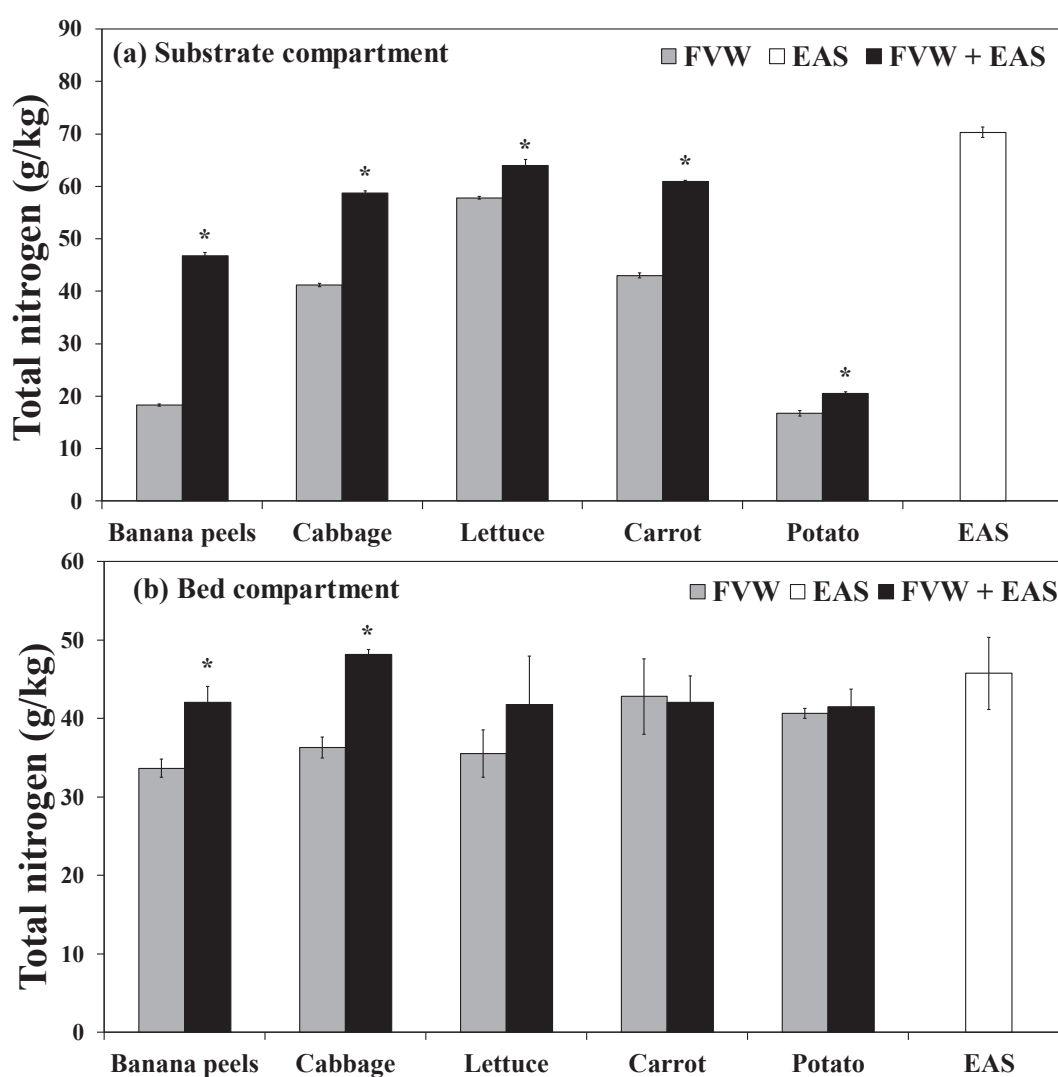


Fig. 4.8 Total nitrogen in the (a) substrate compartment and the (b) bed compartment after vermicomposting. Data are presented as mean and standard deviation, $n = 3$. EAS: excess activated sludge. The asterisk (*) denotes the difference between vermicomposting of FVW and FVW + EAS is statistically significant at 0.05 level.

nitrogen content in the bed compartment of the treatment for the mixture of carrot with EAS may be associated with the faster growth rate of earthworms (27.7 mg/worm/day) recorded in this reactor due to that a part of nitrogen in the initial substrate is also transformed into the earthworm body (Hobson et al., 2005; Viji and Neelananarayanan, 2015). Previous studies also reported that the denitrification process within the earthworms' digestive tract could be a possible reason for the lower nitrogen content after vermicomposting (Hobson et al., 2005). The excessive growth rate of earthworms may

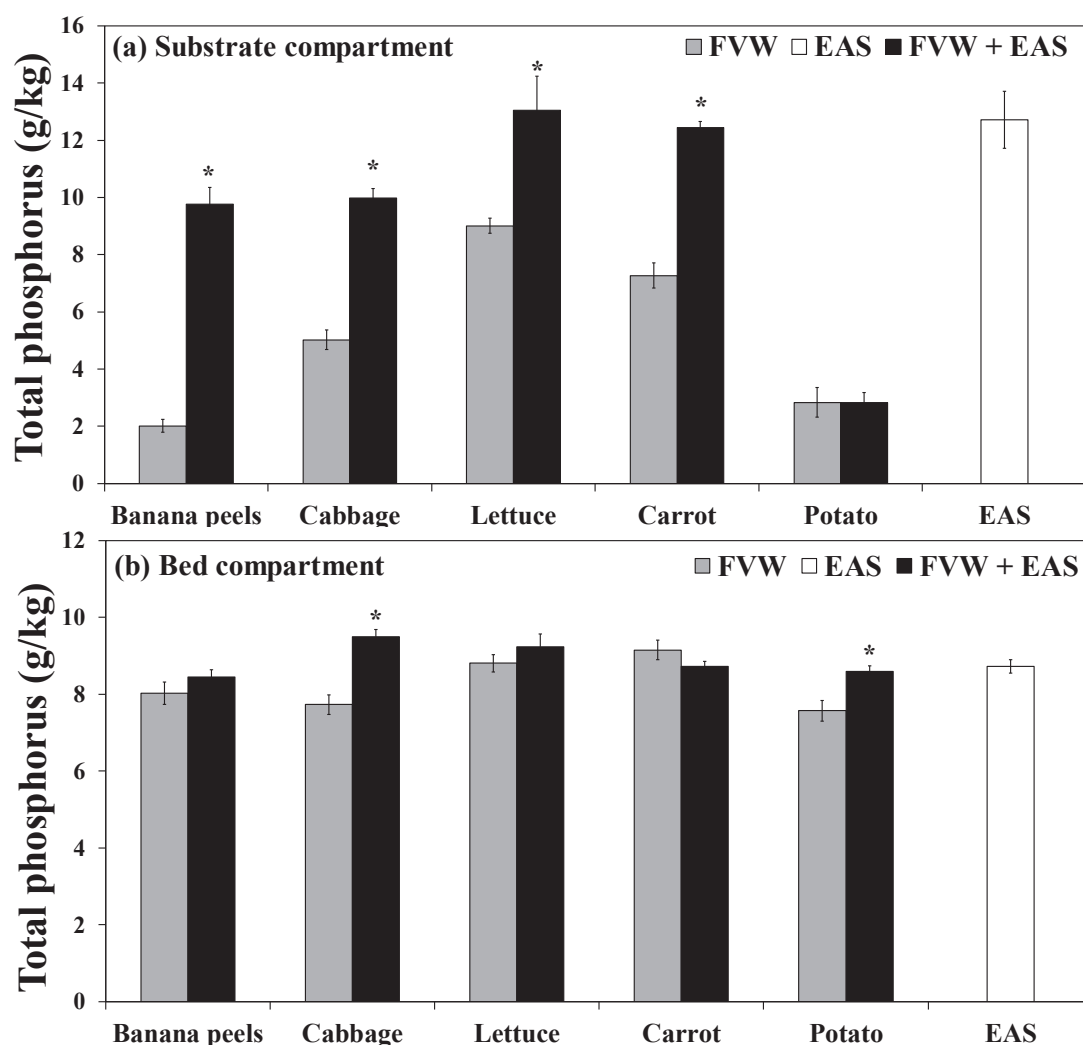


Fig. 4.9 Total phosphorus in the (a) substrate compartment and the (b) bed compartment after vermicomposting. Data are presented as mean and standard deviation, $n = 3$. EAS: excess activated sludge. The asterisk (*) denotes the difference between vermicomposting of FVW and FVW + EAS is statistically significant at 0.05

lead to higher consumption of nitrogen and faster denitrification process in earthworms' digestive tract.

Total phosphorus showed similar results with the total nitrogen, as could be seen from and the activity of microorganisms, thus enhanced the mineralization and mobilization of phosphorus. The earthworm gut can produce a considerable amount of alkaline phosphatases, an essential enzyme involved in biogeochemical cycle of phosphorus in soils (Suthar, 2010). Earlier study also suggested that the increase in phosphorus content is a consequence of the remarkable microbial activity on the ingested material as it passes along the worm's alimentary canal, resulting in phosphorus enrichment (Suthar, 2008a). In addition, it is proved that the extent of total phosphorus after vermicomposting is highly relied on the phosphorus contained in the initial substrates (Huang et al., 2012). The content of total phosphorus in different levels among different vermicomposting treatments is closely related to the quality of organic wastes as substrate (Suthar, 2010). Similar with the result of total nitrogen, a lower total phosphorus content was also recorded in the treatment for carrot with the addition of EAS. Excessive growth of earthworms and the lower microbial activity may be the possible reasons for the lower phosphorus content. Garg and Gupta (2011) documented that the microorganisms play an important role in the enhancement of phosphorus.

The obtained results of this study indicated that the addition of EAS can be considered as a feasible option to enhance the performance of vermicomposting process for treatment of FVW judging from the aspects of organic matter destabilization efficiency, microbial and earthworms' activity, and the fertilizer value of the final product. However, for overall evaluation of the effect of EAS addition, systematic evaluation from the viewpoints of gas production and economic potential are important. For gas production, Lim et al. (2016) mentioned that the production of gas during the vermicomposting

process could lead to secondary pollution on environment. The addition of EAS to FVW for combined treatment may increase the CO₂ production; at the same time, it may also lead to emission of such gases as N₂O, NH₃, H₂S if the process is not well controlled, for instance, if anoxic or anaerobic environment occurred in some part of the reactor. This is very likely because different from FVW, EAS is mainly consisted of microorganisms that contain relatively higher content of nitrogen and sulfur. Therefore, intensive study on gas production process is very important and will be investigated in coming study. On the other hand, vermicomposting is reported as a sustainable process which have a high economic potential compared to other methods (Lim et al., 2016). The addition of EAS could benefit to the sustainable process because EAS, as a waste, can be converted to useful material. However, it may also increase the cost for maintenance and operation of the whole process (collection and transportation of EAS, mixing EAS with FVW). Since many factors affect the economic potential of the process, intensive studies are necessary, which will be conducted in coming studies. Obviously, from the point of utilization value of final product, a positive effect on the economic potential could be achieved by the addition of EAS. Despite this, further study is required to clarify the optimum addition rate of EAS for the vermicomposting of FVW, thus avoiding the excessive intake of nutrients by earthworms which leads to lower nutrient contents in the final products like what occurred in the treatment for the carrot.

4.4 Summary

The addition of EAS could be a feasible option for the enhancement of vermicomposting for treating FVW. The EAS:

- 1) promoted the growth and cocoons production of earthworms;
- 2) promoted the microbial activity in the treatments for banana peels, cabbage, lettuce, and potato, while inhibited the microbial activity in the carrot treatment;
- 3) enhanced the decomposition efficiency of FVW;
- 4) improved the contents of nitrogen and phosphorus in the final products after vermicomposting.

However, the optimum addition rate of EAS into the vermicomposting of FVW should be clarified in future study.

Chapter 5 Antibiotic resistance genes during vermicomposting of excess activated sludge and fruit and vegetable waste

5.1 Background and objective

Antibiotic resistance genes (ARGs), as a newly emerging contaminant, are of great concern worldwide since their proliferation in the environment can bring about serious environmental and public health issues (Peng et al., 2017; Huang et al., 2020). Improper handling of organic waste, including excess activated sludge (EAS) and fruit and vegetable waste (FVW), is reported to be one of the most important routes that spread ARGs to the soil and water environments (Liu et al., 2018; You et al., 2018). EAS, the main byproduct of biological wastewater treatment process, is generated in large quantities and has been recognized as a significant reservoir for many types of ARGs (Cui et al., 2019; Kui et al., 2020). The most frequently detected ARGs in EAS are tetracycline resistance genes and sulfonamide resistance genes (G. Cui et al., 2019). These genes can be actively expressed and transcribed via horizontal gene transfer involving the mobile genetic elements, such as plasmids and integrons (Liu et al., 2019). Different from EAS, FVW is another type of organic waste generated in large quantities. The generation of FVW occurs during the entire food supply chain and its consumption process (Sharma and Garg, 2017). Improper treatment and disposal of FVW is also a great issue faced by many developing countries. FVW is characterized with large water content and rich biodegradable organic compounds (Li et al., 2020a). ARGs are also detected in FVW, which include the original ones existed in the waste material and also some newly emerging ones induced if landfill is used for its treatment and disposal. The leachate from FVW during landfilling is a likely pathway to spread ARGs to the surrounding

environments (Zhang et al., 2016a). Earlier studies have pointed out that sustainable treatment methods for organic waste, such as composting and anaerobic digestion, cannot completely eliminate ARGs (Su et al., 2015; Zhang et al., 2016; Xu et al., 2019); and ARGs remaining after treatment could enter the agricultural system when the treated organic waste is applied as organic fertilizers or soil modifiers, leading to further development of antibiotic resistance in the environment (J. Zhang et al., 2018). A long term application of organic waste-based fertilizers in agricultural fields could increase the occurrence and dissemination of ARGs not only in the soil but also in the final agricultural products, including vegetation and crops (Ding et al., 2019; Murray et al., 2019).

Vermicomposting is an eco-friendly biological treatment method for organic waste. This method has been attracting researchers' attention in recent years due to its effectiveness in converting organic waste into organic fertilizers having higher utilization value through the joint action of earthworms and microorganisms (Yadav and Garg, 2019). So far, many research results on vermicomposting treatment of different types of organic waste (including EAS and FVW) have been reported (Khatua et al., 2018; Karmegam et al., 2019; Rini et al., 2020). For instance, using fresh EAS as the target for treatment, Fu et al. (2015a) indicated that stabilization and beneficial final product could be achieved through vermicomposting after palletization pretreatment for the sludge. In another study, Castillo-González et al. (2019) conducted vermicomposting treatment for food and vegetable waste mixed with buffalo dung, and found that the total nitrogen and potassium as well as the total available phosphate increased significantly in the final product of vermicomposting that applied the conventional composting as the pretreatment.

In addition to the confirmed efficiency in stabilization of bioavailable organic constituents in the waste, vermicomposting is also reported to be capable of eliminating ARGs contained in the waste. Huang et al. (2018) reported that tetracycline resistance

genes and the mobile genetic element gene (*intl 1*) in EAS of a municipal wastewater treatment plant were significantly reduced in their abundances after vermicomposting. In another recent study, Huang et al. (2020) pointed out that vermicomposting can selectively eliminate the relative abundances of some tetracycline and sulfonamide resistance genes in EAS. These effects were confirmed based on the observed difference on the species and abundances of ARGs in the final products between vermicomposting and conventional composting. However, information on the behavior of ARGs in different organic waste, such as different types of FVW, and that on the role of earthworms in regulating the fate and behavior of ARGs in the waste materials remain unclear; for which, academic studies are highly required.

Another fact worthy of special mention with respect to vermicomposting treatment of EAS or FVW is that pretreatment is generally necessary in order to tackle the problems associated with the feature of higher water content of the waste, such as drying the fresh FVW to lower the leachate (Huang et al., 2013), mixing EAS with other bulking materials rich in carbon to increase the C/N ratio (Suthar, 2009b), and pre-composting of food and vegetable waste to reduce the effect of toxic substances (Sharma and Garg, 2017). The pretreatment methods can ameliorate the living environment for earthworms; but they lengthen the time needed for treatment and also lower the nutrient value of the final product (Li et al., 2020a). In a recent study for treatment of FVW, Li et al. (2020) mixed EAS into FVW and treated the mixed substrate using a separate operation system that packed substrate (FVW and EAS) and bed material (supporting layer for earthworms) in two different compartments. Mixing EAS into FVW not only reduced the C/N ratio of FVW but also brought about a large number of bacteria into the vermicomposting system, hence enriching the whole bacterial community involved in breaking down and degrading FVW. The nutrient value of the final product, regarding the content of nitrogen and

phosphorus, was also enhanced apparently. However, this approach may bring about a new problem that must be addressed and solved; the problem of ARGs, since mixing EAS into FVW can introduce ARGs and mobile genetic elements in the EAS to the vermicomposting system. For this novel mixed treatment, research on the fate of ARGs has not yet been conducted and the mechanisms including the role of earthworms are not yet identified.

For the novel separate operation system that has a substrate compartment designed atop a bed compartment, the main advantage is its capability to prevent the leachate in the initial hydrolysis process of FVW and EAS from penetration to the lower part where earthworms prefer to stay and excrete cast. In this system, substrate intake by earthworms generally takes place at times when they move to the substrate compartment. The separation of the substrate from the bed material could also prevent the formation of anaerobic or anoxic environment along the vertical direction of the reactor, which are detrimental to the activity of earthworms. Bacteria in vermicomposting is greatly affected by the activity of earthworms through the gut associated process (GAP) and the cast associated process (CAP) (Domínguez et al., 2017). The fate of ARGs in the system can thus vary because bacterial species and densities involved in hosting and transferring ARGs may differ. An earlier study reported that the gut associated process (GAP) responded to the direct effect of gut bacteria by ingestion, digestion and assimilation of the waste materials; while, the cast associated process (CAP) was more closely associated with the function of bacteria in the cast from the gut (Gómez-Brandón et al., 2012). The novel vermireactor with separate substrate and bed compartments can make possible the deeper exploration of the fate of ARGs in vermicomposting treatment of FVW and EAS, and can generate data for better understanding of the mechanisms.

Accordingly, the main objective of this study was to investigate the fate of ARGs

brought into the system from EAS for treatment of different types of FVW using novel vermireactors that consist of substrate and bed compartments. For this, eleven vermireactors were established and five types of FVW (banana peels, cabbage, lettuce, carrot and potato) and a representative EAS were used as the substrate for separate and mixed treatment (the mixture of each FVW with EAS). For ARGs, the widely detected *tet G*, *tet M*, and *sul I* in wastewater and natural environment were targeted, together with the well detected mobile genetic element gene (*intl I*). In addition, 16S rDNA in both substrate and bed compartments as well as in the fresh cast of earthworms were also quantified in order to evaluate the fate of ARGs from the relative abundance levels. To clarify the role of earthworms, the changes in the abundance of each targeted gene in the fresh cast derived from earthworms before and after vermicomposting were also investigated. To our best knowledge, this is the first study that investigated the fate of ARGs and *intl I* in the substrate and bed material separately, and is also the first study that tried to clarify the changes of ARGs and *intl I* in the fresh cast of earthworms before and after introduced to treatment for FVW, EAS and their mixtures. The present study also provides new insights for controlling the risk of the final products used as fertilizers or soil modifiers.

5.2 Materials and methods

5.2.1 Vermicomposting process and sample collection

5.2.1.1 Vermireactors and materials

Vermicomposting experiments were performed using eleven novel vermireactors designed by Li et al. (2020a). As shown in **Fig. 4.1, Chapter 4**, each reactor had two compartments, a substrate compartment and a bed compartment, with both being separated by a plastic plate opened with holes for the earthworms to move between the

two compartments. *Eisenia fetida* was used as the earthworm species due to its wide tolerance against environmental variables (Huang et al., 2012; Li et al., 2020a). Five types of FVW (banana peels, cabbage, lettuce, carrot and potato) from a supermarket and the dewatered EAS from a slaughterhouse wastewater treatment plant in Gifu, Japan were used as the substrates. The FVW was cut into pieces with a width of about 1 cm (and a thickness of 2 mm for carrot and potato) before the experiment. For the initial bed material, a mixture of soil with the final product from our previous study was used to avoid the threat to earthworms' survival from leachate generated during vermicomposting. The general properties of the FVW, EAS and bed material used in this study before vermicomposting are shown **Table 4.1, Chapter 4**.

For each bed compartment, 100 g (wet basis) bed material and 10 adult earthworms (individual weight: 350–500 mg) were added. For the substrate compartments of six vermireactors, 100 g (wet basis) of banana peels, cabbage, lettuce, carrot, potato, and EAS were filled respectively. For the remaining five substrate compartments, 100 g (wet basis) of the mixture of each FVW type and the EAS with a ratio of 3:2 on wet mass basis were filled respectively. Vermicomposting was conducted under the dark condition at 25 °C until the substrate had been completely treated. The details of the vermireactors used in this study are shown **Fig. 4.2, Chapter 4**.

5.2.1.2 Collection of samples (substrate, bed, and fresh cast)

Samples from both substrate and bed compartments of each vermireactor were homogenized and collected separately after vermicomposting. The collected samples were separated into two parts: one part was used immediately for the measurement of water content and dehydrogenase activity (DHA), and the remaining part was frozen dried by using a freeze-vacuum dryer. The dried samples were pulverized and stored at –25 °C for further analysis. The initial fresh cast sample was obtained from earthworms cultured

in the initial bed material before vermicomposting based on the method of earlier studies (Aira et al., 2016; G. Cui et al., 2019). After vermicomposting, the fresh cast samples derived from earthworms in different vermireactors were also collected using the same method. Briefly, the earthworms from each treatment were washed and wiped carefully before collecting fresh cast (Li et al., 2020a). The cleaned earthworms were placed in different sterile plastic petri dishes respectively and then were kept in dark at 25°C for 5 hours to empty the fresh cast inside the gut of earthworms. After that, the fresh cast in the petri dishes after removing earthworms were frozen dried and then stored at -25°C for further analysis.

5.2.2 DNA extraction and determination of target genes

The frozen dried samples of each treatment were used for extracting and purifying total genomic DNA by the DNA extraction kit (PowerSoil, MOBIO, USA) following the manufacturer's manual. The tetracycline resistance genes (*tet G*, *tet M*), sulfonamide resistance gene (*sul I*), the mobile genetic element (integrase class 1 gene: *intl I*), and the 16S rDNA gene were quantified by the *q*PCR with the utilization of SYBR® Premix Ex Taq™ (TP800 TaKaRa, Japan) (Li et al., 2020b). The reason for selecting these ARGs for the present study is because they were highly detected in EAS (G. Cui et al., 2019) due to the wide use of tetracycline and sulfonamide (Luo et al., 2010). The gene of *intl I* is one of the most widely detected mobile gene that closely associate with the proliferation of ARGs via horizontal gene transfer (Liu et al., 2019). The relative abundance of ARGs and *intl I* against the total bacteria was calculated by dividing the absolute abundance with the copy numbers of 16S rDNA. The standard curves of *tet G*, *tet M*, *sul I* and *intl I* were constructed by the TA-cloning method (Cui et al., 2018). Each gene was quantified in triplicate for each sample. The primer information and *q*PCR conditions are given as

Table 3.2, Chapter 3. The absolute abundances of the targeted ARGs, *intl 1* and 16S rDNA in the substrate and bed materials, as well as in the fresh cast before vermicomposting are displayed in **Table 5.1**.

5.2.3 Analysis for physicochemical and microbial parameters

The general physicochemical and microbial parameters for substrates and bed materials were analyzed following the methods in literature (Li et al., 2020a). Briefly, water content was determined by drying the samples to a constant weight at 105 °C (at least 5 h) in an oven, pH and electrical conductivity (EC) were measured by using the liquid solution adjusted by adding each pulverized dry sample with deionized water (w/v = 1/10) after shaken for 2 hours. Total carbon and total nitrogen were analyzed by the dry combustion method (CN CORDER SUMIGRAPH NC-22F; SHIMADZU). Total phosphorus was analyzed by using the molybdenum blue-absorption method with a spectrophotometer at the designated wavelength of 880 nm. Dehydrogenase activity (DHA) was measured by using the triphenyl tetrazolium chloride (TTC) method.

5.2.4 Statistical analysis

One-way analysis of variation (ANOVA) at the 95% confidence level was conducted to assess the differences of the abundances for all target genes in the substrate compartment, bed compartment and fresh cast samples of different treatments by using Statistics 21 software. The significant differences of the abundances for all target genes before and after vermicomposting in different treatments were also analyzed individually by *t*-test with the 95% confidence level (Statistics 21 software).

Table 5.1 Initial absolute abundances of ARGs, *intl 1* and 16S rDNA in substrate and bed materials, and those in the fresh cast derived from earthworms before vermicomposting. Data are presented as mean and SD, n = 3. EAS: excess activated sludge; SD: standard deviation.

		<i>tet G</i> (copies/g-dry)		<i>tet M</i> (copies/g-dry)		<i>sul 1</i> (copies/g-dry)		<i>intl 1</i> (copies/g-dry)		16S rDNA (copies/g-dry)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Substrate	Banana peels	3.8E+06	7.0E+05	7.8E+04	1.4E+04	2.0E+06	3.9E+05	1.6E+06	1.6E+05	1.3E+09	8.7E+07
	Cabbage	4.1E+05	3.1E+05	1.4E+04	9.3E+03	6.4E+04	1.8E+04	2.1E+05	5.7E+04	9.8E+09	2.2E+09
	Lettuce	4.6E+05	1.8E+05	2.5E+04	1.4E+04	5.7E+04	2.6E+04	1.4E+05	3.5E+04	1.9E+10	1.8E+08
	Carrot	9.4E+05	9.1E+05	1.9E+04	1.3E+04	9.4E+05	2.1E+05	8.1E+06	1.6E+06	1.7E+10	1.2E+09
	Potato	8.5E+05	6.3E+05	1.0E+04	6.0E+03	6.1E+04	3.8E+04	3.6E+04	2.9E+04	1.5E+10	5.6E+08
	EAS	1.4E+08	1.2E+07	2.1E+06	3.4E+05	7.4E+07	2.4E+07	3.8E+07	1.2E+07	2.4E+10	2.2E+09
Initial bed material		7.2E+07	7.7E+06	3.0E+06	3.3E+05	3.2E+07	5.9E+06	8.7E+06	2.3E+06	2.8E+10	2.9E+09
Initial fresh cast		1.2E+07	7.2E+05	5.8E+05	1.3E+05	3.5E+06	2.5E+05	1.6E+06	1.0E+05	2.7E+11	4.4E+10

5.3 Results and discussion

5.3.1 Absolute abundances of ARGs in substrate compartment

The changes in the absolute abundances of ARGs and *intl 1* in the substrate compartments before and after vermicomposting are shown in **Fig. 5.2**. Before vermicomposting, the highest absolute abundance values were recorded for the EAS ($1.4\text{E} + 08$, $2.1\text{E} + 06$, $7.4\text{E} + 07$, and $3.8\text{E} + 07$ copies/g-dry for *tet G*, *tet M*, *sul 1*, and *intl 1*, respectively). For all five different types of FVW, the absolute abundances of ARGs and *intl 1* were detected in lower levels compared with those in EAS. In addition, no significant dissimilarity was observed among different FVW types, except for *intl 1* in carrot ($P < 0.05$). The occurrence of ARGs in FVW was probably owing to the fact that fruit and vegetable are normally cultivated in open fields susceptible to contamination by bacteria via soil, organic fertilizer and water for irrigation (Larrañaga et al., 2018). For ARGs and *intl 1* in the mixtures of EAS and FVW, the absolute abundance levels were obviously higher than those in the FVW alone ($P < 0.05$), indicating that mixing EAS to FVW introduced, to a greater extent, the genes and *intl 1* to the FVW from FVW.

After vermicomposting the abundance of ARGs and *intl 1* in EAS decreased to levels markedly lower as compared to the abundance before vermicomposting ($P < 0.05$), a result comparable to many recent studies on vermicomposting of the EAS. Huang et al. (2018) reported that tetracycline resistance genes in EAS were reduced significantly after vermicomposting, with the reduction ranging from 39.6% to 94.1%. Xia et al. (2019) also found a significant reduction of *intl 1* gene in EAS after treated by earthworms. In contrast to the treatment for EAS, the treatments for FVW showed significant abundance increases of ARGs and *intl 1*, except the treatment for carrot ($P < 0.05$). The extent of increases in the treatments for FVW alone was similar with the reported ones for treatment of FVW by conventional composting (Liao et al., 2019; Liang et al., 2020). For ARGs and *intl 1*

in the substrate compartment treating the mixtures of FVW and EAS, the elimination effect differed obviously among the studied types of FVW. The ARGs and *intl 1* presented in cabbage, carrot and potato added respectively with EAS decreased obviously after vermicomposting ($P < 0.05$). However, no obvious changes were found in the treatment for banana peels mixed with EAS.

For better understanding of the behavior of ARGs and *intl 1*, the changes of total bacteria reflected by the copy numbers of 16S rDNA were also investigated, as could be seen in **Fig. 5.2**. Similar with the changes of ARGs and *intl 1*, the abundances of 16S rDNA were significantly decreased in the substrate compartment of the treatments for FVW added with EAS and the EAS alone after vermicomposting. The largest reduction was observed in the treatment of EAS in a range from $2.44\text{E} + 10$ to $5.70\text{E} + 09$ copies/g-dry. Our previous study also reported a significant reduction of the 16S rDNA after vermicomposting of EAS and found that the reduced 16S rDNA was probably caused by the lysis of the total bacteria including the host bacteria harboring ARGs and *intl 1*, thus leading to the reduction of ARGs and *intl 1* during vermicomposting (Cui et al., 2018). In contrast, the changes of 16S rDNA in the treatments for different FVW were varied with the type of FVW. For banana peels, cabbage, and lettuce, the 16S rDNA significantly increased in the substrate compartments after vermicomposting ($P < 0.05$). The increase of 16S rDNA is in line with a previous study and can be explained as the reason that the increased available nutrients generated by decayed FVW could enhance the growth of bacteria (Wang et al., 2017). For two types of root vegetation (carrot and potato), however, the copy numbers of 16S rDNA were decreased remarkably after vermicomposting ($P < 0.05$). The observed dissimilar in the changes of 16S rDNA in different substrates was probably owing to that the different decomposition efficiency of substrates could closely influence the variation of available nutrients released from waste materials (Li et al.,

2020a), thus influencing the activity and density of bacteria (**Table 5.2**) and the growth of earthworms (**Fig. 4.3, Chapter 4**). Moreover, the bacterial community can be regulated during the vermicomposting process, which may also account for the changes of ARGs and *intl 1* (Huang et al., 2020). It is worth to notice that even if the abundances of 16S rDNA were decreased in the substrate compartments of the treatments for carrot and potato, the absolute abundances of ARGs and *intl 1* were slightly increased. It is probably due to that the root of vegetation can easily uptake the bacterial pathogens and accumulate the antibiotic resistance bacteria in the internal plant tissues (Hirneisen et al., 2012), thus further serving as a basic carrier for ARGs to induce the systemic resistance (Wei et al., 2020).

5.3.2 Absolute abundances of ARGs in bed compartment

Materials in the bed compartments after vermicomposting are considered as the final products which can be used as organic fertilizers. The changes in the absolute abundances of ARGs and *intl1*, as well as the total bacteria reflected by the copy numbers of 16S rDNA in the bed compartments before and after vermicomposting are displayed in **Fig. 5.3**. Relatively higher absolute abundances of ARGs and *intl 1* were detected in the initial bed material, as cloud be seen in **Table 5.1**. ARGs and *intl 1* in the initial bed material came from the soil used for earthworms as a supporting bed, providing a strong evidence for the wide proliferation of ARGs in the soil environment (Gao et al., 2020). After vermicomposting, for most bed compartments, no significant changes ($P < 0.05$) were found in the absolute abundance values of the ARGs and *intl 1* if compared to the initial bed material. For the treatment of cabbage, the absolute abundances of *tet G*, *tet M*, and *sul 1* in the final bed material were attenuated significantly ($P < 0.05$). In contrast, the absolute abundances of *tet G*, *sul 1*, and *intl 1* in the bed compartment for the treatment

of EAS were enriched obviously ($P < 0.05$). For the treatments of cabbage added with EAS and potato, the absolute abundances of the *tet G* gene in their bed materials after vermicomposting were becoming significantly higher ($P < 0.05$). The results revealed that even if abundant ARGs and *intl 1* were introduced into the vermicomposting system from EAS, they were not remaining or transferred into the final products after vermicomposting. Considering the changes in the absolute abundances of ARGs and *intl 1* in both the substrate and bed compartments together, it is reasonable to infer that the targeted genes in the organic wastes were significantly eliminated by vermicomposting (except the treatments for FVW alone).

The changes of 16S rDNA were very slight in the final bed materials of all treatments, and were found to be closely linked with the changes of the ARGs and *intl 1*. The most significant 16S rDNA increase in the bed material was observed for the treatment for potato (from $2.8E + 10$ copies/g-dry to $3.7E + 10$ copies/g-dry). The increased 16S rDNA was probably caused by the effect of earthworms in enriching the agricultural probiotics in the final vermicomposting product (Huang et al., 2018). In contrast, a significant decrease from $2.8E + 10$ copies/g-dry to $1.8E + 10$ copies/g-dry was detected in the bed compartment of the treatment for cabbage ($P < 0.05$). These results again revealed that the significant difference of the ARGs and *intl 1* could be explained by the changes of 16S rDNA (G. Cui et al., 2019), suggesting the elimination of ARGs and *intl 1* was achieved probably by maintaining the total bacteria through earthworms' gut. The possible mechanism will be further discussed later.

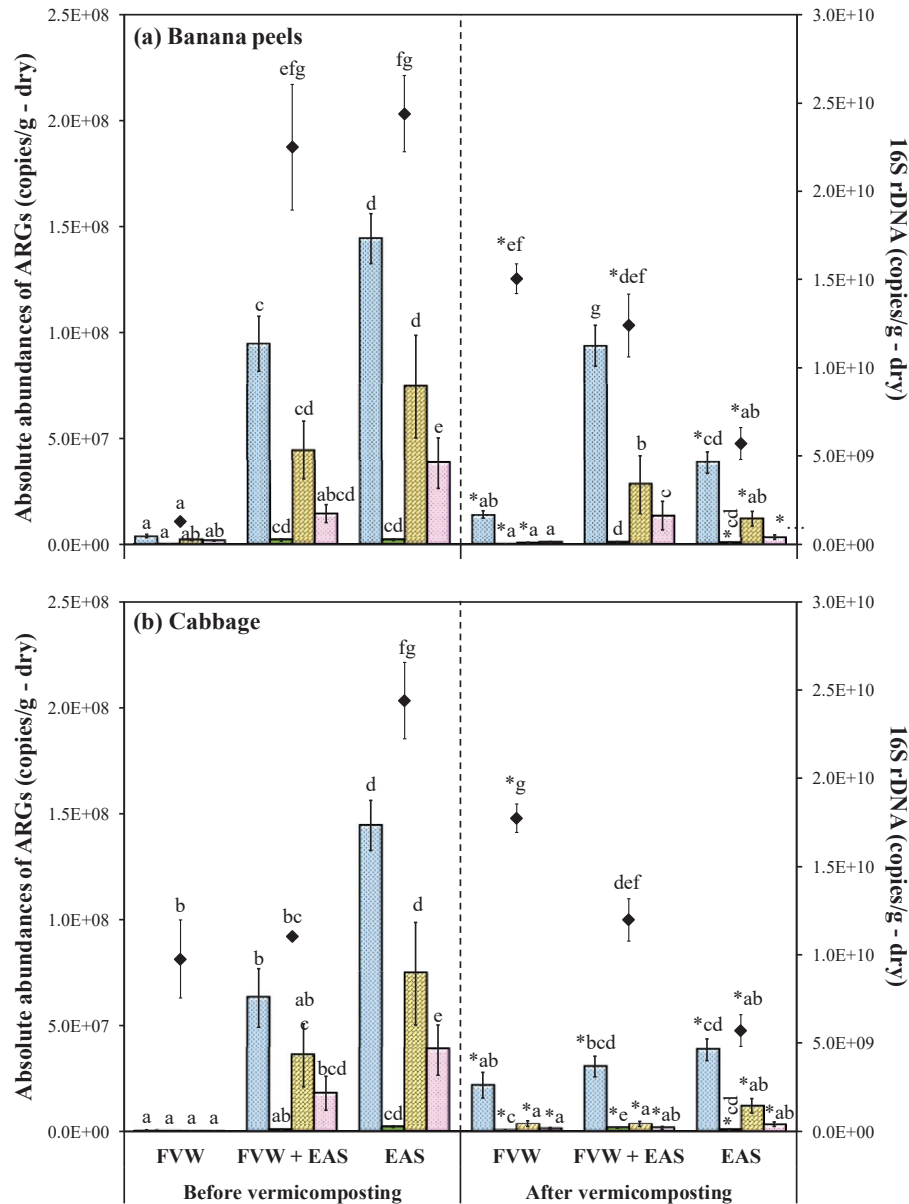


Fig. 5.2 Changes in absolute abundances of ARGs, *intl 1* and 16S rDNA in the substrate compartments after vermicomposting of FVW, FVW + EAS and EAS: (a) Banana peels, (b) Cabbage, (c) Lettuce, (d) Carrot, and (e) Potato. Data are presented as mean and standard deviation, $n = 3$. The asterisk (*) denotes the difference before and after vermicomposting is statistically significant (t -test: $P < 0.05$). The different letters indicate that the difference for a target (ARGs and 16S rDNA) is significant among the treatments based on the One-way ANOVA (Tukey's HSD test: $P < 0.05$). FVW: fruit and vegetable waste; EAS: excess activated sludge.

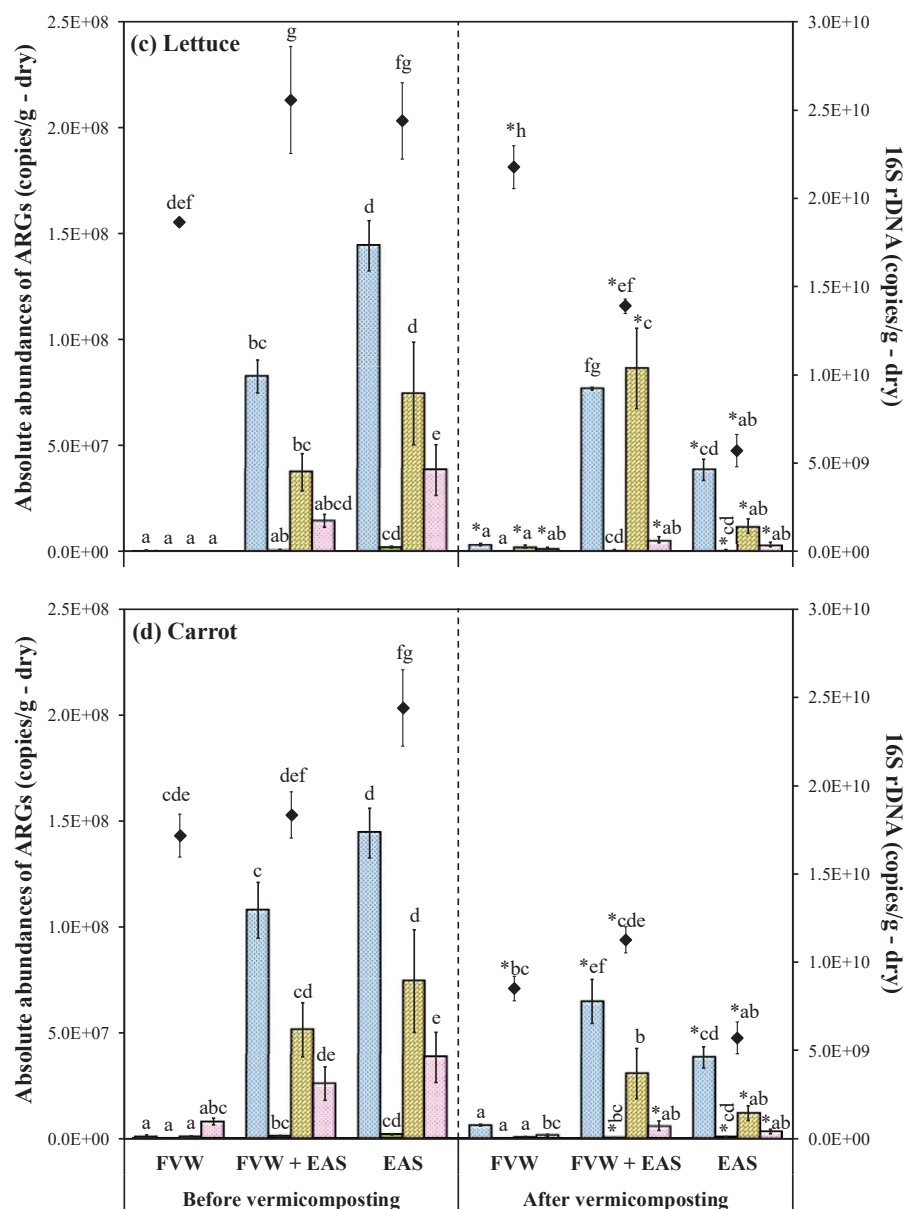


Fig. 5.2 Changes in absolute abundances of ARGs, *intl 1* and 16S rDNA in the substrate compartments after vermicomposting of FVW, FVW + EAS and EAS: (a) Banana peels, (b) Cabbage, (c) Lettuce, (d) Carrot, and (e) Potato. Data are presented as mean and standard deviation, $n = 3$. The asterisk (*) denotes the difference before and after vermicomposting is statistically significant (t -test: $P < 0.05$). The different letters indicate that the difference for a target (ARGs and 16S rDNA) is significant among the treatments based on the One-way ANOVA (Tukey's HSD test: $P < 0.05$). FVW: fruit and vegetable waste; EAS: excess activated sludge. **(Continued)**

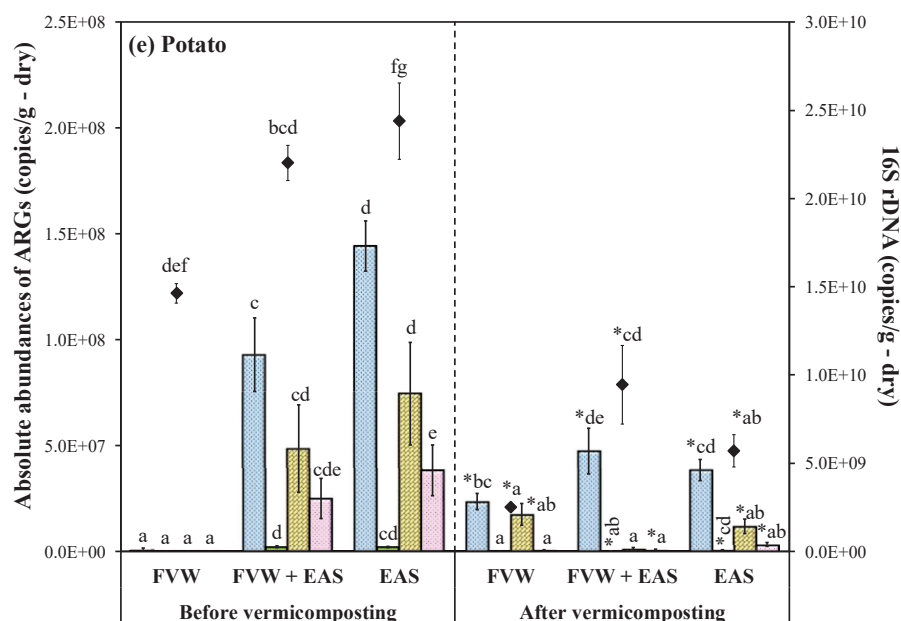


Fig. 5.2 Changes in absolute abundances of ARGs, *intl 1* and 16S rDNA in the substrate compartments after vermicomposting of FVW, FVW + EAS and EAS: (a) Banana peels, (b) Cabbage, (c) Lettuce, (d) Carrot, and (e) Potato. Data are presented as mean and standard deviation, $n = 3$. The asterisk (*) denotes the difference before and after vermicomposting is statistically significant (t -test: $P < 0.05$). The different letters indicate that the difference for a target (ARGs and 16S rDNA) is significant among the treatments based on the One-way ANOVA (Tukey's HSD test: $P < 0.05$). FVW: fruit and vegetable waste; EAS: excess activated sludge. **(Continued)**

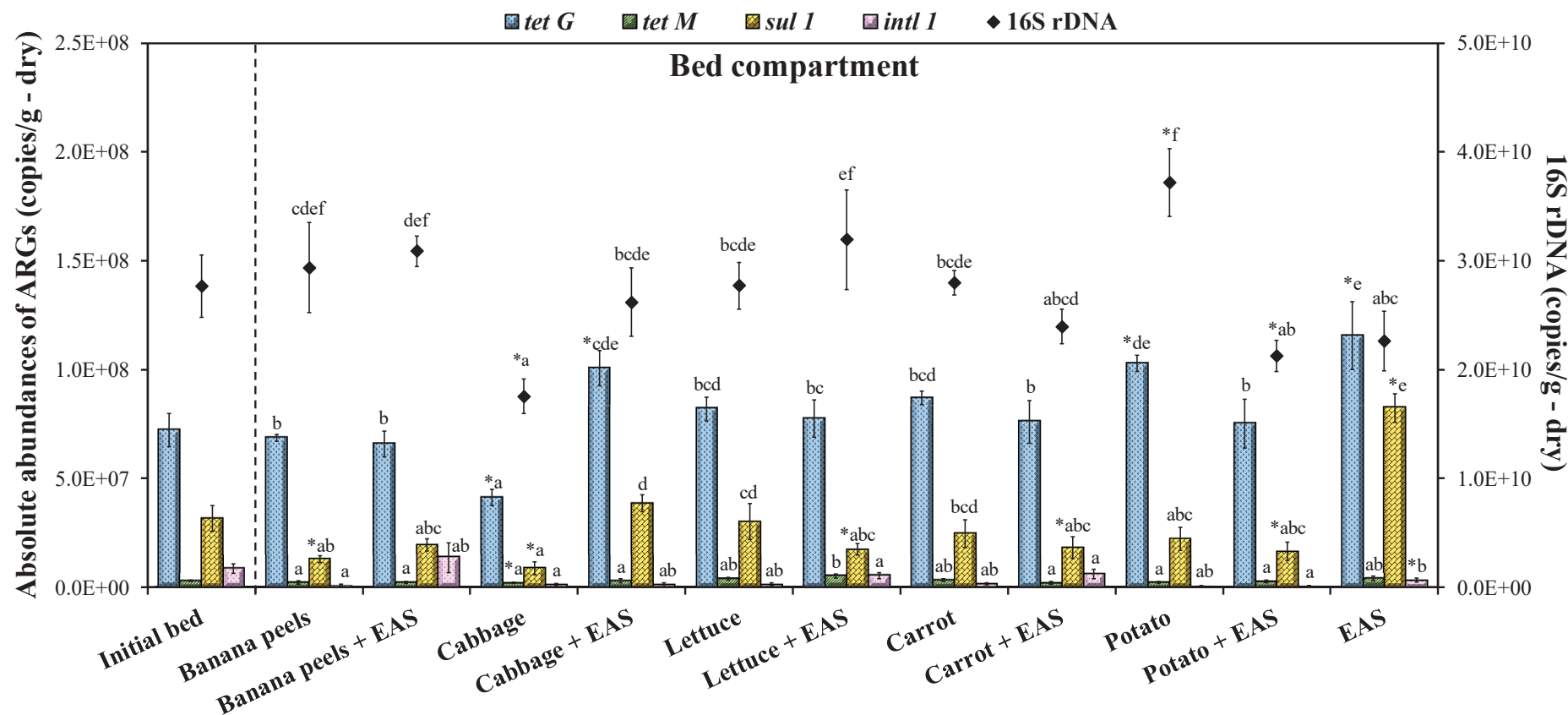


Fig. 5.3 Changes in absolute abundances of ARGs, *intl 1* and 16S rDNA in the bed compartments after vermicomposting. Data are presented as mean and standard deviation, $n = 3$. The asterisk (*) denotes the difference before and after vermicomposting is statistically significant (t -test: $P < 0.05$). The different letters indicate that the difference for a target (ARGs and 16S rDNA) is significant among the treatments based on the One-way ANOVA (Tukey's HSD test: $P < 0.05$). EAS: excess activated sludge.

Table 5.2 Properties of substrate and bed material in each treatment after vermicomposting. The asterisk (*) denotes the difference between treatment for FVW and FVW + EAS after vermicomposting is statistically significant (*t*-test: $P < 0.05$). Data are presented as mean \pm SD, $n = 3$. Values in parentheses show the changing extent (%) before and after vermicomposting, with the positive values indicating increase and the negative ones indicating reduction after vermicomposting. FVW: fruit and vegetable waste; EAS: excess activated sludge; SD: standard deviation.

Substrate compartment	Total carbon (mg/g)	Total nitrogen (mg/g)	Total phosphorus (mg/g)	Dehydrogenase activity [$\mu\text{g-TF}/(\text{g} \cdot \text{h})$]
Banana peels	430.8 \pm 7.8 (4.2 %)	18.3 \pm 0.2 (74.3 %)	2.0 \pm 0.0 (53.8 %)	363.4 \pm 63.0
Banana peels + EAS	393.0 \pm 2.8* (-7.4 %)	46.8 \pm 0.3* (38.5 %)	9.8 \pm 0.2* (63.3 %)	499.4 \pm 99.2*
Cabbage	354.2 \pm 1.1 (-8.4 %)	41.2 \pm 0.3 (43.1 %)	5.0 \pm 0.1 (66.7 %)	114.5 \pm 9.5
Cabbage + EAS	368.3 \pm 1.6 (-9.2 %)	58.8 \pm 0.3* (11.6 %)	10.0 \pm 0.1* (58.7 %)	1430.1 \pm 114.5*
Lettuce	459.9 \pm 1.0 (22.1 %)	57.8 \pm 0.3 (70.5 %)	9.0 \pm 0.1 (100.0 %)	4086.7 \pm 606.1
Lettuce + EAS	404.3 \pm 3.0* (-0.3 %)	64.0 \pm 1.2* (9.4 %)	13.0 \pm 0.1* (34.0 %)	786.3 \pm 82.4*
Carrot	425.7 \pm 5.5 (10.7 %)	43.0 \pm 0.4 (186.7 %)	7.3 \pm 0.0 (217.4 %)	7223.7 \pm 37.8
Carrot + EAS	395.5 \pm 2.9* (-2.3 %)	61.0 \pm 0.2* (32.3 %)	12.5 \pm 0.0* (56.3 %)	2782.0 \pm 58.1*
Potato	408.7 \pm 1.6 (-2.0 %)	16.8 \pm 0.5 (68.0 %)	2.8 \pm 0.0 (115.4 %)	3395.3 \pm 58.7
Potato + EAS	361.8 \pm 6.0* (-13.5 %)	20.5 \pm 0.3* (-27.3 %)	2.8 \pm 0.0 (-37.8 %)	57.5 \pm 17.0*
EAS	376.2 \pm 3.1 (-10.9 %)	70.3 \pm 1.0 (-0.6 %)	12.7 \pm 0.1 (-1.6 %)	2127.3 \pm 396.1

Table 5.2 Properties of substrate and bed material in each treatment after vermicomposting. The asterisk (*) denotes the difference between treatment for FVW and FVW + EAS after vermicomposting is statistically significant (*t*-test: $P < 0.05$). Data are presented as mean \pm SD, $n = 3$. Values in parentheses show the changing extent (%) before and after vermicomposting, with the positive values indicating increase and the negative ones indicating reduction after vermicomposting. FVW: fruit and vegetable waste; EAS: excess activated sludge; SD: standard deviation.

(Continued)

Bed compartment	Total carbon (mg/g)	Total nitrogen (mg/g)	Total phosphorus (mg/g)	Dehydrogenase activity [$\mu\text{g-TF}/(\text{g} \cdot \text{h})$]
Banana peels	331.7 \pm 11.5 (-0.4 %)	33.7 \pm 1.2 (-17.6 %)	8.0 \pm 0.3 (14.3 %)	64.3 \pm 14.4
Banana peels + EAS	330.6 \pm 20.4 (-0.7 %)	42.1 \pm 2.0* (2.9 %)	8.5 \pm 0.2 (21.4 %)	111.4 \pm 16.5*
Cabbage	307.3 \pm 13.5 (-7.7 %)	36.3 \pm 1.4 (-11.2 %)	7.7 \pm 0.3 (10.0 %)	405.3 \pm 53.8
Cabbage + EAS	364.4 \pm 9.1* (9.4 %)	48.2 \pm 0.6* (17.8 %)	9.5 \pm 0.2* (35.7 %)	0.0 \pm 0.0*
Lettuce	363.4 \pm 25.5 (9.1 %)	35.5 \pm 3.0 (-13.2 %)	8.8 \pm 0.2 (25.7 %)	87.1 \pm 4.3
Lettuce + EAS	367.8 \pm 38.6 (10.5 %)	41.8 \pm 6.2 (2.2 %)	9.2 \pm 0.3 (31.4 %)	0.0 \pm 0.0*
Carrot	324.8 \pm 29.6 (-2.5 %)	42.8 \pm 4.8 (4.6 %)	9.2 \pm 0.3 (31.4 %)	181.2 \pm 2.0
Carrot + EAS	338.4 \pm 19.1 (1.6 %)	42.0 \pm 3.4 (2.7 %)	8.7 \pm 0.1 (24.3 %)	0.0 \pm 0.0*
Potato	334.1 \pm 16.4 (0.3 %)	40.7 \pm 0.6 (-0.5 %)	7.6 \pm 0.3 (8.6 %)	188.6 \pm 20.7
Potato + EAS	323.3 \pm 14.0 (-2.9 %)	41.5 \pm 2.2 (1.5 %)	8.6 \pm 0.2* (22.9 %)	77.4 \pm 22.6*
EAS	331.7 \pm 35.9 (-0.4 %)	45.8 \pm 4.6 (12.0 %)	8.7 \pm 0.2 (24.3 %)	39.0 \pm 11.6

5.3.3 Absolute abundances of ARGs in fresh cast

The changes of ARGs, *intl 1* and 16S rDNA in the fresh cast samples derived from the earthworms in all treatments before and after vermicomposting are shown in **Fig. 5.4**. In general, the absolute abundances of all target genes were remarkably higher in the fresh cast samples obtained after vermicomposting ($P < 0.05$). The observed changes were more apparent in the bed compartments. Among all fresh cast samples after vermicomposting, the most apparent enrichment of ARGs and *intl 1* was found for earthworms from the treatment for carrot, followed by the treatment for potato with the addition of EAS. The enrichment of ARGs in the fresh cast samples supports the previous study on the changes of ARGs in the cast of earthworms after incubation in the antibiotic-amended soil (Pu et al., 2020), which revealed apparent increases in the abundance of ARGs; but is not in agreement with the findings of our previous study on the gut digestion of earthworms fed with EAS for 8 hours (G. Cui et al., 2019). In our previous study, a decrease in the abundance of ARGs and *intl 1* in the fresh cast derived from earthworms after feeding EAS was reported. However, it needs special mention that the reported decrease was based on the result of comparison with the abundance level in the initial EAS to be fed as the substrate. In the present study, the abundance levels of ARGs and *intl 1* contained in the organic wastes used as the initial substrates (FVW + EAS and EAS) were in about 8 orders of magnitude (**Fig. 5.2**). ARGs and *intl 1* were in the abundance levels of about 7 orders of magnitude in the fresh cast samples derived from earthworms in the treatments of different organic wastes (except for carrot and the potato with addition of EAS), suggesting that considerable numbers of genes in the organic waste can be significantly eliminated after gut digestion of earthworms (G. Cui et al., 2019). Contrary to our hypothesis, the absolute abundances of ARGs and *intl 1* in the fresh cast of earthworms from the treatment for EAS were not higher than those in the fresh cast

derived from the treatments for FVW. Moreover, no significant differences were found among the fresh cast samples derived from different treatments regarding the absolute abundances of ARGs and *intl 1*, except for the treatment of carrot and potato with the addition of EAS. This finding could be explained as one of the possible reasons for the enrichment of bacteria in the gut of earthworms (Chao et al., 2019; Wang et al., 2019).

Similar with the changes of ARGs and *intl 1*, the total bacteria represented by the copy numbers of 16S rDNA in the fresh cast samples also increased for all treatments after vermicomposting ($P < 0.05$), which is in agreement with the result reported by Rudi et al. (2009) that vermicomposting significantly increased the density of bacteria in the fresh cast of earthworms. For the observed increase in copy numbers of 16S rDNA, two potential explanations are conceivable: the first one is that the earthworms changed from a starved state to a fed state if organic waste is fed during the vermicomposting process, thus leading to abundant bacteria in their gut (Rudi et al., 2009); and the second one is that the gut of earthworm can result in an increase of vegetative cells and the germination of spores for the bacterial community (Huang et al., 2012). Different from our result, Wang et al. (2019) reported a significant decrease of total bacteria represented by the copy numbers of 16S rDNA in the fresh cast of earthworms after vermicomposting by comparing the numbers in the initial substrate. In addition, Gómez Brandón et al. (2011) reported a reduction of bacterial numbers in the fresh cast compared with the substrates (cow, horse and pig manure); while, no differences regarding the bacterial community structure were found between fresh cast samples derived from the different substrates. The obtained results indicated that the gut of earthworms plays a bottleneck role in regulating the bacteria in the vermicomposting system and could favor the existence of a reduced bacterial population in the organic waste (Gómez Brandón et al., 2011), thereby eliminating the ARGs and *intl 1* introduced into the vermicomposting system.

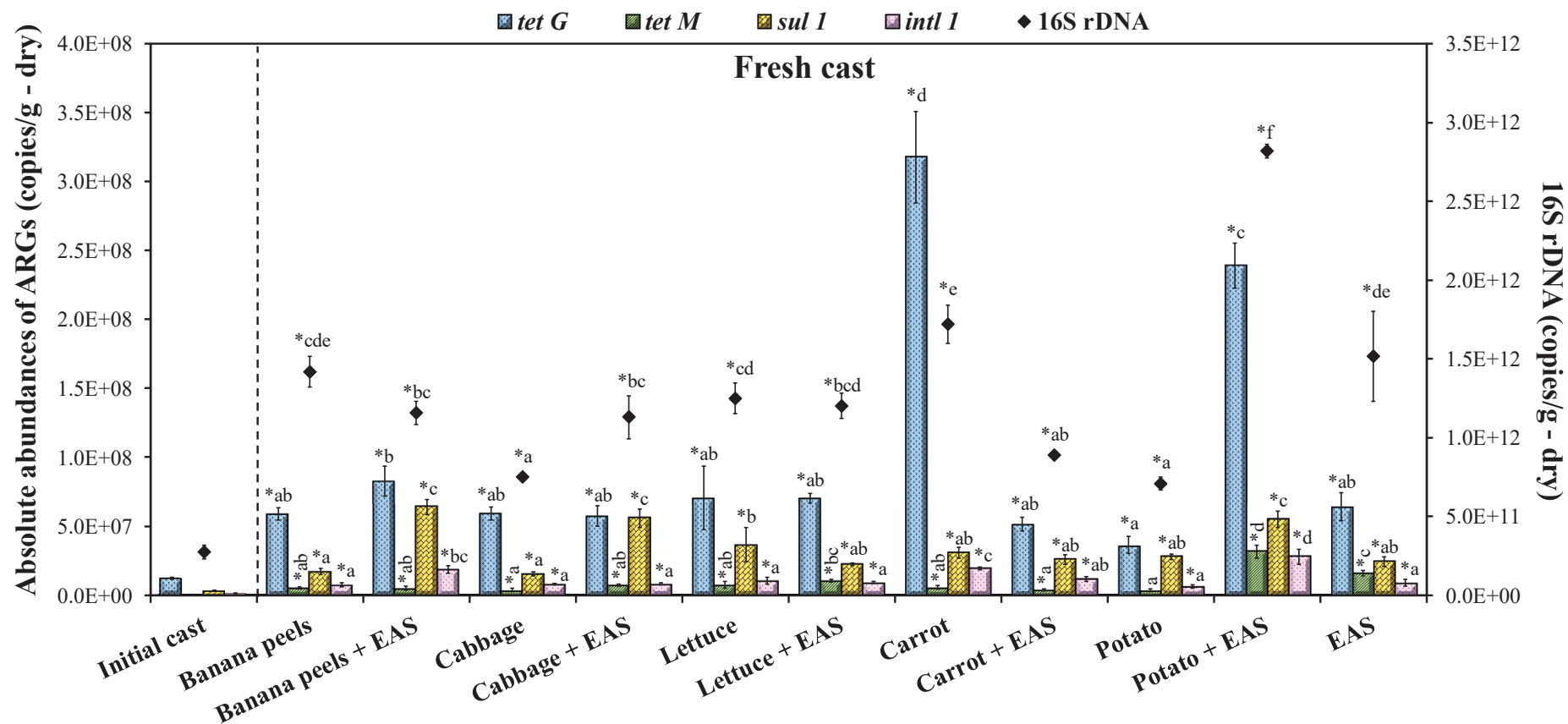


Fig. 5.4 Changes in absolute abundances of ARGs, *intl 1* and 16S rDNA in the fresh cast samples of earthworms after vermicomposting. Data are presented as mean and standard deviation, $n = 3$. The asterisk (*) denotes the difference before and after vermicomposting is statistically significant (t -test: $P < 0.05$). The different letters indicate that the difference for a target (ARGs and 16S rDNA) is significant among the treatments based on the One-way ANOVA (Tukey's HSD test: $P < 0.05$). EAS: excess activated sludge.

5.3.4 Relative abundances of ARGs after vermicomposting

The relative abundances of ARGs and *intl 1* in the substrate and bed compartments, as well as the fresh cast samples after vermicomposting were shown in **Fig. 5.5**. The relative abundances of ARGs and *intl 1* are defined as the ratio of antibiotic resistance bacteria to the total bacteria, and their changes can be caused by the variation in the numbers of possible host bacteria harboring the ARGs and *intl 1* and can indicate the transfer potential of ARGs among bacteria (Resende et al., 2014; Sun et al., 2016). In general, the relative abundances of ARGs and *intl 1* in the fresh cast samples were much lower than those in the substrate and bed compartments of all treatments after vermicomposting (about 1-3 orders of magnitude), except for the *sul 1* gene in FVW. It is revealed that the gut of earthworms can significantly change the bacterial composition with a remarkable decrease in the abundances of possible host bacteria. As a result, the bacterial composition in the fresh cast samples was distinctly different from that of the substrate bed materials (Rudi et al., 2009; Pu et al., 2020). Moreover, the relative abundances of ARGs and *intl 1* in the fresh cast samples derived from different treatments for treating different organic wastes were similar with each other, suggesting that the bacteria in earthworms' gut could be resembled by defining a shared core gut bacteria, even when assembling in the different bacterial environments (Berg et al., 2016). On the other hand, the unexpected higher relative abundances of ARGs and *intl 1* were observed in the bed materials after vermicomposting (final product used as organic fertilizer), indicating a potential risk of proliferation of ARGs in the agricultural field. It is worth to be noticed that the initial bed material used in the present study contained relatively higher amounts of ARGs and *intl 1* (**Table 5.1**), however, the relative abundances of ARGs and *intl 1* in the bed materials after vermicomposting were maintained in a contract level for all treatments, except the treatment of EAS (as shown in supplementary material). The relative abundances of

ARGs and *intl 1* in the final products are likely to be greatly affected by the ARGs and *intl 1* presented in the initial bed material rather than those in the organic waste because bacteria in the final product of vermicomposting are closely associated with the bacteria in the initial bed material (Suthar, 2009a). The results suggested that it is necessary to pay attention to ARGs and *intl 1* in the material used as the initial bed for vermicomposting.

5.3.5 Practical implications and future perspectives

In the present study, the highly detected ARGs (*tet G*, *tet M*, and *sulI*) and mobile genetic element (*intl 1* gene) in the FVW, EAS, and the mixture of FVW and EAS are confirmed to be effectively eliminated by vermicomposting using a novel vermireactor. The earthworms' gut is a hospitable micro-environment for increasing the number of total bacteria, including the host bacteria harboring ARGs simultaneously inside the gut by ingesting substrates (Chao et al., 2019). However, the increasing extent for host bacteria carrying ARGs seems to be larger than that for other bacteria, thereby leading to the elimination of ARGs during vermicomposting. Vermicomposting is suggested as a feasible approach for controlling the ARGs and *intl 1* during the treatment process for organic wastes (FVW, FVW + EAS, and EAS), especially for those rich in ARGs (FVW + EAS and EAS). According to the present study, four key points need to be emphasized for better controlling of ARGs during the practical application: 1) for the operation system, a separate substrate and bed compartments system is highly recommended since it can provide a better environment for earthworms by avoiding the risk of toxic substances generated by organic waste, the activity of earthworms is closely linked with the elimination of ARGs and the treatment efficiency of the waste material; 2) for the composition of organic waste, the waste used as substrate is closely associated with the decomposition efficiency of the waste material and the growth of earthworms, and

treating FVW mixed with EAS could lead to a better growth of earthworms (as shown in supplementary information) and a higher utilization value for the final product as fertilizers (**Table 5.2**); 3) for the density of earthworms, since the gut of earthworms is the main force for the elimination of ARGs, increasing the density of earthworms can probably improve the removal efficiency for ARGs; and 4), for the selection of bed material, since the ARGs existing in the initial bed material used as a supporting layer for earthworms can greatly affect the ARGs in the final product of vermicomposting, the potential mobility of ARGs from the final product to the soil environment or the vegetation needs to be clarified through further study.

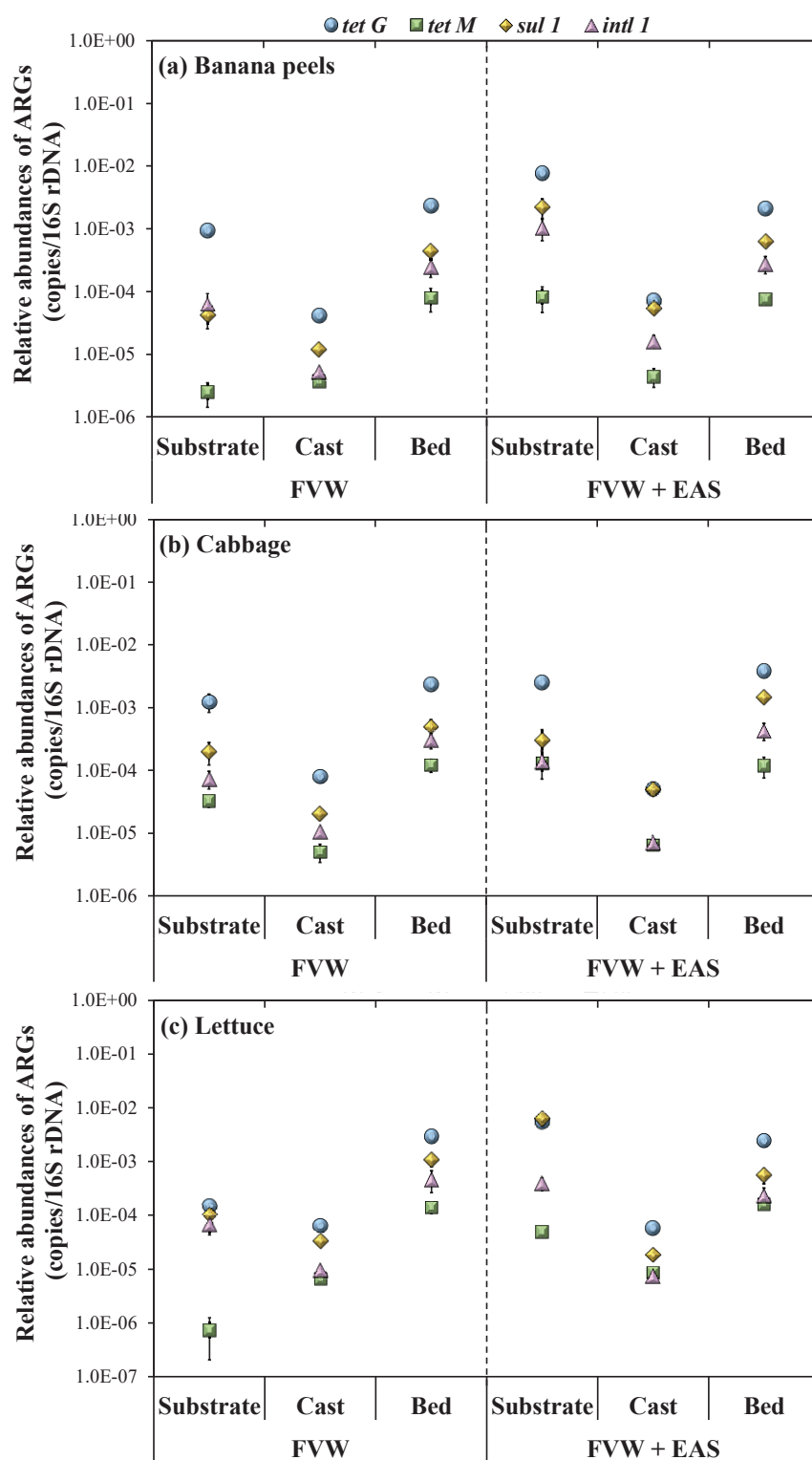


Fig. 5.5 Relative abundances of ARGs, *intl 1* and 16S rDNA in the substrate, bed material and the fresh cast of earthworms after vermicomposting of FVW, FVW + EAS and EAS: (a) Banana peels, (b) Cabbage, (c) Lettuce, (d) Carrot, (e) Potato, and (f) EAS. Data are presented as mean and standard deviation, n = 3. FVW: fruit and vegetable waste; EAS: excess activated sludge.

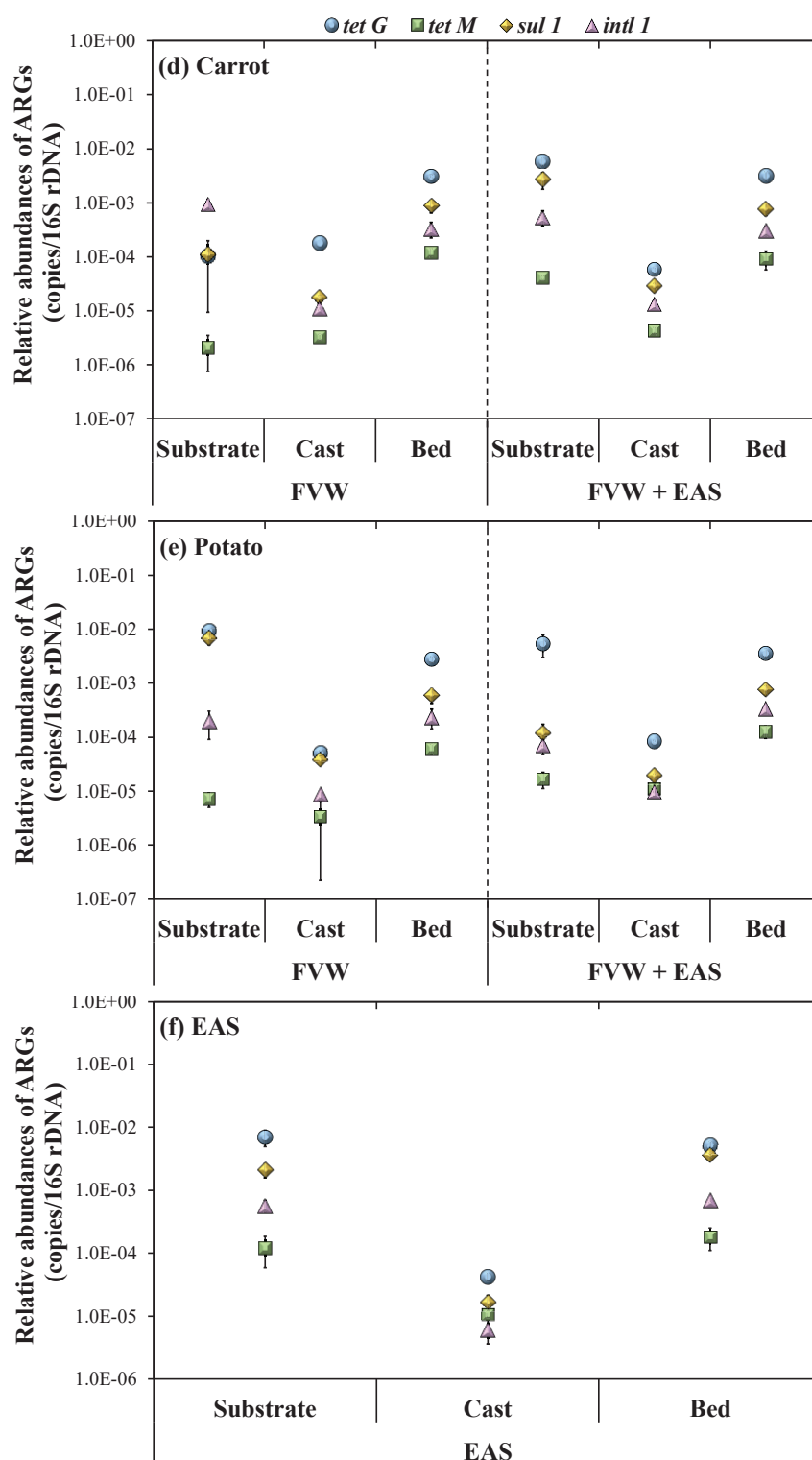


Fig. 5.5 Relative abundances of ARGs, *intl 1* and 16S rDNA in the substrate, bed material and the fresh cast of earthworms after vermicomposting of FVW, FVW + EAS and EAS: (a) Banana peels, (b) Cabbage, (c) Lettuce, (d) Carrot, (e) Potato, and (f) EAS. Data are presented as mean and standard deviation, n = 3. FVW: fruit and vegetable waste; EAS: excess activated sludge. **(Continued)**

5.4 Summary

Elimination of antibiotic resistance genes (ARGs) from excess activated sludge (EAS) added for effective treatment of different fruit and vegetable waste (FVW) by using a novel vermireactor that consists of substrate and bed compartments was investigated. ARGs (*tet G*, *tet M* and *sul I*) and mobile genetic element gene (*intl I*) were targeted and, through quantitative analysis of their abundances in both the compartments and the fresh cast of earthworms, significant reductions in substrate compartments were confirmed for the treatments for FVW added with EAS and EAS alone even if the reduction extents differed among the types of FVW. The gut of earthworms played a significant role in selectively reducing the host bacteria harboring ARGs and *intl I* through activation of non-antibiotic resistance bacteria or digestion of the resistance bacteria. The present study provided an insight for proper controlling of ARGs during vermicomposting of FVW and EAS. However, abundant ARGs and *intl I* with a contract level of those in the initial bed material were detected in all final products from different vermireactors, suggesting a possible risk for the proliferation of ARGs in the environment still exist if applied as fertilizers or soil modifiers.

Chapter 6 Conclusion

The fate and behavior of ARGs during water and solid waste treatment were investigated in this study in order to provide new insights for eliminating the proliferation of ARGs in the environment. For this, the study was carried out by three systematic investigations: 1) Clarification the fate and behavior of eARGs and iARGs in sludge with different settleability; 2) Enhancement of vermicomposting efficiency for FVW and EAS through clarifying the effect of EAS on vermicomposting of FVW; and 3) Clarification of fate and behavior of ARGs from EAS added for more effective treatment of FVW and the role of earthworms during vermicomposting. Based on the obtained results, main conclusions can be drawn as follows.

For clarification of the fate and behavior of eARGs and iARGs in sludge with different settleability, sludge from six household WWTFs was sampled and fractionated using a newly designed settling tube into fractions with different settleability, and eARGs and iARGs in the obtained fractions were evaluated by targeting on the widely detected *tet G*, *tet M* and *sul I* in water environment based on the PMA-*q*PCR method. The well-reported mobile genomic element *intl 1* and total bacterial 16S rDNA were also quantified. For the sludge fraction of lower settleability, more damaged bacterial cells were observed and the distribution of relatively higher percentages of eARGs than iARGs were found. For the sludge fraction of higher settleability, which contained flocs with larger sizes formed by both intact and damaged bacterial cells, the relative abundances of ARGs and *intl 1* were found apparently lower even if the presence percentages of eARGs were comparatively higher. The result indicates that the low settling sludge possesses higher transfer potential for ARGs and thus suggest that enhancing the settleability of the sludge flocs is important for mitigating the spread of ARGs.

To enhance the treatment efficiency of FVW and EAS by vermicomposting, a novel vermireactor consists of substrate and bed compartments was designed and used to treat

different FVW (banana peels, cabbage, lettuce, carrot, and potato) with and without the addition of EAS. The addition of EAS with higher bacterial density and activity, as well as rich nitrogen and phosphorus promoted the growth and cocoon production of earthworms. The increase of the activity of bacteria in the system of vermicomposting was also achieved, which contributed to the enhanced decomposition for FVW. The addition of EAS also improved the content of nitrogen and phosphorus in the final products and significantly lowered their C/N ratio values.

For clarification of the fate and behavior of ARGs from EAS introduced for more effective treatment of FVW and the role of earthworms, vermicomposting treatment of different FVW (banana peels, cabbage, lettuce, carrot, and potato) and EAS was conducted using novel vermireactors. ARGs (*tet G*, *tet M* and *sul I*) and the mobile genetic element gene (*intl I*) were targeted together with 16S rDNA. In addition, the targeted genes in fresh cast derived from earthworms were also quantified. Significant changes of ARGs and *intl I* occurred in all substrate compartments. In the substrate compartment, the ARGs greatly decreased in the treatments of FVW added with EAS and the treatment of EAS alone; however, in all bed compartments, no significant changes of ARGs were observed. In the fresh cast of earthworms, absolute abundance of ARGs and *intl I* increased, however, the relative abundance reduced significantly as a result of enrichment of total bacteria in the cast by a magnitude of 2 orders.

The findings of this study could provide two suggestions for better controlling ARGs during water and solid waste treatment in order to mitigate the proliferation of ARGs in the environment: 1) Reduction of ARGs especially eARGs in the treated wastewater can be achieved through the enhancement of sludge settleability by improving the sludge properties; and 2) Elimination of ARGs in solid waste such as EAS could be achieved by treating solid waste via vermicomposting before disposal.

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