Journal of Environmental Management 230 (2019) 102–109

Contents lists available at ScienceDirect

Journal of Environmental Management

journal homepage: www.elsevier.com/locate/jenvman

Research article

Dynamics of oxytetracycline, sulfamerazine, and ciprofloxacin and related antibiotic resistance genes during swine manure composting

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ARTICLE INFO

Keywords:
Veterinary antibiotic
Oxytetracycline
Sulfamerazine
Ciprofloxacin
Antibiotic resistance genes
Swine manure composting

ABSTRACT

Understanding the dynamics of veterinary antibiotic and related antibiotic resistance genes (ARGs) during swine manure composting is crucial in assessing the environmental risk of antibiotics, which could effectively reduce their impact in natural environments. This study investigated the dissipation of oxytetracycline (OTC), sulfamerazine (SM1) and ciprofloxacin (CIP) as well as the behaviour of their corresponding ARGs during swine manure composting. These antibiotics were added at two concentration levels and two different methods of addition (single/mixture). The results indicated that the removal efficiency of antibiotics by composting were ≥85%, except for the single-SM1 treatment. The tetracycline resistance genes (TRGs) encoding ribosomal protection proteins (RPP) and efflux pump (EFP) and fluoroquinolone resistance genes (FRGs) could be effectively removed after 42 days. On the contrary, the TRGs encoding enzymatic inactivation (EI) and sulfonamide resistance genes (SRGs) were enriched up to 31-fold (*sul* 2 in single-low-SM1). Statistical analyses indicated that the behaviour of these class antibiotics and ARGs were controlled by microbial activity and significantly influenced by environmental factors (mainly C/N, moisture and pH) throughout the composting process.

1. Introduction

In recent years, the environmental pollution of antibiotics and antibiotic resistance genes (ARGs) has become a serious concern worldwide, due to the extensive use of veterinary antibiotics in livestock farming to control diseases and promote growth. As the largest antibiotic-consuming country in the world, it is estimated that more than half of the total usage (over 84,000 tons) was applied in the livestock and poultry industry in China for 2013 (Zhang et al., 2015). However, as much as 30–90% of the applied antibiotics are excreted in their parent compounds or primary metabolites through manure and urine (Sarmah et al., 2006). Those non-metabolized antibiotic residues in the animal manure become a significant source of antibiotics and induce the development of antibiotic resistant microbes in the environment (Guo et al., 2018).

Tetracyclines (TCs), fluoroquinolones (FQs) and sulfonamides (SAs) are three commonly used and frequently detected classes of veterinary antibiotics in China. According to the previous reports, the highest detected concentrations of ciprofloxacin (CIP), sulfamerazine (SM1) and oxytetracycline (OTC) in swine manure from different regions in China reached as high as 10, 17 and 354 mg kg^{-1}, respectively (Chen et al., 2012; Hou et al., 2015; Wang et al., 2017). The presence of residual antibiotics in manures can produce selective pressure for antibiotic resistant bacteria and ARGs that can enter the environment through a variety of pathways, especially land application of animal manure (He et al., 2016; Joy et al., 2013; Tang et al., 2015). The spread of these corresponding ARGs caused by discharge of swine waste has been frequently reported (Brooks et al., 2014; He et al., 2016). The tetracycline resistance genes (TRGs, including encoding ribosomal protection proteins (RPP), efflux pump (EFP), and enzymatic inactivation (EI) proteins), the sulfonamide resistance genes (SRGs) and fluoroquinolone resistance genes (FRGs) are commonly present in swine manure and manure-amended soil (Tang et al., 2015; Wang et al., 2015a, 2016a). These ARGs can be easily disseminated by means of bacterial reproduction and horizontal gene transfer (HGT) among bacteria in the environment. The transfer of ARGs, especially through HGT, may confer a novel antibiotic resistance in another organism, which could be a source of potential harm to the health of people and/or the environment (Keese, 2008).

Due to the production of a large amount of swine manure every year

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https://doi.org/10.1016/j.jenvman.2018.09.074
Received 14 October 2017; Received in revised form 10 September 2018; Accepted 22 September 2018
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in China, it can be assured that swine manure is an important source of antibiotics and ARGs in elevated levels (Cheng et al., 2013; Hou et al., 2015). Moreover, because of the direct application of swine manure as fertilizer by local farmers, farmland is vulnerable to contamination by antibiotics and ARGs through direct application of swine manure (Joy et al., 2013; Tang et al., 2015). Therefore, it is urgent to significantly reduce or cut-off antibiotics and ARGs applications into the environment from swine manure. Previous studies have shown that aerobic composting could effectively remove residual antibiotics in swine manure (Arikan et al., 2009; Selvam et al., 2012b). Selvam et al. (2012b) found that chlortetracycline and sulfadiazine were completely removed from the composting mass within 21 and 3 d, respectively; whereas, 17–31% of the spiked ciprofloxacin remained in the composting mass. However, norfloxacin and ofloxacin were detected as the dominant antibiotics in swine thermophilic compost samples with concentrations of 1192 and 1802 $\mu g\,kg^{-1}$, respectively, which were significantly higher than those in the corresponding manures, due to the high adsorption of fluoroquinolones by organic matter (Xie et al., 2016). The presence of antibiotics can generate a selective pressure for ARGs, and thus there must be certain relationships between some ARGs and concentrations of antibiotics (Chao et al., 2016). Several studies have shown that the composting process is effective for reducing the abundance of several ARGs (Selvam et al., 2012a; Wallace et al., 2018; Wang et al., 2012), but other results suggested that the thermophilic composting of swine manure failed to prevent the proliferation of ARGs (Wang et al., 2015b). However, little information is available regarding the fate of oxytetracycline, sulfamerazine and ciprofloxacin and related ARGs when present during aerobic swine manure composting. Exploring the dynamic relationship between those antibiotics and related ARGs is important to effectively reduce the residual antibiotics and ARGs during composting.

In the present study, we hypothesized that antibiotic concentrations would have an important influence on the abundance of ARGs during composting. Therefore, the major objectives of this study were (1) to quantify the dynamic changes of three antibiotics and their corresponding ARGs and (2) to determine the relationships between residual antibiotics, their corresponding ARGs and physicochemical conditions during thermophilic aerobic composting of swine manure. Three typical antibiotics, including OTC, SM1 and CIP were selected for this study because they are commonly used in swine production in China.

2. Materials and methods

2.1. Reagents and chemicals

OTC, SM1 and CIP were obtained from Dr. Ehrenstorfer (Augsburg, Germany) (Table S1). The isotopes of norfloxacin-d5, sulfadiazine-d4 and tetracycline-d6 were purchased from WITEGA Laboratories (Berlin, Germany), C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada) and TRC, Toronto Research Chemicals (Toronto, Canada), which were used as the internal standards of CIP, SM1 and OTC, respectively. Methanol (MeOH) and acetonitrile (ACN) (HPLC grade) were acquired from Fisher Science Co. Milli-Q water from a Milli-Q Advantage A10 system (Millipore, USA) was used when ultrapure water was required.

2.2. Composting operation

The swine manure was collected from a large-scale farm in Daxing District of Beijing, China. The fresh manure was spread, and air dried (15–20 °C) to a water content < 30%, crushed, and sieved through a 5-mm mesh, then swine samples were analyzed for physicochemical properties including antibiotics. The manure had a pH of 7.57, total organic carbon (TOC) of 29.62%, total nitrogen (TN) of 2.50%. The concentrations of CIP and SM1 in the collected swine manures were not detected and OTC was below the method quantification limit (MQL) according to the limits described in subsection 2.3. The wheat straw and saw dust had total organic carbon contents of 44.63% and 49.27%, and total nitrogen contents of 1.13% and 0.09%, respectively.

The composting experiment was performed in an organic fertilizer plant at Daxing district of Beijing, China. Bubble boxes were used as composting units (Fig. S1). The swine manure with a moisture content of less than 30% was pre-spiked with OTC, SM1 and CIP separately (single-compound treatments) and together (mixture-compounds treatments) at two concentration levels: high (200 $mg\,kg^{-1}$ OTC, 10 $mg\,kg^{-1}$ SM1, 10 $mg\,kg^{-1}$ CIP or 200 $mg\,kg^{-1}$ OTC + 10 $mg\,kg^{-1}$ SM1 + 10 $mg\,kg^{-1}$ CIP) and low (20 $mg\,kg^{-1}$ OTC, 1 $mg\,kg^{-1}$ SM1, 1 $mg\,kg^{-1}$ CIP or 20 $mg\,kg^{-1}$ OTC + 1 $mg\,kg^{-1}$ SM1 + 1 $mg\,kg^{-1}$ CIP). To achieve the most homogeneous distribution, the target antibiotics in aqueous solution were sprinkled over the swine manures through manual sprayer, stirring constantly. Then spiked swine manures were allowed to equilibrate for 2 days and stirred regularly during this period. Meanwhile, the initial concentrations of target antibiotics in the composting were confirmed by the measured value after this period. A control treatment was also prepared without the addition of antibiotics. There were 9 treatments in total. For each treatment, wheat straw (8.76 kg, dw) and saw dust (3.75 kg, dw) were mixed with swine manure (15 kg, dw) to adjust the C/N to ~25 and moisture content to ~55%. The mixture of these raw materials was then put into a bubble box and the volume of mixtures was about 137 L (Fig. S1). Each treatment was repeated in triplicate. Considering the future use of the compost, the moisture was not re-adjusted during the composting process. The composts were allowed to process for 42 days, and turned every day during the first week and every two days after the first week to maintain aerobic conditions and homogeneity of the materials. During the composting period, a temperature detector continuously sensed the temperature in the centre of composting pile continuously. As shown in Fig. S1, composite samples of compost were prepared by mixing three subsamples, respectively, taken from the bottom, middle and top layers of each pile on days 0, 3, 5, 7, 17, 21, 28, and 42. One part of each composite sample was used for the measurement of moisture, wheat seed germination index (GI) immediately after preparation, one part was freeze-dried for the analysis of the pH, TOC, TN and targeted antibiotics, and the rest of the part was stored at −80 °C for later ARGs abundance analyses. The detailed processes of physicochemical and GI analysis of compost are summarized in the Supporting Information.

2.3. Determination of antibiotics

The targeted antibiotics were extracted from freeze-dried composite samples (Ho et al., 2013) and determined by an Agilent 1200 series HPLC equipped with a Sunfire C18 column (150 × 4.6 mm, 3.5 μm, Waters, USA) and an Agilent 6410 Triple Quadrupole mass spectrometer. Ultrapure water containing 0.1% formic acid (v/v) (A) and ACN (B) were used as the mobile phase at a total flow rate of 0.4 $mL\,min^{-1}$. A linear gradient elution was conducted as follows: 0 min, 8% B; 0–12 min, linear increase to 55% B; 12–13 min, linear decrease to 8% B; 13–16 min, 8% B. The MS parameters were listed in Table S2. The recovery and method quantification limit (MQL) of target antibiotics ranged from 62% to 89%, and 7.6 to 13.7 $\mu g\,kg^{-1}$ (dw), respectively (Table S3). The detailed procedures of antibiotic analysis were described in the Supporting Information.

All the samples were analyzed in triplicate and procedural blanks (prepared without the targeted antibiotics or without the compost samples) were run to detect the possible sources of interference, and the relative standard deviation was less than 10%. Dissipation kinetics of the three antibiotics was calculated using the first-order kinetic model:

$$\frac{dC}{dt} = -kC \tag{1}$$

where C represents the concentration of target antibiotic at the time t, and k represents the rate constant. The half-life ($t_{1/2}$) of the antibiotics

was calculated using $t_{1/2} = \ln 2/k$.

2.4. DNA extraction and quantitative real-time PCR

DNA from 0.2 g of composted samples was extracted using the PowerSoil DNA isolation kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manual. The extracted DNA were quantified using a spectrophotometer, and stored at −80 °C until further analysis.

After a preliminary screening, seven tetracycline resistance genes (TRGs), including four RPP (*tet* M, *tet* O, *tet* Q and *tet* W), two EFP (*tet* G and *tet* K) and one EI (*tet* X), four sulfonamide resistance genes (SRGs), including *dfr* A1, *dfr* A7, *sul* 1 and *sul* 2, two fluoroquinolone resistance genes (FRGs), including *qep* A and *qnr* B, and 16S rDNA were quantified by quantitative real-time PCR (qPCR) (Table S4). The qPCR was performed by Wcgene Biotech, Shanghai, China using an ABI VIIA™ 7 Real-Time PCR system (Applied Biosystems, Life Technologies, Foster City, CA, USA) and described in detail in the Supporting Information.

To evaluate the reduction of ARGs in manure composts, an eliminate rate (*ER*) was defined:

$$(ER)_{RA}(\%) = [(RA_{D0} - RA_{D42})/RA_{D0}] \times 100 \quad (2)$$

$$(ER)_{AA}(\%) = [(AA_{D0} - AA_{D42})/ AA_{D0}] \times 100 \quad (3)$$

where $(ER)_{RA}$ and $(ER)_{AA}$ are the eliminate rate of relative abundances and absolute abundances of target ARGs, respectively; RA_{D0} and RA_{D42} are the relative abundances of target ARGs on day 0 (D0) and day 42 (D42), respectively; AA_{D0} and AA_{D42} are the absolute abundances of target ARGs on day 0 (D0) and day 42 (D42), respectively.

2.5. Statistical analysis

Pearson's correlation analysis (SPSS software, Windows version 16.0, SPSS Inc.) was performed in order to determine relationships between antibiotics, ARGs and chemical conditions in the composting process. Redundancy analyses (RDA) of the correlations between ARGs and environment factors were performed using Canoco 4.5 for Windows.

3. Results and discussion

3.1. Evaluation of composting efficacy

Temperature is one of the key indicators for quality and progress in composting (Hassen et al., 2001). As shown in Fig. 1a, the entire composting process can be divided into four phases according to the temperature change of the composting piles, namely, mesophilic phase (25–40 °C, days 0–1), thermophilic phase (35–65 °C, days 2–6), cooling phase (second mesophilic phase, 65-40 °C, days 7–21) and maturation phase (40-25 °C, days 22–42) (Diaz et al., 2007). One of the most important aspects of the total nutrient balance is the ratio of organic carbon to total nitrogen (C/N). As shown in Fig. 1b, the C/N ratios for all the treatments gradually decreased as composting proceeded, with a relatively faster decrease in the active thermophilic phase. This is due to the carbon loss as CO_2 caused by microbial mineralization of carbon compounds. At the end of composting, the C/N ratios of composts for different treatments were around 20, indicating the maturity of compost (Wang et al., 2015b). The lower C/N ratios (c.a. 16) in two SM1 treatments implies relatively stronger activity and more biomass of bacteria than fungi in those two treatments (Malik et al., 2016; Wang et al., 2015b). Water is essential for all microbial activity and should be present in appropriate amounts throughout the composting cycle. Due to the high rates of heat generation, evaporative cooling removed large amounts of water vapour during the composting process. The moisture contents decreased constantly and ranged from 34.1 to 41.9% (w/w) at the end of composting (Fig. 1c), which could effectively prevent biological activity in stabilized composts. In all the treatments, the pH

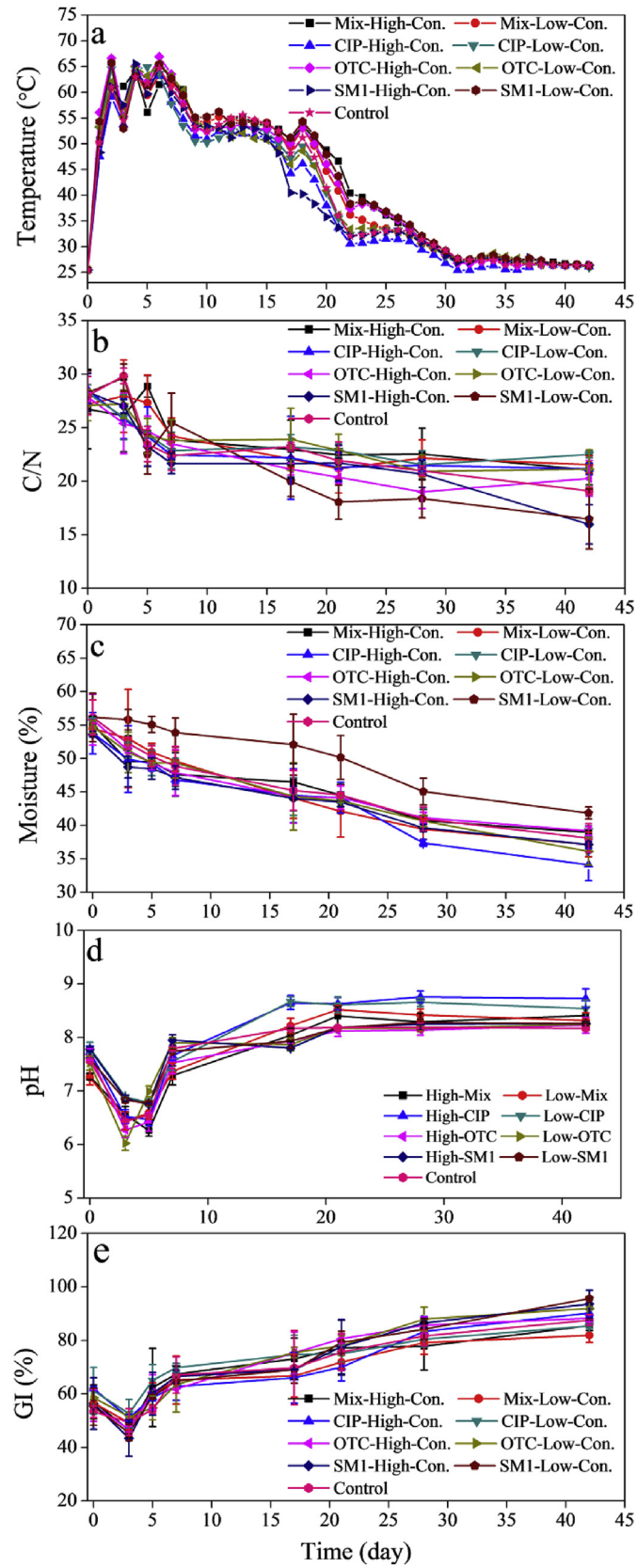

Fig. 1. Variations of temperature (a), C/N (b), moisture (c), pH (d) and GI (e) over the composting time. Error bars represent the standard deviations (n = 3).

increased from 6.02 to 6.78 in five days and gradually increased to ~8.5 on day 42 (Fig. 1d).

GI is a practical index that reflects the maturity of composting (Wang et al., 2015b). As shown in Fig. 1e, the GIs reached minimum values on day 3, ranging from 43.4% to 51.5%. This implies that the thermophilic phase could not eliminate all harmful substances in the composting piles and thus the wheat seed germination was inhibited. At the end of composting, the GIs of all 9 treatments were higher than 80%, indicating a better maturity of every composting pile. There was a slight inhibition of the seed germination by the mixture additions of three antibiotics (Mix-High-Con., 85.5%; Mix-Low-Con., 81.9%) and the addition of low concentration of CIP (85.4%) when compared to the control (87.6%). Similar results have been reported previously for other antibiotics (Selvam et al., 2012b). However, the addition of these antibiotics did not retard the composting process, which agreed with previous observations (Wang et al., 2015b).

3.2. Dissipation of antibiotics during composting

In the control swine manures, CIP and SM1 were not detected and OTC was below the MQL, indicating the background concentrations of these antibiotics in the collected swine manure were low.

Overall, the dissipation patterns of CIP in the four treatments (i.e. Single-High-Con, Single-Low-Con, Mix-High-Con and Mix-Low-Con) were similar, with a sharp decrease during the first 7 days and then a gradual decrease afterwards (Fig. 2a). The dissipation kinetics of CIP in

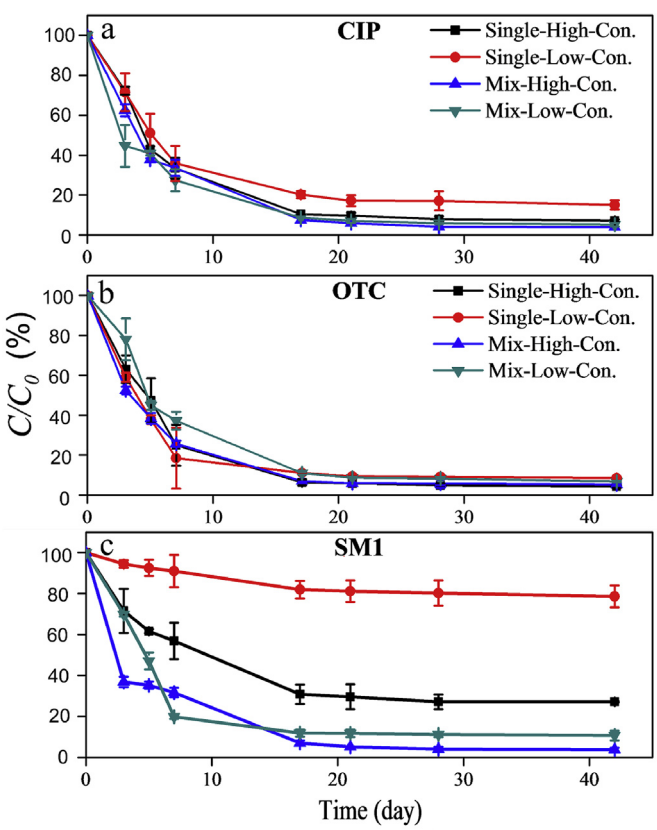

Fig. 2. Changes in the concentrations of (a) ciprofloxacin (CIP), (b) oxytetracycline (OTC) and (c) sulfamerazine (SMI) during swine manure composting. 100% refers to the initial antibiotic concentrations of (a) CIP at 3.65, 0.46, 3.56 and 0.44 mg kg^{-1} (dw) for Single-High-Con., Single-Low-Con., Mix-High-Con., and Mix-Low-Con., respectively; (b) OTC at 77.00, 6.87, 79.00 and 6.90 mg kg^{-1} (dw) for Single-High-Con., Single-Low-Con., Mix-High-Con., and Mix-Low-Con., respectively; (c) SM1 at 4.44, 0.46, 4.42 and 0.45 mg kg^{-1} (dw) for Single-High-Con., Single-Low-Con., Mix-High-Con., and Mix-Low-Con., respectively.

four treatments (Table S5) indicated a relatively faster dissipation of CIP in the mixture addition than single addition. At the end of composting, the percentage dissipation ($\Delta m^{a}_{0\text{-}42d}$ (%)) of CIP was significantly lower in the Single-Low-Con treatment than other three treatments (Table S5). Selvam et al. (2012a) reported the removal percentages of 69.0% and 82.9% with an initial concentration of 1 and 10 mg kg^{-1} of CIP after 56 days of swine manure composting, respectively, which was only mixed with sawdust as the supplementary material. The higher dissipation percentages of CIP in the present study indicated that composting conditions were favorable for the biodegradation of CIP.

The dissipation pattern of OTC is similar to CIP. The removal rates of OTC were up to 91.8–96.0% after 42 d of composting (Fig. 2b and Table S5). This indicated that OTC could be effectively dissipated during manure composting processes, no matter whether it existed in the swine manure alone or mixed with other antibiotics. Wu et al. (2011) reported that 92% of OTC with an initial concentration of 1.6 mg kg^{-1} was dissipated within 52 d of composting, which agrees well with our study. Generally, OTC and CIP have inhibition on the microbial community and activity, thus the biodegradation of OTC and CIP was restrained in some degree. However, the high dissipation rates of OTC and CIP occurred in the composting. This result may be attributed to the different ways of degradation of OTC and CIP during the composting processes. Wang et al. (2015b) have shown that OTC was degraded by both abiotic and biotic processes.

In contrast to CIP and OTC, SM1 was less degraded, especially in the single-compound addition treatments with low initial concentrations (Fig. 2c and Table S5). Several studies have found that biodegradations of sulfonamides were preceded by a lag phase (6–50 d) due to microbial adaptation (Ingerslev and Halling-Sørensen, 2000; Pérez et al., 2005). Other studies have found limited or no degradation of sulfonamides in manure or marine sediment (Dolliver et al., 2008; Hektoen et al., 1995), which are consistent with the results from our study. However, in the co-presence of CIP and OTC, the dissipation of SM1 in swine manure was promoted during composting (Table S5). The underlying mechanisms for this promoted dissipation of SM1 are not clear in this study. It was supposed that the changes of C/N from the extra addition of CIP and OTC might facilitate the dissipation of SM1 by the adapted microbes consortium during the composting compared with the single addition of SM1 (Fig. 1b). Further study is needed to clarify this reason responsible for the enhanced dissipation of SM1 in the co-presence with CIP and OTC during swine manure composting. Selvam et al. (2012b) had also reported that sulfadiazine was completely removed from the compost that was spiked with this antibiotic together with chlortetracycline and ciprofloxacin.

With regression coefficients (R^2) ranging from 0.83 to 0.94 (Table S5), the strong linear regression indicates that the dissipation data of each antibiotic in swine manure fitted the first-order kinetic model (Eq. (1)) well. Our results further supported previous observations that the dissipation of antibiotics followed the first-order kinetics during the composting process (Ho et al., 2013; Ramaswamy et al., 2010). In this study, all of the targeted antibiotics showed a relatively fast dissipation during composting with the half-life ($t_{1/2}$) ranging from 7 to 17 days (Table S5), except for the single addition of SM1 at low concentration ($t_{1/2}$ = 88 days). In general, the dissipation rate constants (k values) of targeted antibiotics obtained from the treatments with high levels of antibiotics were greater than those from the low levels (Table S5). This result was consistent with a previous study (Selvam et al., 2013). They demonstrated the simple first-order kinetics of the dissipation of tetracycline during composting, with $t_{1/2}$ values of 11.75 and 11.23 days and k values of 0.0507 and 0.0527 day^{-1} for the swine manure treatments spiked with initial concentration of 10 and 100 mg kg^{-1} of tetracycline, respectively.

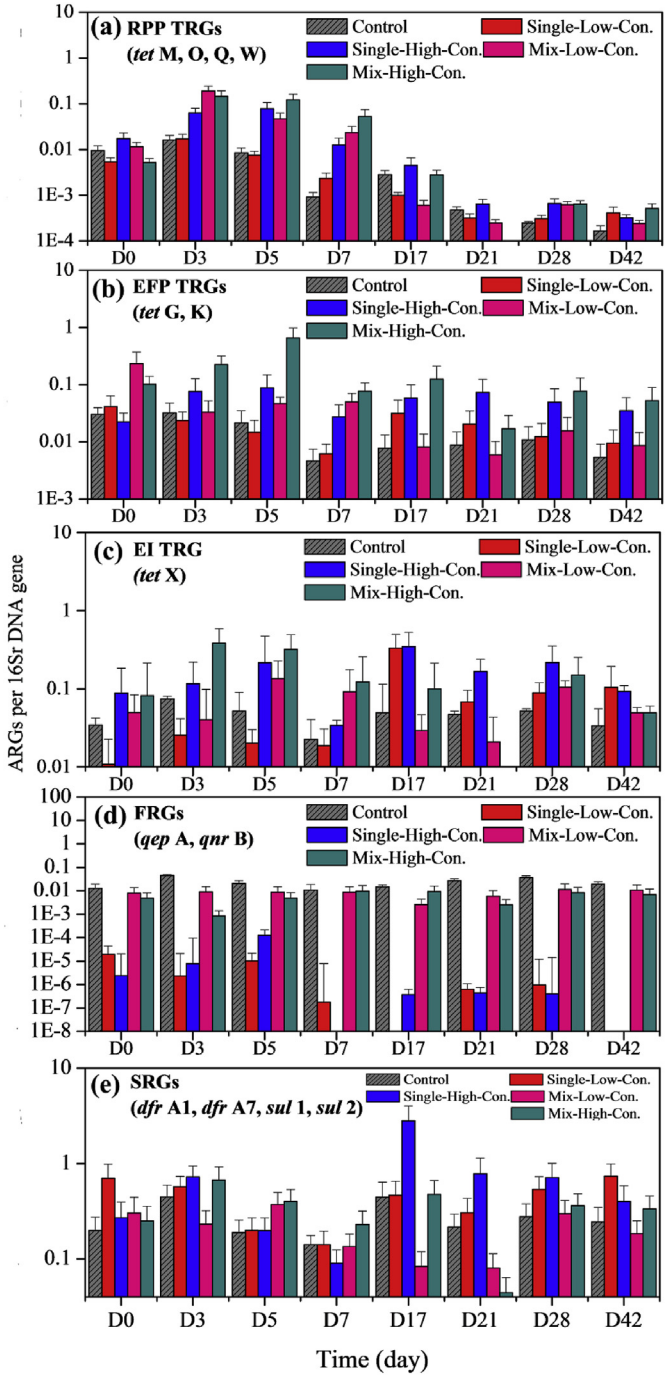

Fig. 3. Variation of the relative abundance of target ARGs – (a) RPP TRGs, (b) EFP TRGs, (c) EI TRG, (d) FRGs and (e) SRGs over the composting time. The data on day 0 (D0) represent the initial relative abundance of target ARGs for the control and 3 different treatments spiked with the single and mixture of OTC, CIP and SM1; error bars represent the standard deviations (n = 3).

3.3. Behaviour of target ARGs during composting

The average absolute abundances of ARGs (including TRGs, SRGs and FRGs) in all compost samples ranged from 3.90×10^6 to 9.90×10^7 copies g^{-1} (dw), and the variation of the absolute abundances of target ARGs over the composting period in the control and different treatments spiked with the single OTC, CIP and SM1 and mixture (OTC + CIP + SM1) were shown in Fig. S3. In order to minimize the effect of the variation in DNA extraction among different samples, the absolute abundances of ARGs were normalized to those of 16S rDNA genes, which were referred as relative abundances (Fig. 3). The results indicated that the relative abundances of target ARGs were generally greater in the compost samples spiked with high concentrations of single antibiotic or mixture of antibiotics, except for the FRGs that generally exhibited opposite variation patterns between high and low levels of CIP. In addition, at some points, gene copies were higher in the control treatment when compared with antibiotic spiked treatments. Thus, beside the antibiotic selection pressure, some other factors might also influence the abundances of ARG (saw in Fig. 4 and discussed in more detail below) (Selvam et al., 2012a).

The RPP TRGs (summation of *tet* M, *tet* O, *tet* Q and *tet* W) initially increased from mesophilic phase to thermophilic phase, dominated in the thermophilic phase, sharply decreased in the cooling phase, and then remained nearly constant in the maturation phase (Fig. 3a). This result indicated that a large number of the tetracycline resistant bacteria may not be thermophilic and the higher degradation of OTC during the thermophilic phase reduced the OTC selection pressure on the microbes. The EFP TRGs (summation of *tet* G and *tet* K), EI TRG (*tet* X), and SRGs (summation of *dfr* A1, *dfr* A7, *sul* 1 and *sul* 2) had the same trends as the RPP TRGs during the initial mesophilic phase and thermophilic phase, but increased with decreasing temperature in cooling phase from day 7, and then remained nearly constant in the maturation phase (Fig. 3b, c, and e). Compared to the TRGs and SRGs, the relative abundances of FRGs (*qep* A and *qnr* B) were very low, which indicated that the reduced resistance of the bacteria existed in the composting piles (Selvam et al., 2012a).

Similar to the RPP of TRGs, the FRGs (summation of *qep* A and *qnr* B) in the single-CIP treatments initially dominated in the thermophilic phase, and then decreased thereafter and could not be detected in the maturation phase after 42 days (Fig. 3d). By contrast, the FRGs in the treatment with mixture of antibiotics exhibited similar variation patterns in all the composting phases. In this study, the background concentration of CIP in the collected swine manure was below the detectable levels. However, the greater copy numbers of FRGs were found in the control treatment than the treatments spiked with CIP at all the time points. This agrees well with the experimental results reported by Selvam et al. (2012a). Thus, beside the CIP selection pressure, some other factors influence the FRGs copy numbers. More studies are required to reveal the mechanisms of maintenance and spread of resistance genes in natural ecosystems. It is noted that the behaviour of ARGs during composting observed in this study was different from that in a previous study (Wang et al., 2015b). In this study, the required moisture was added once to the composting piles at the beginning of the experiment. The compost moisture decreased constantly over time and only ranged from 34.1% to 41.9% at the end of composting (Fig. 1c). The relatively low compost moisture and high rate of heat generation during composting in this study might effectively prevent the survival of antibiotic resistance bacteria (Diaz and Savage, 2007; Sharma et al., 1997; Zhang et al., 2018). This could lead to the behaviour of ARGs observed in this study to be different from the previous study by Wang et al. (2015b).

As shown in Table S6, in most cases, TRGs including both RPP and EFP and FRGs could be effectively removed after 42 days of composting, indicating the effectiveness of the thermophilic composting as a potential treatment method for eliminating those ARGs in swine manure composts. This result agrees with previous studies that both RPP and EFP *txt* gens could be removed effectively by the thermophilic composting (Selvam et al., 2012a; Yu et al., 2005). In contrast, the thermophilic composting in this study caused amplification of both EI, TRG (*tet* X) and SRGs with the accumulation rates (i.e. negative of *ER*) up to 3156% (*sul* 2 in single-SM1 and low-level treatment). This is different from previous reports (Selvam et al., 2012a). We assume that this could be related to the moisture adjustment during the whole process of composting. Continuous or even frequent moisture adjustment during the composting process, especially in the late stage of composting, may further affect the ARG profiles and biological activity

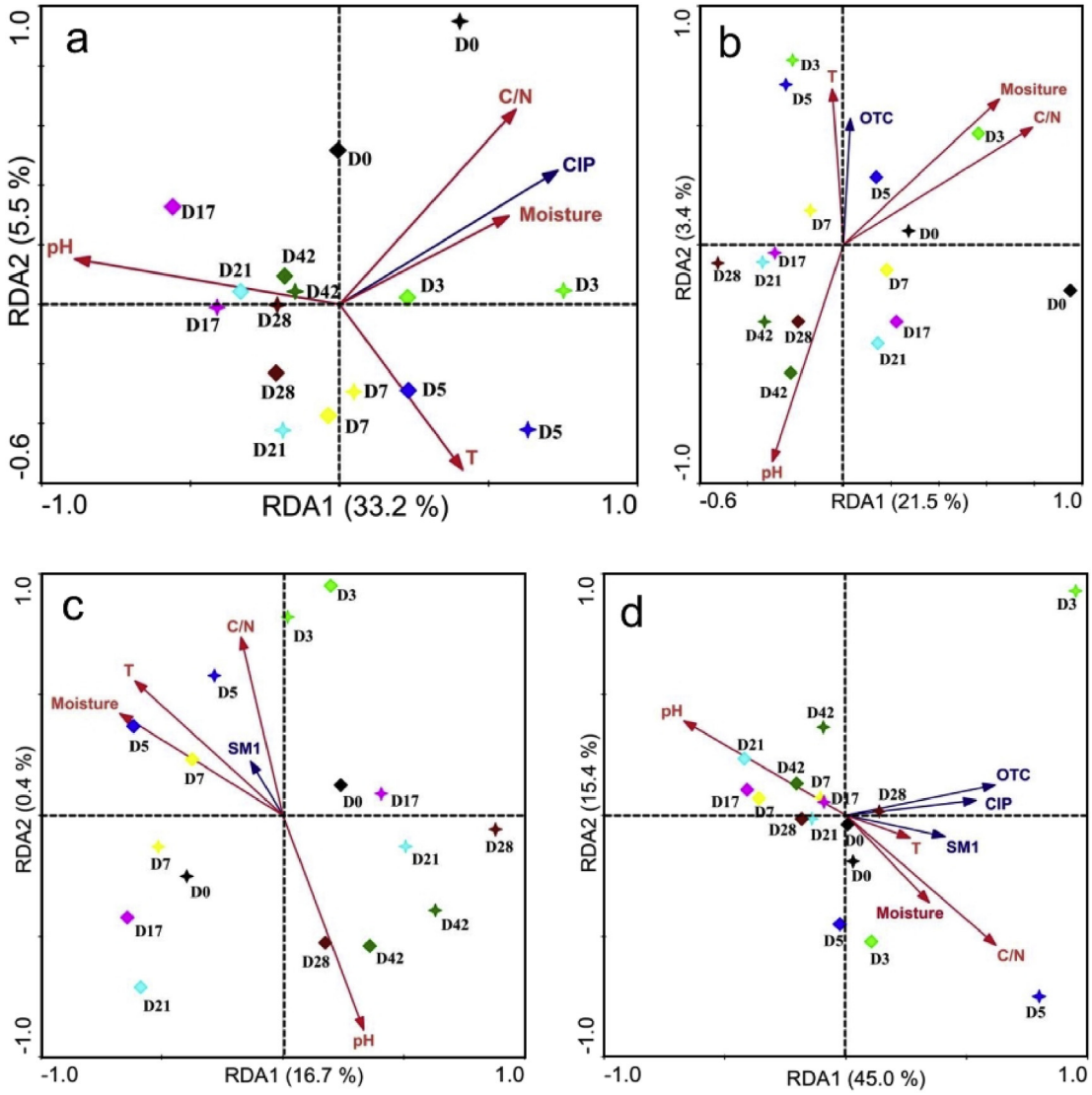

Fig. 4. Redundancy analysis (RDA) of the relationship between environmental factors, i.e., C/N, temperature (T), pH, moisture, and antibiotics, and the corresponding ARG profiles during swine manure composting spiked with CIP (a), OTC (b), SM1 (c) and mixture of those antibiotics (d). The star and diamond symbols denote data obtained from the addition of antibiotics at high and low concentrations, respectively. Black, light green, blue, yellow, pink, sky blue, dark red and dark green denote data collected from days 0, 3, 5, 7, 17, 21, 28 and 42, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

in the stabilized material (Diaz et al., 2007; Lin et al., 2016; Tiquia et al., 1996).

3.4. Relationships between antibiotics, ARG levels and physical-chemical conditions

The relationships between antibiotics, ARG levels and physical-chemical conditions of the composting process were determined based on the Pearson correlation analysis (Tables S7–14). In general, the degradation of targeted antibiotics was positively correlated with C/N and moisture, such as single-high-OTC with C/N ($r = 0.958$, $P < 0.01$) (Table S9), mixture-low-CIP with C/N ($r = 0.807$, $P < 0.01$) (Table S14), single-low-CIP with moisture ($r = 0.946$, $P < 0.01$) (Table S8) and mixture-high-OTC with moisture ($r = 0.882$, $P < 0.01$) (Table S13). Many studies have confirmed that the microbial (specifically bacteria) populations also increase with increasing C/N ratio and moisture content in a certain range during the composting process (Zhang et al., 2018), which coincides with the enhanced biodegradation of antibiotics, indicating that microorganisms play an important role in the degradation of antibiotics. In contrast, the targeted compounds were negatively correlated with pH, such as single-low-CIP ($r = 0.772$, $P < 0.05$) (Table S8) and single-high-OTC ($r = 0.832$, $P < 0.05$) (Table S9). The antibiotics, such as TCs and FQs, have a series of functional groups (e.g., amines, carboxylics and phenolics) allowing these compounds to exhibit different species. For example, there are four species (including H_3OTC^+, $H_2OTC^{\pm}$, $HOTC^-$ and OTC^{2-}) for OTC with the variation of pH (Cheng et al., 2018). Throughout the whole pH changes during the swine manures composting (6.02–8.76) (Fig. 1d), the target compounds (OTC and CIP) predominantly existed as zwitterions rather than cationic species. Therefore, the adsorption mechanism of those antibiotics is more likely to be a complexation reaction on the compost mixture surface rather than electrostatic attraction (Cheng et al., 2018; Gong et al., 2012). In addition, ion exchange also plays a very important role due to the dependence of the antibiotic adsorption on pH.

In general, the relative abundances of ARGs were positively correlated with temperature, C/N, moisture and pH, especially for the OTC treatments (Tables S9, S10, S13 and S14), which implies that the

enhancement of those factors could accelerate the proliferation of ARGs (Song et al., 2016; Wang et al., 2016b). For example, there is a good similarity variation between RPP of TRGs (Fig. 3a) and temperature (Fig. 1a) for the treatment of OTC. This might also well explain why greater abundance of ARGs occurred in the thermophilic phase. Temperature, C/N and moisture played important roles in shaping the microbial communities during the composting, which might directly or indirectly cause the variations of resistance genes.

In addition, positive correlations were found between the levels of antibiotics and ARGs, e.g., single-high-OTC with *tet* K ($r = 0.906$, $P < 0.01$), mixture-high-SM1 with *tet* K ($r = 0.710$, $P < 0.05$) and mixture-low-CIP with *tet* K ($r = 0.989$, $P < 0.01$). The single treatments with addition of CIP or SM1 were not found to be associated with the ARGs investigated, based on the relative abundances of ARGs. Many studies have reported there were significant correlations between ARG abundances and detected antibiotic levels due to the selective pressure for ARGs generated by the presence of antibiotics (He et al., 2014; Wu et al., 2015).

Redundancy analysis (RDA) was conducted to further confirm the relationships between environmental factors (C/N, temperature, pH, moisture and antibiotics) and the ARG profiles during swine manure composting (Fig. 4). The results showed that the selected environmental factors accounted for 39.0%, 25.9%, 21.7% and 69.0% of the variations in the ARG profiles during the composting spiked with CIP, OTC, SM1 and mixture of those compounds, respectively. As shown in Table S15, for OTC additions, the most important explanatory variables were the residual OTC that explained 12.8% and 7.6% in single and mixture treatments, respectively. These results indicate that the TRG profiles were affected greatly by the residual OTC levels due to the selective pressure for TRGs generated by the presence of OTC, as it has been proven previously (Wu et al., 2015). For CIP additions, the results indicated that the ARG profiles were affected greatly by the pH. Generally, organic matter with a wide range of pH (from 3 to 11) can be composted (Bertoldi et al., 1985). In this study, the change of pH levels ranged from 6.02 to 8.76 in the composting, which was unlikely to cause the inhibition of microbes. So the most likely cause could be the variations of other factors, such as the physicochemical characteristics of the composting substrate and conformation of DNA, that were relatively sensitive to the pH during the composting, which elicited the different binding capabilities of ARGs to the substrate surface (Yu et al., 2017; Zhu et al., 2016). For SM1 treatments, the results showed that the ARG profiles were affected greatly by the moisture, which have yet to be validated by Li et al. (2015). In addition, moisture can affect the survival of bacteria (Diaz et al., 2007; Wang et al., 2016a). This might be a key step for indirect influence of the SRGs amplification in this study.

As shown in Fig. 4, the main contributors to the ARG profiles varied among the different phases. In general, moisture and C/N mainly affected the ARG profiles during the thermophilic phase, whereas temperature had the main effects in the mesophilic phase, followed by pH in the maturation phase. It should be noted that the ARG profiles obtained with different treatments were similar within the different phase, especially in the maturation phase. Thus, the composting phase had important effects on the changes in the ARG profiles. The majority of the ARGs were removed in the thermophilic phase and moisture and C/N were the most important environmental factors during this phase. If the moisture and C/N are appropriate during the composting, the temperature rises almost exponentially with time until the thermophilic phase begins at about 65–70 °C, and this period could persist for 1–3 weeks (Diaz et al., 2007). High temperature helps to enhance the elimination of ARGs (Li et al., 2017). Therefore, optimized composting conditions, such as moisture and C/N, may be required to ensure the control of ARGs.

4. Conclusions

The study revealed that single or mixture addition of OTC, CIP and SM1 at different concentrations could affect the removal efficiencies of those antibiotics and the relevant ARGs during the swine manure composting. Overall, all three antibiotics and most of the ARGs could be removed effectively after composting, which is controlled by microbial activities that vary considerably with environmental factors, mainly with C/N, moisture and pH. Furthermore, the main factors varied in different stages of the composting. The thermophilic composting of swine manure could play an important role in effectively removing residual antibiotics and ARGs. However, further optimizations need more studies to increase the removal efficiency in the enriched TRGs during the composting.

Acknowledgements

The work was financially supported by the National Key Technology R&D Program of China (2018YFD0500206), the National Natural Science Foundation of China (31572209 and 31772395) and the China Postdoctoral Science Foundation (2016M600151).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2018.09.074.

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