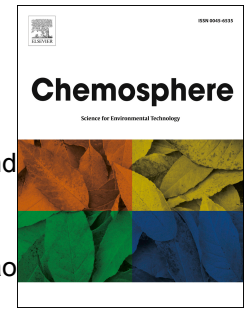
Accepted Manuscript



Effects of two ecological earthworm species on atrazine degradation performance and bacterial community structure in red soil

Zhong Lin, Zhen Zhen, Lei Ren, Jiewen Yang, Chunling Luo, Laiyuan Zhong, Hanqiao Hu, Yueqin Zhang, Yongtao Li, Dayi Zhang

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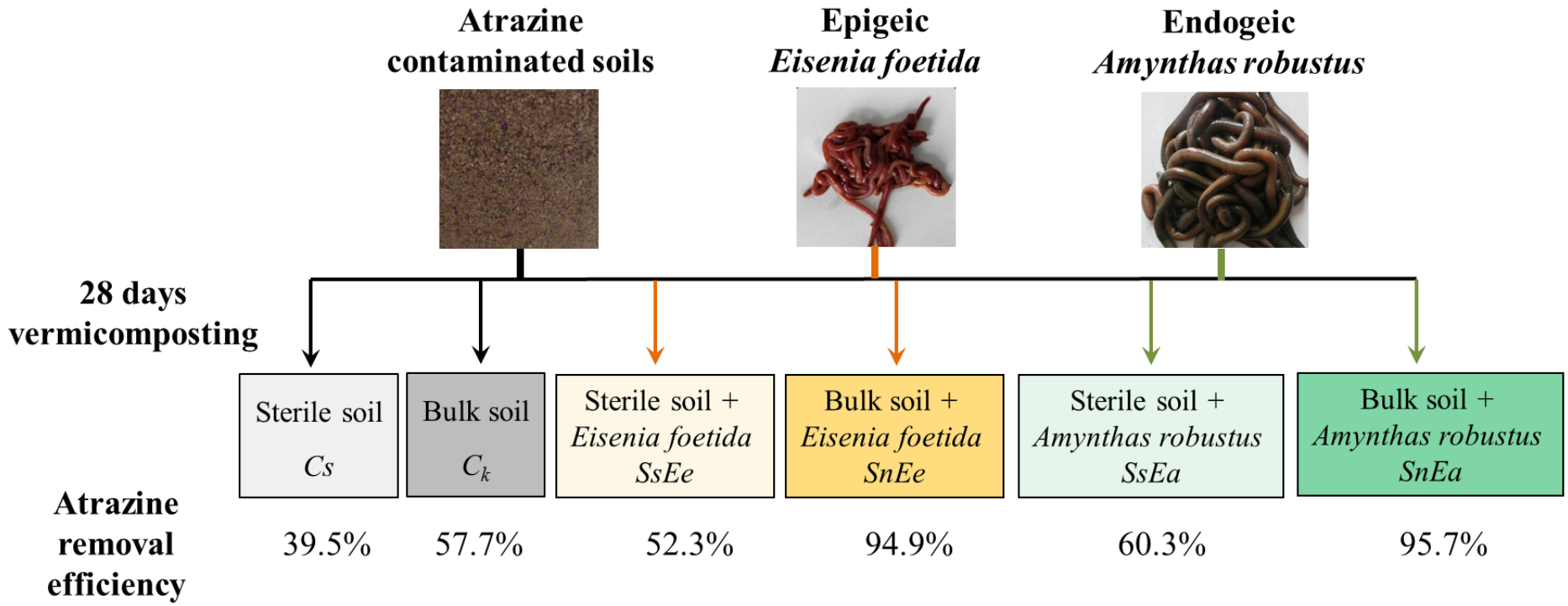
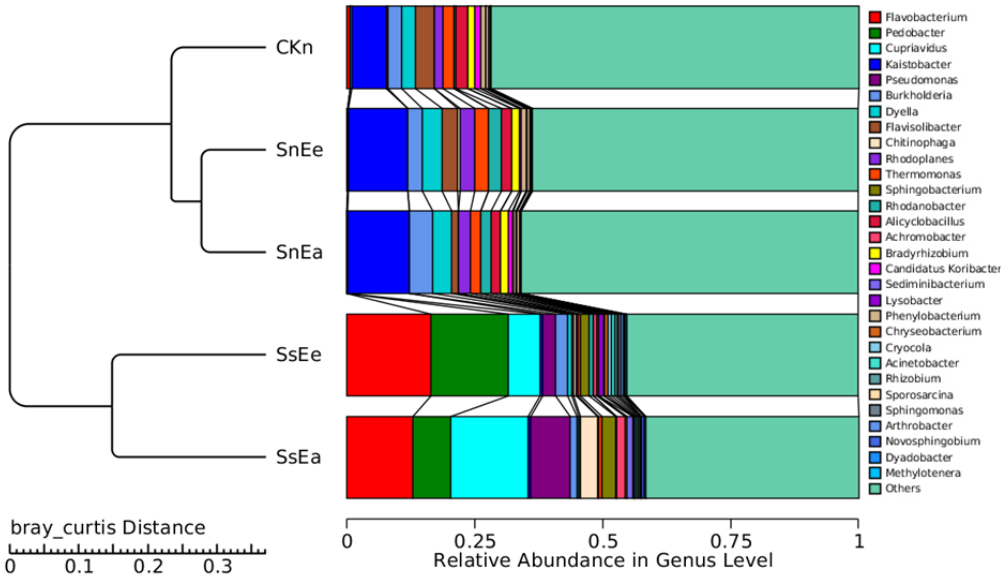
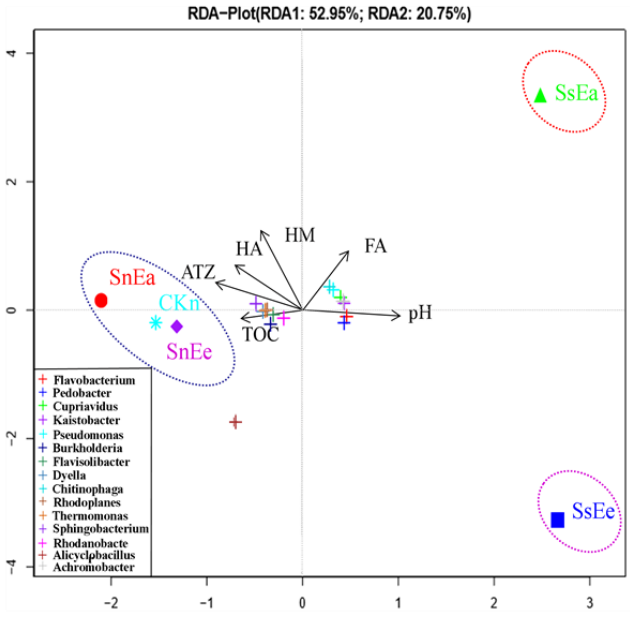

Atrazine contaminated soils
Epigeic Eisenia foetida
Endogeic Amynthas robustus
28 days vermicomposting
Sterile soil Cs
Bulk soil Ck
Sterile soil + Eisenia foetida SsEe
Bulk soil + Eisenia foetida SnEe
Sterile soil + Amynthas robustus SsEa
Bulk soil + Amynthas robustus SnEa
Atrazine removal efficiency
39.5%
57.7%
52.3%
94.9%
60.3%
95.7%


CKn
SnEe
SnEa
SsEe
SsEa
bray_curtis Distance
0
0.1
0.2
0.3
0
0.25
0.5
0.75
1
Relative Abundance in Genus Level
Flavobacterium
Pedobacter
Cupriavidus
Kaistobacter
Pseudomonas
Burkholderia
Dyella
Flavisolibacter
Chitinophaga
Rhodoplanes
Thermomonas
Sphingobacterium
Rhodanobacter
Alicyclobacillus
Achromobacter
Bradyrhizobium
Candidatus Koribacter
Sediminibacterium
Lysobacter
Phenylobacterium
Chryseobacterium
Cryocola
Acinetobacter
Rhizobium
Sporosarcina
Sphingomonas
Arthrobacter
Novosphingobium
Dyadobacter
Methylotenera
Others


RDA-Plot(RDA1: 52.95%; RDA2: 20.75%)
SsEa
SnEa
CKn
SnEe
SsEe
HA
HM
FA
ATZ
pH
TOC
Flavobacterium
Pedobacter
Cupriavidus
Kaistobacter
Pseudomonas
Burkholderia
Flavisolibacter
Dyella
Chitinophaga
Rhodoplanes
Thermomonas
Sphingobacterium
Rhodanobacte
Alicyclpbacillus
Achromobacter
4
2
0
-2
-4
-2
-1
0
1
2
3

Effects of two ecological earthworm species on atrazine degradation performance and bacterial community structure in red soil

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22

Abstract

Vermicomposting is an effective and environmentally friendly approach for eliminating soil organic contamination. Atrazine is one of the most commonly applied triazinic herbicides and frequently detected in agricultural soils. This study investigated the roles and mechanisms of two earthworm species (epigeic *Eisenia foetida* and endogeic *Amynthas robustus*) in microbial degradation of atrazine. Both earthworms accelerated atrazine degradation performance from 39.0% in sterile soils to 94.9%-95.7%, via neutralizing soil pH, consuming soil humus, altering bacterial community structure, enriching indigenous atrazine degraders and excreting the intestinal atrazine-degrading bacteria. *Rhodoplanes* and *Kaistobacter* were identified as soil indigenous degraders for atrazine mineralization and stimulated by both earthworm species. *A. robustus* excreted the intestinal *Cupriavidus* and *Pseudomonas*, whereas *Flavobacterium* was released by *E. foetida*. This study provides a comprehensive understanding of the distinct effects of two earthworm species on soil microbial community and atrazine degradation, offering technical supports to apply vermicomposting in effective soil bioremediation.

Keywords:

Atrazine, earthworm, vermicomposting, soil microbial community

1. Introduction

Pesticides have become an important part of modern agriculture nowadays, particularly for integrated pest management (IPM). As one of the most commonly applied triazinic herbicides, atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) leads to global problem of soil and water pollution owing to its massive usage, high chemical stability, apparent mobility, and significant toxicity to humans and ecosystems (Douglass et al., 2017; Sánchez et al., 2017). Although atrazine has been banned for future use in the European Union, it is allowed in many other countries including the United States and China, with an increasing annual consumption at a rate about 20% (Yue et al., 2017). Due to the high persistence in the environment, atrazine natural attenuation in soils takes extremely long time, normally years or even decades to occur (Domínguez-Garay et al., 2016). The atrazine mass fractions in soils ranged from below detection of limit (<LOD) to several mg/kg. It was reported that the atrazine mass fraction in the Yangtze River Delta agricultural soils ranged from <0.001 to 0.113 mg/kg dry soil mass, with a mean of 0.0057 mg/kg (Sun et al., 2017). Readman *et al.* (1993) also found atrazine mass fraction from <0.001 to 4.9 mg/kg across Mediterranean sediments. The residual atrazine mass fraction was in the range of 0.015-0.55 mg/kg in Iran soils (Dehghani et al., 2010). How to accelerate atrazine degradation rate and improve its remediation performance has caused increasing attentions.

Microorganisms capable of mineralizing atrazine are ubiquitous in atrazine contaminated soils, e. g., *Rhodococcus*, *Pseudomonas*, *Acinetobacter*, *Rhizobium*, *Agrobacterium*, *Xanthomonas* and *Arthrobacter* (Liu et al., 2016; Douglass et al., 2017). However, their degradation efficiencies are relatively low due to their slow

growth, low abundance and poor catabolic activities (Chirnside et al., 2009; Fang et al., 2015; Zhang et al., 2015). Bioaugmentation is an environmentally friendly approach for rapid and cost-effective clean-up of atrazine from the environment (Alekseeva et al., 2011). Currently, most bioaugmentation approaches attempt to add exogenous degrading strains or growth-promoting substrates to improve the abundance or activities of atrazine degraders, and further accelerate atrazine bioremediation (Douglass et al., 2015; Zhang et al., 2015). For instance, *Pseudomonas* sp. ADP and *Chelatobacter heintzii* are used as inoculants in atrazine bioaugmentation, and the functional degradation genes include *atzA*, *atzB*, *atzC*, *atzD*, *atzE*, *atzF* and *trzD* (Monard et al., 2008). The degradation capability of *Pseudomonas* sp. cells are reported to be enhanced by the addition of Layered Double Hydroxide bionanocomposites (Alekseeva et al., 2011). Carbon nanotubes can also enhance the biodegradation rate of atrazine through stimulating bacterial growth and the expressions of degradation genes (Zhang et al., 2015). Nevertheless, the main drawbacks of bioaugmentation lie in the poor environmental adaptability of the inoculated degraders, low utilization of additive substrates, insufficient oxygen supply and poor sustainability (Zhang et al., 2015; Zhu et al., 2017). In addition, atrazine is easily adsorbed by soil organic matters and aggregates, greatly reducing its biological accessibility or bioavailability and inhibiting microbial mineralization (Prado et al., 2014). Hence, bioaugmentation is successful in lab-scale work but always questionable in field trials.

Earthworms represent a dominant fraction of biomass in terrestrial ecosystems, having strong environmental adaptability, reproductive capacity and high resistance (Shan et al., 2014). The bioturbation of earthworms can increase soil aeration, improve the transport and distribution of microorganisms, and enhance the contact

between microorganisms and reactants (Lin et al., 2016b). Additionally, earthworms also increase soil microbial activities via digesting organic matters and improving nutrient availability (Lin et al., 2016a). Thus, vermicomposting treatments with earthworms can ameliorate soil properties, offset the limitations in bioaugmentation, and consequently improve the pollutants removal efficiency (Li et al., 2015; Lin et al., 2016a). Lin *et al*. (2016a) reported earthworms significantly enhance the pentachlorophenol (PCP) degradation by improving soil physicochemical properties and increasing microbial biomass and activities. *Lumbricus terrestris* L. is found to influence the persistence and transport of atrazine in soils, leading to the faster atrazine dissipation and mineralization in vermicomposting treatments (Farenhorst et al., 2000). Despite numerous studies investigating the roles of vermicomposting in enhancing organic pollutants biodegradation, little is known about the effects of different ecological earthworms on atrazine mineralization, via altering soil microbial community structure and encouraging atrazine-degrading microbes.

Herein, this study investigated the roles of two ecologically distinct earthworms (epigeic *Eisenia foetida* and endogeic *Amynthas robustus*) in atrazine degradation in a classic red soil in China. During 28 days vermicomposting treatments, our main aims were: 1) to analyze the atrazine residual mass fractions and degradation efficiencies in vermicomposting treatments; 2) to explore the influence of two earthworms on soil properties and bacterial community structure in red soils; 3) to identify the promoted atrazine degraders by two ecological earthworms during atrazine degradation process.

2. Materials and Methods

2.1 Soil samples

Surface upland soils (0-20 cm) were collected from Arboretum in South China

Agricultural University in Guangzhou, China (23°9′29.32″N, 113°21′12.75″E), which were identified as red soil and no atrazine was detectable. All the soil samples were air dried, passed through a 2-mm sieve, and adjusted to 60% moisture prior to use. Two earthworm species, endogeic *A. robustus* and epigeic *E. fetida*, were purchased from Yingde and Jiangmen (China), respectively. Atrazine (purity>98%), methanol, acetone and ethanol were purchased from Sigma-Aldrich (USA), and all the other chemicals were purchased from Chengshuo Company (China) except for specific statement.

2.2 Experimental design and procedure

Six treatments were set to evaluate atrazine degradation performance in bioremediation and vermicomposting process, as listed in Table 1, including: sterile soil (CKs), original bulk soil (CKn), sterile soil with epigeic *E. foetida* (SsEe), sterile soil with endogeic *A. robustus* (SsEa), bulk soil with epigeic *E. foetida* (SnEe), bulk soil with endogeic *A. robustus* (SnEa). Given the frequently detected atrazine mass fraction in soils (mg/kg level) and lethal dosage for earthworms (LD_{50}=78 mg/kg) (APVMA, 1997), the contamination level of atrazine in our study was set as 10 mg/kg. The preparation of artificial atrazine-contaminated soils followed previous protocol (Lin et al., 2016a), and the final atrazine mass fraction was 10 mg/kg (dry soil mass) for all the treatments. In vermicomposting treatments, the 2 kg soils were further added with 30 epigeic *E. foetida* (0.93±0.13 g) and endogeic *A. robustus* (2.24 ±0.26 g), respectively. Each treatment was carried out in triplicates.

Table 1. Experimental treatments.

Treatment	Note	Soil (kg)	Atrazine mass fraction	Earthworm

			(mg/kg)	(number/treatment)
CKs	Sterile soil	2	10	-
CKn	Bulk soil	2	10	-
SsEe	Sterile soil + *E. foetida*	2	10	30
SsEa	Sterile soil + *A. robustus*	2	10	15
SnEe	Bulk soil + *E. foetida*	2	10	30
SnEa	Bulk soil + *A. robustus*	2	10	15

2.3 Soil property analysis

Soil pH was measured in soil-water slurry (1:2.5, m/m) by a combination glass electrode. Soil total nitrogen was determined by Kjeldahl digestion (Li et al., 2015). After digestion with perchloric acid and hydrofluoric acid, soil total phosphorus and potassium were measured by colorimetric assay (ammonium molybdate) and by atomic absorption spectrometry (AAS), respectively (Chirnside et al., 2009). Soil total organic carbon (TOC) content was determined by dichromate oxidation. The content of fulvic and humic acids and humin were alkaline extracted and determined following standard methods for soil analysis (Swift, 1996). Soil texture was measured according to the United States Department of Agriculture soil textural triangle standard. The determination of soil maximum water holding capacity followed previously described cutting-ring methods (Lin et al., 2016b). The physical properties of the soils included: pH 5.52, TOC 36.60 g/kg, total nitrogen 1.29 g/kg, total phosphorus 1.05 g/kg, and total potassium 11.35 g/kg. The soil contained clay (38.72%), sand (33.36%) and silt (27.92%), classified as clay loam.

Soil basal respiration was determined after 0, 7, 14, 21 and 28 days of atrazine degradation, according to Lin's method (Lin et al., 2016b). The total number of cultivable bacteria, fungi and actinomyces were counted as colony forming units

(CFUs) on agar plates using the dilution plate method. The media used for the enumeration of bacteria, fungi, and actinomyces were beef extract peptone medium, Czapek's medium, and Gause's No. 1 synthetic medium, respectively (Zhen et al., 2014).

2.4 Soil atrazine analysis

Soil atrazine was extracted by methanol batch-extraction method (Lin et al., 2016a). Briefly, the 2.0 g freeze dried soil was transferred into polycarbonate centrifuge tube and added with 20 mL of methanol. After standing for 10 min and subsequently ultrasonic extraction for 15 min, the soil-methanol suspension was centrifuged at 3,200 ×g. The above procedure was repeated 3 times, and the 60 mL of extracts were pooled together. Concentrated by rotary evaporation to 2 mL, the extracts were transferred to solid-phase extraction column (AccuBOND □ ODS-C18 Cartrid-gas 500 mg/6 mL, using 4.5 mL of methanol to activate). It was further eluted with 3 mL of 5% ammonia methanol solution for three times, and the eluent was blown to nearly dry with nitrogen gas and diluted with methanol in 2 mL Agilent spectrum bottle.

Atrazine was quantified using high-performance liquid chromatography (HPLC, Waters 1525/2487, USA), supplemented with a Waters 1525 binary pump, an analytical reversed-phase column (5 μm Pinnacle II C18, 4.6 mm i.d. and 25 cm long, Waters, USA) and a Waters 2487 dual absorbance UV/Vis detector. The mobile phase consisted of 80% methanol and 20% phosphate buffer (10 mmol/L) and was pumped at a rate of 1.0 mL/min. Measurements were taken under isocratic conditions at 25±1°C at a wavelength of 220 nm. The injection volume was 10 μL. Soil atrazine mass fraction was determined according to the standard curves from five external standards.

2.5 Soil microbial community analysis

After 28 days atrazine degradation, soil DNA in each treatment was extracted with PowerSoil DNA extraction kit (MoBio, USA) following manufacturer's instructions. DNA concentrations were determined using an ND-2000 UV-Vis spectrophotometer (NanoDrop Technologies, USA). The hypervariable V4 region of the 16S rRNA gene was subsequently amplified using the primer pair of 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACVSGGGTATCTA AT-3′) with barcode, following previously described protocol (Sengupta and Dick, 2017). Purified PCR amplicons were sequenced by the Illumina HiSeq2500 platform (Novogene, China). All the reads passed the quality filtering, and the reads were discarded if the barcodes were uncorrectable, the bases with Phred Quality Score <19 covered above 30% of the read, or the ambiguous bases were over 5%. Chimeras were removed and the sequences with high quality were clustered into different operational taxonomic units (OTUs) based on 97% similarity using Uparse (http://drive5.com/uparse/). The OTU representative sequences were chosen for taxonomical classification using QIIME pipeline and Ribosomal Database Project (RDP) (Xu et al., 2017).

2.6 Data analysis

One-way analysis of variance (ANOVA) was performed to determine the difference between treatments, and the significant difference ($p<0.05$) was marked with different alphabet letters in figures. All statistical analysis was carried out in SPSS (Version 18.0). Alpha-diversity (observed species, Chao1 and Shannon) was used to estimate the complexity of bacterial community in different samples using QIIME software (http://qiime.org/scripts/alpha_diversity.html). Unweighted Pair-group Method with

Arithmetic means (UPGMA) was performed as the hierarchical clustering method interpreting the metric distance matrix using average linkage and cluster of bacterial genus by QIIME software (http://qiime.org/scripts/upgma_cluster.html). The major bacterial lineages in total sequences (top 10) exhibited the heatmap analysis and species classification tree among different samples, using R software and MEGAN, respectively (http://ab.inf.uni-tuebingen.de/software/megan4/). Canonical correspondence analysis (CCA) was conducted to investigate the impacts of each environmental factor on bacterial community structure using R software.

3. Results

3.1 Atrazine degradation performance

The residual atrazine mass fraction in all the treatments during the 28 days degradation was illustrated in Figure 1. There was no difference among the treatments at 0 and 7 days ($p>0.05$). From 14 days, the residual atrazine in vermicomposting treatments, especially in SnEe and SnEa treatments with bulk soils, was significantly lower than that in CKs and CKn (no earthworm). After 28 days biodegradation, atrazine residue in CKn (4.23 mg/kg) was significantly lower than CKs (6.05 mg/kg), proving the functions of soil indigenous microorganisms in atrazine mineralization. In sterile soil treatments, the atrazine mass fraction in SsEe and SsEa was 4.77 and 3.97 mg/kg, both significantly lower than that in CKs (7.05 mg/kg, $p<0.05$). Thus, additional 1.28 and 2.08 mg/kg of atrazine was removed by epigeic *E. foetida* and endogeic *A. robustus*, respectively. The results indicated that earthworms themselves could enhance the atrazine abiotic degradation, possibly attributing to their intestinal flora capable of mineralizing atrazine directly. In bulk soil treatments, the residual atrazine was much lower, 0.51 mg/kg in SnEe and 0.43 mg/kg in SnEa. It suggested

that soil indigenous microorganisms contributed to the majority of atrazine degradation, and earthworms could promote their activities and thus accelerate atrazine mineralization.

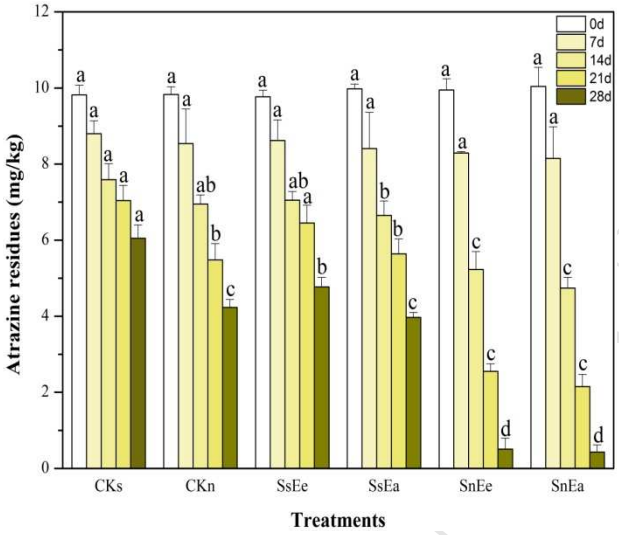


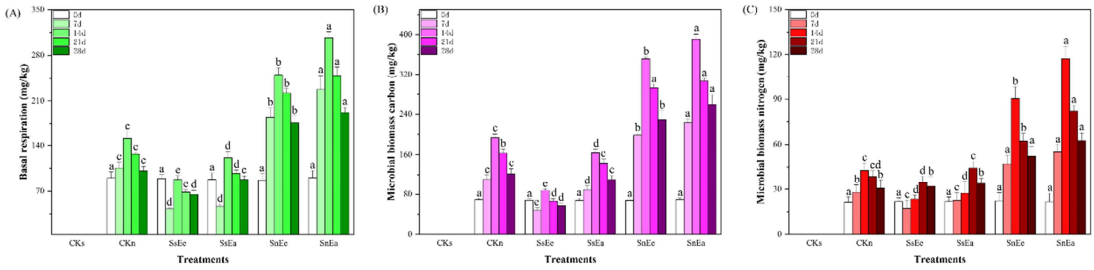
Figure 1. Soil residual atrazine mass fraction during atrazine degradation process in different treatments. Bars with different lower-case letters (a, b and c) refer to significant differences (ANOVA, Duncan's test, $p<0.05$) among the treatments, where the same letter indicates no significant difference.

3.2 Soil microbial profiles

After 28 days vermicomposting, the pH value in SnEe and SnEa treatments was 6.36 and 6.61, respectively, both significantly higher than that in SsEe and SsEa treatments, followed by CKs and CKn (Table 2). The soil TOC was highest in CKn and CKs (22.67 g/kg and 23.42 g/kg, Table 2), about 10% more than those in vermicomposting treatments (SsEe, SsEa, SnEe and SnEa). Table 2 also showed the significant decrease of humic acid and humin in both vermicomposting treatments (SnEe and SnEa comparing to CKn; SsEe and SsEa comparing to CKs), whereas fulvic acid remained

244 the same. The results fitted well with the declining soil TOC, attributed to the
245 consumption of humic acid and humin by both earthworms.

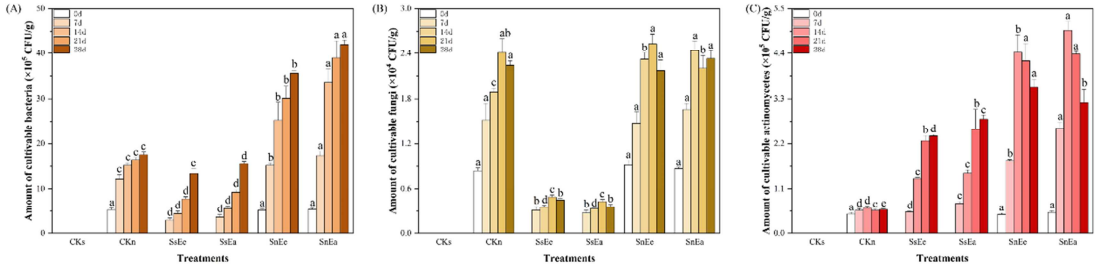
246 Soil basal respiration, microbial biomass carbon and microbial biomass nitrogen
247 varied across different treatments throughout the atrazine degradation process, as
248 shown in Figure 2. They all peaked on Day 14 and then decreased until the end of
249 experiment. The highest soil basal respiration rate (Day 14, Figure 2A) was observed
250 in SnEe (249.55 mg/kg) and SnEa (306.9 mg/kg), significantly higher than CKn
251 (151.12 mg/kg), SsEe (87.15 mg/kg) and SsEa (121.05 mg/kg). Similarly, the highest
252 microbial biomass carbon was found in SnEa on day 14 (390.85 mg/kg), followed by
253 SnEe (351.57 mg/kg), CKn (193.57 mg/kg), SsEa (163.85 mg/kg) and SsEe (87.53
254 mg/kg) (Figure 2B). The SnEa treatment also had the highest microbial biomass
255 nitrogen (117.37 mg/kg, Figure 2C), showing no significant difference with SnEe
256 (90.27 mg/kg), but remarkably higher than CKn (42.90 mg/kg), SsEa (27.34 mg/kg)
257 and SsEe (23.36 mg/kg). Our results suggested that both soil microbial activities and
258 biomass were enhanced by earthworms.



259

260 **Figure 2.** Soil basal respiration (A), microbial biomass carbon (B) and microbial
261 biomass nitrogen (C) during atrazine degradation process in different treatments. Data
262 are mean ± standard deviation (SD, n=3). Bars with different lower-case letters (a, b
263 and c) refer to significant differences (ANOVA, Duncan's test, $p<0.05$) between

264 treatments, where the same letter indicates no significant difference.



265

266 **Figure 3.** Colony forming units (CFU) of cultivable bacteria (A), fungi (B) and
267 actinomyces (C) during atrazine degradation process in different treatments. Data are
268 mean±SD (n=3). Bars with different lower-case letters (a, b and c) refer to significant
269 differences (ANOVA, Duncan's test, $p<0.05$) between treatments, where the same
270 letter indicates no significant difference.

271

272

273 **Table 2.** Soil properties after 28 days atrazine degradation.

Treatment	pH	TOC (g/kg)	Fulvic acid (mg/kg)	Humic acid (mg/kg)	Humin (mg/kg)
CKs	5.27±0.25 d	22.67±0.66 a	6.24±0.17 b	7.91±0.51 a	8.78±0.56 a
CKn	5.37±0.27 d	23.42±0.54 a	7.71±0.18 a	7.51±0.57 a	9.23±0.19 a
SsEe	5.67±0.17 c	18.77±0.62 c	7.11±0.41 ab	2.33±0.68 bc	7.54±0.29 b
SsEa	5.93±0.04 bc	19.14±0.71 c	6.94±0.43 ab	1.97±0.38 c	6.11±0.42 c
SnEe	6.36±0.11ab	21.06±0.89 b	7.97±0.28 a	3.11±0.79 b	6.45±0.17 c
SnEa	6.61±0.30 a	20.06±1.17 bc	8.21±0.70 a	2.43±0.50 bc	6.17±0.39 c

274 Note: Bars with different lower-case letters (a, b, c and d) refer to significant differences (ANOVA, Duncan's test, $p<0.05$) between treatments,

275 where the same letter indicates no significant difference.

Figure 3 illustrated the number of bacteria, fungi and actinomyces during atrazine degradation in different treatments. Cultivable bacteria showed a dramatic increase from 0 to 28 d (Figure 3A), and more cultivable bacteria were found in vermicomposting treatments with bulk soils (SnEe and SnEa) than bulk soils (CKn), all significantly higher than those in vermicomposting with sterile soils (SsEe and SsEa). After 28 days degradation, the number of cultivable bacteria was 42.01×10^5 CFU/g in SnEa, 35.57×10^5 CFU/g in SnEe, 17.54×10^5 CFU/g in CKn, 15.58×10^5 CFU/g in SsEa and 13.41×10^5 CFU/g in SsEe. Cultivable fungi increased from 0 to 21 d (Figure 3B), and then kept stable until the end of degradation. There was no difference among bulk soil treatments (CKn, SnEe and SnEa) throughout the degradation process, and they were all significantly higher than those in sterile soil treatments (SsEe and SsEa, $p<0.05$). The number of cultivable fungi after 28 days degradation was 2.33×10^4 CFU/g, 2.17×10^4 CFU/g, 2.24×10^4 CFU/g, 0.36×10^4 CFU/g and 0.45×10^4 CFU/g in SnEa, SnEe, CKn, SsEa and SsEe treatments, respectively. Similarly, cultivable actinomyces kept stable in CKn, increased in SsEe and SsEa treatments, but peaked on 14 d in SnEa and SnEe treatments (Figure 3C). On day 28, cultivable actinomyces in SnEe and SnEa treatments were 3.58×10^5 CFU/g and 3.21×10^5 CFU/g, much higher than those in SsEe (2.39×10^5 CFU/g) and SsEa (2.81×10^5 CFU/g) treatments. They were about 5-7 times higher than that in CKn treatment (0.58×10^5 CFU/g). Our results indicated that both earthworms significantly promoted the numbers of cultivable bacteria and actinomyces, potentially altered microbial community structure and encouraged their functions in atrazine degradation. The majority of cultivable fungi were from bulk soils, and vermicomposting did not show remarkable impacts on soil fungal communities.

3.3 Soil bacterial community structure

A total number of 188,454 high quality reads were obtained from all the treatments after filtering low quality reads and chimaeras, and trimming the adapters, primers and barcodes. In each treatment, the effective reads were 37,611 for CKn; 37,650 for SsEe; 39,636 for SsEa; 33,090 for SnEe and 34,728 for SnEa. The OTU numbers in CKn, SnEe and SnEa were 1,030, 962 and 1,054, significantly higher than those in SsEe (642) and SsEa (671), as listed in Table S1. Rarefaction curves of observed species and Shannon index approached the plateau from less than 5,000 tags per sample (Figure S1), suggesting a sufficient sequencing depth for microbial community analysis. To evaluate microbial community diversity, the alpha-diversity indices were calculated and listed in Table S1. Shannon and Chao 1 indices in SnEe and SnEa treatments were similar as that in CKn treatment, all significantly higher than vermicomposting treatments with sterile soils (SsEe and SsEa, $p<0.05$). Accordingly, the observed species in SnEe, SnEa and CKn treatments were also higher than that in SsEe and SsEa. The results revealed that the diversity and richness of bacterial community in bulk soils were significantly improved by both earthworms. In sterile soil treatment with earthworms, the majority of detected OTUs came from earthworm intestinal bacteria which were less abundant than soil indigenous bacteria.

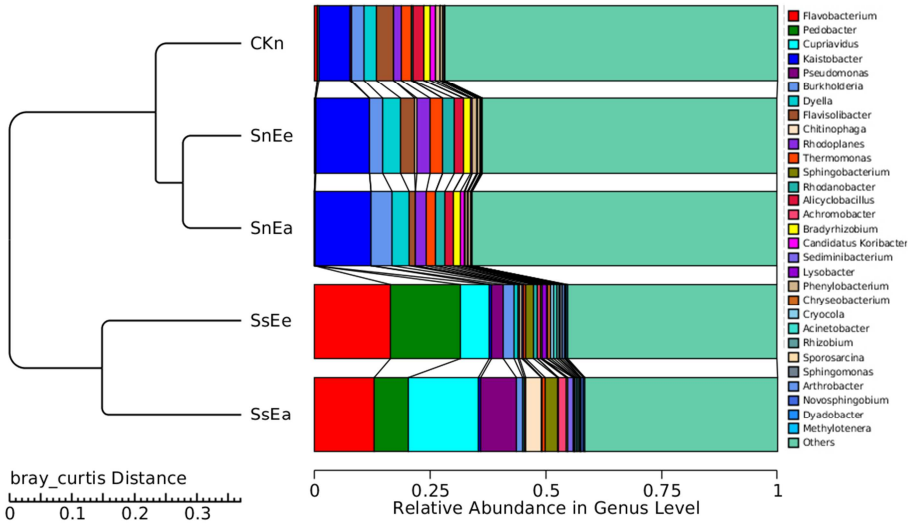


Figure 4. Soil microbial community barplot with cluster tree at the genus level. Taxonomic classification of 97% sequence identity is classified to the genus level using RDP classifier.

The taxonomic information at the genus level in different treatments was shown in Figure 4. The cluster tree was applied to identify the similarity of bacterial community structures among the five treatments, and two clusters were well separated from each other, suggesting two distinct types of bacterial communities. Generally, the microbial communities in CKn and bulk soil treatments with earthworms (SnEe and SnEa) were clustered together, indicating a higher community similarity among these treatments. The bacterial assemblages in the three treatments were predominated by *Kaistobacter*, *Burkholderia*, *Dyella*, *Flavisolibacter*, *Rhodoplanes*, *Thermomonas* and *Alicyclobacillus*. On the contrast, SsEe and SsEa were clustered together, and the dominant bacterial genera included *Flavobacterium*, *Pedobacter*, *Cupriavidus*, *Pseudomonas*, *Burkholderia*, *Sphingobacterium* and *Achromobacter*. The heatmap plots of the soil microbial communities based on Bray-Curtis distance (Figure S2) illustrated a similar cluster and similarity between these treatments.

Figure 5 illustrated that, in the treatments with bulk soils (CKn, SnEe and SnEa), the relative abundance of *Flavisolibacter* (3.71%, 3.03% and 1.37%), *Rhodoplanes* (1.68%, 2.89% and 2.33%) and *Kaistobacter* (6.73%, 11.50% and 12.20%) was higher than that in treatments with sterile soils (SsEe and SsEa) (Figure S3). Additionally, the relative abundance of *Rhodoplanes* and *Kaistobacter* was significantly higher in SnEe and SnEa treatments than that in CKn, whereas there was no difference for *Flavisolibacter*. The results indicated that they were soil indigenous bacteria, and earthworms could enrich *Rhodoplanes* and *Kaistobacter*. *Flavobacterium* (16.52% and 13.02%), *Pedobacter* (15.11% and 7.40%), *Cupriavidus* (6.26% and 14.97%), *Chitinophaga* (0.58 %and 3.32%) and *Pseudomonas* (2.57% and 7.71%) were only dominant in SsEe and SsEa treatments, but they were rare genera in CKn, SnEe and SnEa, suggesting their main originality from earthworm gut (Figure S3D and S3E). Higher relative abundance of *Flavobacterium* and *Pedobacter* was found in SsEe treatment (epigeic *E. foetida*), whereas *Cupriavidus*, *Chitinophaga* and *Pseudomonas* were enriched in SsEa treatment (endogeic *A. robustus*). It suggested the different intestinal bacteria in the two ecological earthworms. *Burkholderia* and *Dyella* were predominant in all the treatments, and their relative abundance was higher in SnEe (3.00% and 3.72%) and SnEa (4.47% and 3.63%) than those in CKn (2.80% and 2.57%). It hinted that the two bacterial genera were from both bulk soils and earthworm guts, and they were encouraged by earthworms during vermicomposting process.

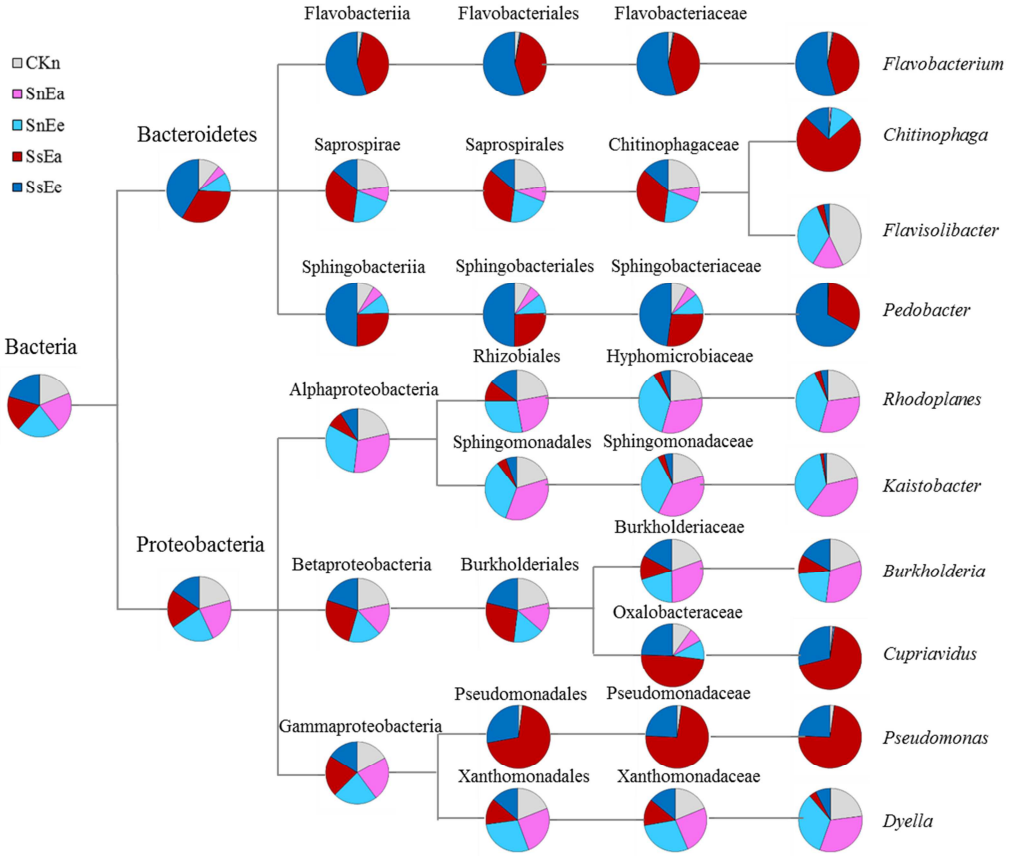
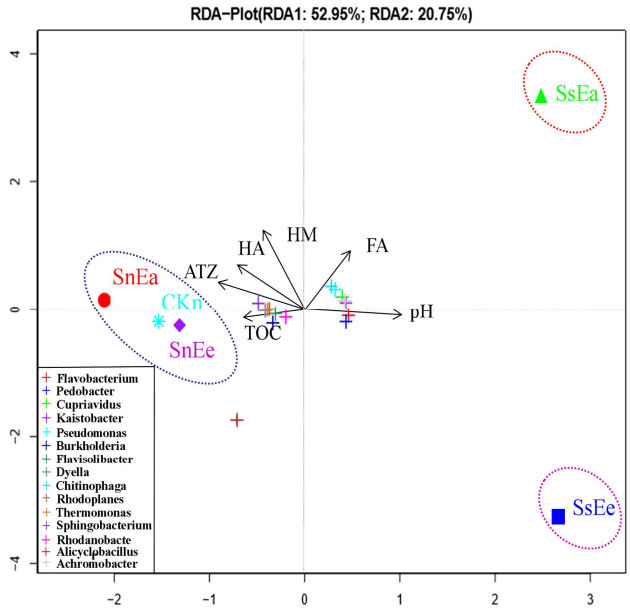


Figure 5. The species classification tree and relative abundance of the top 10 bacterial lineages. *Flavisolibacter*, *Rhodoplanes* and *Kaistobacter* had higher relative abundance in bulk soil treatments (CKn, SnEe and SnEa) than sterile soil treatments (SsEe and SsEa). *Flavobacterium*, *Pedobacter*, *Cupriavidus*, *Chitinophaga* and *Pseudomonas* were dominant genera only in SsEe and SsEa treatments. *Burkholderia* and *Dyella* were predominant in all the treatments.

3.4 Correlations between bacterial community and environmental factors

The ordination diagram of canonical correspondence analysis (CCA) illustrated a clear correlation between microbial community structure and environmental factors (Figure 6). Soil pH and atrazine content were highly correlated with the first CCA axis and accounted for 52.95% of the total variation observed in bacterial community

structure. Humin and fulvic acid were correlated with the second CCA axis, explaining 20.75% of the total variation. The impact of each environmental factor on microbial community structure was represented by the length of the arrow, and the cosine angle between the arrows showed their relationship (smaller angle indicated more significant correlation). Thus, soil residual atrazine was significantly positively correlated with humin ($p<0.01$), humic acid ($p<0.05$) and TOC ($p<0.05$), but negatively correlated with pH ($p<0.01$). They were key environmental factors affecting atrazine degradation performance. Meanwhile, the bacterial community groups of bulk soil treatments (CKn, SnEa and SnEe) were discriminated from those of sterile soil treatments (SsEa and SsEe) by the first CCA axis. Accordingly, the top 10 abundant bacterial genera were also separated into two groups by the first CCA axis. *Kaistobacter*, *Burkholderia*, *Dyella*, *Flavisolibacter* and *Rhodoplanes* were clustered in Group 1 close to bulk soil treatments, whereas *Flavobacterium*, *Pedobacter*, *Cupriavidus*, *Pseudomonas* and *Chitinophaga* were grouped together with relative closer distance to sterile soil treatments. It is noteworthy that *Flavobacterium* and *Pedobacter* leaned towards SsEe treatment, while *Cupriavidus* and *Pseudomonas* trended towards SsEa treatment, consistent with our results from species classification tree (Figure 5).



386

387 **Figure 6.** Canonical correspondence analysis (CCA) of 16S rRNA gene and
388 environmental factors. Arrows indicate the direction and magnitude of environmental
389 factors associated with bacterial community structure.

390 ## 4. Discussion

391 In the present study, the roles of two ecological earthworm species were investigated
392 in soil atrazine vermicomposting degradation. The significant lower residual atrazine
393 in CKn than CKs after 28 days degradation highlights the major contribution of the
394 indigenous microorganisms to atrazine mineralization. In sterile soil treatments (SsEe
395 and SsEa), both endogenic and epigeic earthworms could eliminate atrazine alone
396 without soil microorganisms, but the atrazine removal efficiency was relatively lower
397 than that in vermicomposting treatments with bulk soils (SnEe and SnEa, Figure 1).
398 Our findings suggest earthworms can accelerate atrazine degradation rate in soils.
399 Here, atrazine had significantly positive correlation with humic acid, TOC and humin,
400 but was negatively correlated with pH (Figure 6). The results showed that earthworm
401 treatments could neutralise soil pH and consume humic acid and humin (Table 2),

which are the key environmental factors for atrazine degradation (Hickman and Reid, 2008; Indraratne et al., 2008; Wang et al., 2011). The acid dissociation constant (pKa) of atrazine is 1.68, and the ionized atrazine fraction therefore increases at higher soil pH and principally raises atrazine availability to microorganisms. Andleeb *et al*. (2016) reported higher atrazine degradation rate at pH=7 than pH=5, attributing to the change of atrazine mobility and distribution under different pH conditions (Deng et al., 2017). Hence, earthworms could enhance atrazine degradation by neutralizing soil pH. Additionally, soil organic matters (fulvic acid, humic acid and humin), which have the strong hydrogen bonding and van der Waals force, can strongly sorb atrazine in soils and decrease its availability (Delwiche et al., 2014; Sagarkar et al., 2014). The consumption of humic acid and humin by earthworms in this study therefore releases atrazine from soil aggregates and consequently accelerates its biodegradation. Our findings are consistent with Luepromchai's work that the enhanced removal of polychlorinated biphenyl (PCB) in vermicomposting is attributed to the increasing PCB availability and the abundance of PCB-degraders (Luepromchai et al., 2002). Besides, dechlorination of soil dichlorodiphenyltrichloroethane (DDT) and PCP is also enhanced by earthworm-stimulated aerobic degradation by soil microorganisms (Lin et al., 2012; Lin et al., 2016a; Lin et al., 2016b).

The abundance and activities of atrazine-degraders are key to the successful atrazine mineralization. In the present study, earthworms encouraged cultivable bacteria and actinomyces (Figure 3), thus improving soil basal respiration and microbial biomass (Figure 4) and increasing the soil microbial richness and evenness (Table S1 and Figure S1). It might be explained by earthworm bioturbation which augments soil porosity and oxygenation, increasing microbial activities and access to atrazine (Chachina et al., 2016). In addition, the mucus, urine and cast of earthworms are

nutritionally rich and can also stimulate soil indigenous microorganisms and aid in atrazine biodegradation (Schaefer and Juliane, 2007). The species classification tree and CCA diagram revealed that the *Flavisolibacter*, *Rhodoplanes* and *Kaistobacter* were soil indigenous bacteria, and of them, the relative abundance of *Rhodoplanes* and *Kaistobacter* was increased in vermicomposting treatments (Figure 5 and 6). Most of the identified genera have the ability to degrade various organic pollutants in previous research, such as atrazine, PCP, chlorinated phenols and polycyclic aromatic hydrocarbons (PAHs) (Alekseeva et al., 2011; Bernard et al., 2012; Lin et al., 2016b). Ailijiang *et al*. (2016) demonstrated that *Rhodoplanes* is an aromatic degrader of PAHs and phenol, and Yang *et al*. (2014) also found the enhanced soil nonylphenol removal in earthworm treatments stimulating *Rhodoplanes*. *Kaistobacter* belonging to the family *Sphingomonadaceae* has higher abundance in PCB contaminated soils and is responsible for PCB dechlorination (Yu et al., 2017). *Sphingomonadaceae* can be stimulated by vermicomposting to accelerate the removal of organic contaminants from soils (Rodriguez-Campos et al., 2014). *Flavisolibacter* has been previously reported as an indicator sensitive to carbamazepine (Thelusmond et al., 2016) or triclosan (Ma et al., 2017) in soils, but their functions remain unclear. Our work for the first time hints its important roles in atrazine biodegradation.

Additionally, the intestinal flora of earthworms may also contain atrazine degraders, which might be released to soils through excretion and consequently accelerate atrazine mineralization (Li et al., 2015). The distinct separation of bacterial community groups between bulk soil and sterile soil treatments (Figure 6) suggested the excretion of earthworm intestinal flora, which are significantly different from soil indigenous bacteria. From the species classification tree and CCA diagram (Figure 5

and 6), the dominant genera (*Flavobacterium*, *Pedobacter*, *Cupriavidus*, *Chitinophaga* and *Pseudomonas*) in vermicomposting treatments with sterile soils were different with the treatments with bulk soils. The five bacterial genera are intestinal microorganisms in earthworm guts. In addition, *Flavobacterium* and *Pedobacter* had the higher relative abundance in SsEe treatment, whereas *Cupriavidus*, *Chitinophaga* and *Pseudomonas* were enriched in SsEa treatment. The results indicated distinct intestinal microorganisms are excreted by epigeic and endogeic earthworms. Different earthworm species have distinct gut microbial communities, which are affected post-exposure to various types and concentrations of organic pollutants (Tejada et al., 2016; Ma et al., 2017). Our findings are similar as Bernard's work that endogeic earthworms affect bacterial communities and organic matter metabolism, strongly stimulating the growth of several bacterial families, such as *Flavobacteriaceae*, *Chitinophagaceae* and *Sphingobacteriaceae* (Bernard et al., 2012). *Flavobacterium* is reported to metabolize atrazine, deisopropylatrazine and deethylatrazine (la Cecilia and Maggi, 2016) and can be stimulated in vermicomposting treatments for enhanced PCP biodegradation in red soils (Lin et al., 2016b). *Pedobacter* is an antibiotic resistant bacteria (Woegerbauer et al., 2015), but there is no previous report showing its capability in mineralizing organic pollutants. *Cupriavidus* is detected in the earthworm gut and capable of accelerating PCP degradation, the relative abundance of which is enriched by earthworms (Li et al., 2015). Zhu's work (2017) shows that *Chitinophaga* is a cellulose degrader applied in soil bioremediation via biochar-microbe interactions. *Pseudomonas* is recognized as a model strain for completely mineralizing atrazine (Alekseeva et al., 2011), and Monard *et al*. (2011) found *Pseudomonas* is one of the most dominant atrazine degraders in earthworm burrow linings. Prior to this study, only intestinal flora of

individual earthworm species is linked to atrazine metabolism, and our work for the first time proves the distinct intestinal atrazine degraders in different earthworm species during the same atrazine degradation process.

Burkholderia and *Dyella* were both identified as dominant bacterial genera in all the treatments, and their relative abundance in SnEe and SnEa was higher than that in CKn. They are possibly from both bulk soil and earthworm gut, and can be enriched in vermicomposting (Figure 5). Fang *et al*. (2015) reported some atrazine-degrading bacterial genera in soils, including *Arthrobacter*, *Burkholderia* and *Methylobacterium*. *Burkholderia* is also identified as PCP degrader and can be stimulated by vermicomposting (Li et al., 2015). *Dyella* is capable of mineralizing biphenyl and PCP in soils (Li et al., 2009; Lin et al., 2016a), and vermicomposting can enhance its activities during the bioremediation process (Lin et al., 2016a). Although this work cannot link *Burkholderia* and *Dyella* to atrazine metabolism directly, our findings strongly hint their functional roles in atrazine vermicomposting degradation, which has not been reported previously.

5. Conclusion

In the present study, we analyzed atrazine residuals, soil properties and microbial community structure after 28 days vermicomposting treatments. The results indicated that both *endogeic A. robustus* and *epigeic E. foetida* earthworms accelerated atrazine mineralization from 39.0% to 94.9%-95.7% by increasing the abundance and activities of indigenous atrazine-degrading bacteria and releasing the intestinal flora to soils. Soil bacterial community structure was also significantly altered by earthworms, which could neutralize soil pH and consume humus, consequently increasing atrazine availability in soils and accelerating atrazine biodegradation. Some atrazine degraders

(*Rhodoplanes*, *Kaistobacter*, *Cupriavidus*, *Pseudomonas* and *Flavobacterium*) were promoted by two ecological earthworm species during atrazine degradation process. For the first time, our work revealed different mechanisms of ecological earthworm species in accelerating atrazine degradation. The findings suggested that vermicomposting is an effective approach to accelerate soil atrazine degradation and has good potential in the bioremediation of atrazine contaminated soils. Further studies are suggested to address the atrazine metabolic pathway and the speciation of atrazine metabolites in vermicomposting treatments, for better understanding the mechanisms of enhanced atrazine degradation by different earthworm species.

Acknowledgements

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Highlights

1. Enhanced atrazine bioremediation by two ecological earthworm species.
2. Vermicomposting neutralizes soil pH and consumes organic matter.
3. Altered soil microbial communities in vermicomposting.
4. Different intestinal atrazine-degrading bacteria excreted by each earthworm species.