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Behavior of antibiotics and antibiotic resistance genes in eco-agricultural system: A case study

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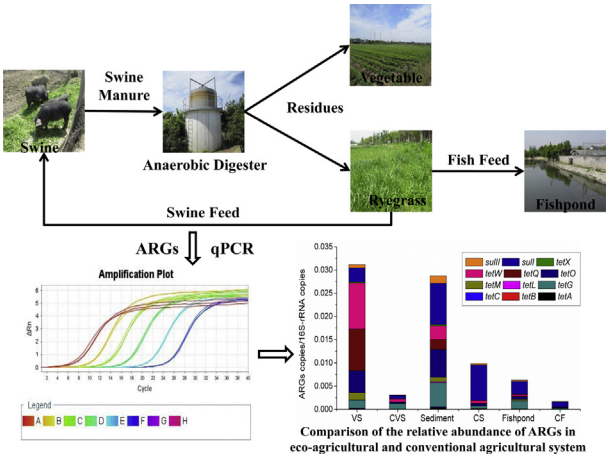
HIGHLIGHTS

- *Tet*Q had the highest relative abundance and *tet*G was the most persistent gene.
- The anaerobic digestion has no effective removal of most ARGs.
- The abundance of ARGs in soils and fishpond was higher than that of control system.
- Positive correlations were observed between the total ARGs and TN, TP and TOC.

GRAPHICAL ABSTRACT



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ABSTRACT

This study aims to determine abundance and persistence of antibiotics and antibiotic resistance genes (ARGs) in eco-agricultural system (EAS), which starts from swine feces to anaerobic digestion products, then application of anaerobic digestion solid residue (ADSR) and anaerobic digestion liquid residue (ADLR) to the soil to grow ryegrass, one of swine feed. Oxytetracycline had the highest concentration in manure reaching up to 138.7 mg/kg. Most of antibiotics could be effectively eliminated by anaerobic digestion and removal rates ranged from 11% to 86%. ARGs abundance fluctuated within EAS. *TetQ* had the highest relative abundance and the relative abundance of *tetG* had the least variation within the system, which indicates that *tetG* is persistent in the agricultural environment and requires more attention. Compared to the relative abundance in manure, *tetC* and *tetM* increased in biogas residue while three ribosomal protection proteins genes (*tetO*, *tetQ*, *tetW*) decreased ($p < 0.05$), with other genes showing no significant change after anaerobic fermentation ($p > 0.05$). Most ARGs in downstream components (soils and fishpond) of EAS showed significantly higher relative abundance than the control agricultural system ($p < 0.05$), except for *tetG* and *sulI*.

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1. Introduction

The spread and evolution of antibiotic resistance genes (ARGs), and their acquisition by bacterial pathogens are challenging life-saving antibiotic therapies [1]. Large amounts of antibiotics are applied in agricultural production systems, particularly in intensive animal farming, and are causing amplification of ARGs in the environment [2]. ARGs in microorganisms could be transferred to indigenous environmental bacteria by horizontal gene transfer [3]. Transmission of ARGs on mobile genetic elements from animal husbandry to human microbiomes could be caused by contact with animals, their direct surroundings, excretions or produce [4]. Public health risks of a possible transfer of resistant zoonotic agents from animals to humans led to a change in policies through a ban on the use of antibiotics as growth promoters in European Union, and through introduction of monitoring systems of resistance in food animals in many countries [5].

China is the largest antibiotics producer and consumer in the world and annual usage of antibiotics is approximately 210 million kg, with 46.1% being used in livestock industries [6]. The use of antibiotics in animal husbandry for disease treatment and growth promotion is unmonitored, which has caused high concentrations of antibiotic residues in animal manures. The majority of antibiotics is poorly absorbed in animal gut and is excreted into the environment by feces and urine [7], then persists and accumulates in soils after repeated manure application [8].

It is estimated that production of livestock and poultry feces was as high as 3.26 billion ton in China in 2009 [9]. To recover resources, such as nutrients from livestock manure and to control pollution, development of eco-agricultural system (EAS) has been considered as an environment-friendly approach. In such a system, concepts of sustainable development and circular economy are adopted [10]. Recycling of livestock and poultry manure using biogas technology is one of the prevailing models for EAS in China [11]. "Three in One" pattern, such as "Pig-Biogas-Fruit", "Pig-Biomass-Vegetable", "Pig-Biomass-Grain", is very popular in rural areas, where livestock breeding is combined with biogas system to treat animal manures. Biogas is used as bioenergy for households and anaerobic digestion residue is used to grow fruit trees, vegetables and cereals. Green food can be developed from this close-loop production system [12]. However, behaviors of antibiotics and ARGs in the recycling process of material and energy of EAS have never been studied. Therefore, this study focuses on an EAS in Hangzhou area, eastern China. We chose a classic model of recycling swine feces, and investigated abundance and distribution of ARGs in every link of the system, from livestock manures to anaerobic digestion solid residue (ADSR) and anaerobic digestion liquid residue (ADLR), then vegetable, ryegrass fields and fish pond. Manure has become a reservoir of resistant bacteria and antibiotic compounds [13], and its application to agricultural soils is assumed to increase antibiotic resistance genes and selection for resistant bacteria populations in soils [14–16]. The genome location of resistance genes is likely to shift toward mobile genetic elements such as broad-host-range plasmids, integrons, and transposable elements [3,17]. Horizontal transfer of these elements to bacteria adapted to soil or other habitats supports their environmental transmission independent of the original host [18,19]. This study aims to address the following questions:

(1) What kinds of antibiotics and ARGs occur in each component of the EAS including livestock manures, ADSR and ADLR, anaerobic digestion residue-amended soil and fish pond?
(2) How do ARGs associate with antibiotics and nutrient substance flow within the EAS?
(3) Do the contamination related to the presence of ARGs in the EAS elevate compared to a control agricultural system?

Twelve ARGs (*tetA*, *tetB*, *tetC*, *tetG*, *tetL*, *tetM*, *tetO*, *tetQ*, *tetW*, *tetX*, *sulI* and *sulII*) and one genetic element associated with ARGs [class 1 integron (*intI1*)] were selected, since these genes reflect different resistance mechanisms encoding for ARGs. Specifically, *tetA*, *tetB*, *tetC*, *tetG* and *tetL* code for energy-dependent efflux proteins which export tetracyclines out of the cell; *tetM*, *tetO*, *tetQ* and *tetW* code for ribosomal protection proteins; *tetX* encodes for tetracyclines inactivating enzymes [3]; *sulI* and *sulII* code for dihydropteroate synthases, which are insensitive to sulfonamides [20]. Eight related antibiotics [Tetracycline (TC), Chlortetracycline (CTC), Oxytetracycline (OTC), Sulfadiazine (SD), Sulfamethoxazole (SMX), Sulfamerazine (SM1), Sulfamethazine (SM2) and Trimethoprim (TMP)] were chosen due to their widely application in agricultural activities.

2. Materials and methods

2.1. Description of eco-agricultural system

A total of 36 samples was collected in 2013 from an EAS in Hangzhou area, eastern China. The flow of the system is depicted in Fig. 1, excrement from breeding swine enter biogas digesters, and after anaerobic digestion biogas can be a source of household fuel and ADSR and ADLR are used to grow vegetables and ryegrass, the ryegrass could be used to feed swine and raise fish. There are 20 hectares vegetable soils and 2 hectares ryegrass soils, the ADSR was used as fertilizer in soils once a year and the ADLR was used to irrigate soils once a week. The sampled soils were fertilized with ADSR and ADLR for 8 years. A vegetable soil and fishpond from a near-by farmhouse, which is independent from the eco-agricultural system and never used antibiotics, was chosen as a control system.

2.2. Sample collection and chemical analyses

Feed, manure, ADSR, ADLR, fertilized soils [vegetable soil (VS) and ryegrass soil (RS)] and fishpond in the system were sampled (triplicates). Random manure samples were taken from freshly excreted feces. ADSR samples were collected as soon as it was separated from the digestion broths. Soil samples were collected from the top 0–10 cm of the surface fertilized soil. After process of vacuum freeze-drying, samples were passed through a 2 mm sieve and stored at −80 °C for DNA extraction. The ADLR samples and effluent after underground pipeline for irrigation were collected in triplicate from storage tanks, respectively. As for fishponds, Lagoons were sampled in triplicate at 20 and 150 cm below the surface for liquid and sediment, respectively. The sediment in fishponds was preprocessed as soil samples above. Total nitrogen and total phosphorus in samples were determined by parallel analysis according to soil agrochemical analysis [21], and organic matters in samples were determined by the $K_2Cr_2O_7$ oxidation method.

2.3. DNA extraction and PCR

Liquid samples were concentrated using a vacuum filtration apparatus onto 0.22-μm filters until the filter clogged, after which the filters were stored at −80 °C until DNA extraction was performed. Total DNA was extracted using an UltraClean Water DNA Kit (Mo Bio Laboratories, Carlsbad, CA, USA). DNA extraction was performed following the manufacturer's protocol. Quality and concentration of the purified DNA were determined by spectrophotometer analysis (NanoDrop ND-2000c, Thermo Fisher Scientific, Waltham, MA, USA) and 1.5% agarose gel electrophoresis.

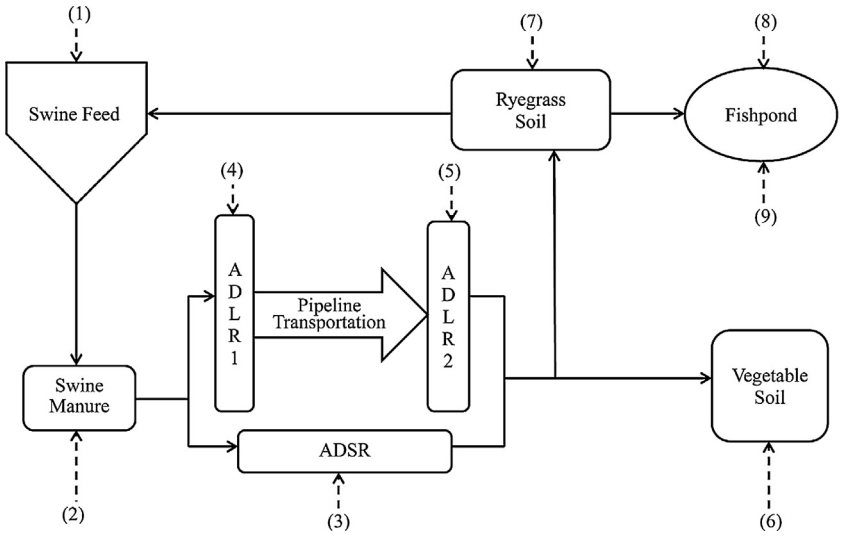

Fig. 1. The flow diagram of the eco-agricultural system and sampling sites location.
ADSR = anaerobic digestion solid residue; ADLR = anaerobic digestion liquid residue. Sampling sites: (1) swine feed, (2) swine manure, (3) ADSR, (4) ADLR, (5) ADLR after pipeline transportation, (6) vegetable soil, (7) ryegrass soil, (8) fishpond, (9) sediment of the fishpond.

Table 1
Correlation analysis of ARG copy numbers and antibiotic residue concentrations in all samples.

	TC	CTC	OTC	TTC	SM1	SM2	SMX	SD	TS	TMP
*tet*A	**0.470**	**0.622**	**0.489**	**0.492**	0.002	0.007	**0.530**	**0.312**	**0.524**	0.035
*tet*B	**0.543**	**0.816**	**0.798**	**0.799**	−0.011	-0.007	**0.854**	**0.282**	**0.843**	0.018
*tet*C	−0.005	−0.045	−0.099	−0.099	−0.052	−0.050	−0.098	0.058	−0.098	−0.037
*tet*G	**0.508**	**0.702**	**0.600**	**0.600**	0.0001	0.005	**0.642**	**0.314**	**0.635**	0.032
*tet*L	**0.530**	**0.757**	**0.680**	**0.683**	−0.003	0.001	**0.732**	**0.307**	**0.723**	0.028
*tet*M	0.086	0.055	−0.057	−0.055	−0.035	−0.032	0.051	0.131	−0.051	−0.014
*tet*O	**0.499**	**0.822**	**0.909**	**0.910**	−0.033	−0.029	**0.969**	0.205	**0.956**	−0.011
*tet*Q	**0.486**	**0.813**	**0.915**	**0.915**	−0.036	−0.033	**0.975**	0.192	**0.961**	−0.015
*tet*W	**0.514**	**0.830**	**0.897**	**0.898**	−0.028	−0.024	**0.960**	0.223	**0.944**	−0.004
*tet*X	**0.498**	**0.680**	**0.565**	**0.568**	0.001	0.006	**0.611**	**0.314**	**0.604**	0.034
*tet*T	**0.512**	**0.829**	**0.900**	**0.900**	−0.028	−0.025	**0.960**	0.220	**0.946**	−0.007
*sul*I	**0.427**	**0.550**	**0.393**	**0.397**	0.001	0.007	**0.429**	**0.302**	**0.425**	0.034
*sul*II	**0.491**	**0.666**	**0.546**	**0.549**	0.002	0.007	**0.590**	**0.314**	**0.584**	0.035
*sul*T	**0.446**	**0.578**	**0.433**	**0.436**	0.002	0.007	**0.471**	**0.307**	**0.466**	0.035

In each cell, the value indicates the Pearson Correlation Coefficient (r). Bold values indicated statistical significance ($p < 0.05$). TC = tetracycline; CTC = chlortetracycline; OTC = oxtetracycline; TTC = total tetracycline; SM1 = sulfamerazine; SM2 = sulfamethazine; SMX = sulfamethoxazole; SD = sulfadiazine; TS = total sulfonamides; TMP = trimethoprim.

Twelve ARGs (*tetA*, *tetB*, *tetC*, *tetG*, *tetL*, *tetM*, *tetO*, *tetQ*, *tetW*, *tetX*, *sulI* and *sulII*) and *intI1* were investigated. The PCR assays for DNA extracted from all samples were conducted in a 25 μL volume reaction, the PCR mixture consisted of 2.5 μL 10× PCR Buffer (Mg^{2+} Free); 0.5 μL dNTP mixture (10 mM each); 1.5 μL $MgCl_2$ (25 mM); 1 μL of each primer; 1.5U of Taq DNA Polymerase; and 1 μL of template (the concentration of DNA was between 34.5 ng/μL and 228.3 ng/μL). Primers targeting the twelve ARGs were selected from published literature [22–27], with their amplification sizes and conditions listed in Table S1. PCR products were analyzed by electrophoresis on a 1.5% agarose gel in 1 × TAE buffer. To ensure reproducibility, duplicate PCR reactions were performed for each sample. Sterile water was used as the negative control in every run.

2.4. Real-time qPCR

Real-time qPCR was applied to quantify the presence of *tetA*, *tetB*, *tetC*, *tetG*, *tetL*, *tetM*, *tetO*, *tetQ*, *tetW*, *tetX*, *sulI*, *sulII*, and *intI1*. 16S rRNA was also quantified to correct for variance in the abundance of ARGs caused by differences in background bacterial abundance and DNA extraction efficiency.

The qPCR amplification and quantification were conducted using a Step One Plus real-time PCR system (ABI, USA). The following PCR protocol was followed: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, 30 s at different annealing temperatures (Table S1), extension for 30 s at 72 °C and a fluorescence acquisition step at 72 °C, then a final melt curve stage with temperature ramping from 60 to 95 °C. A 15 μL reaction mixture consisted of 7.5 μL of SYBR Premix Ex Taq™ (TaKaRa, Japan), 0.3 μL of ROX reference dye, 0.2 μM concentration of each primer, 2 μL of template DNA, and 4.6 μL of ddH_2O. Additional information on the qPCR primers is given in Supporting Information (Table S1).

The number of target gene copies in unknown samples was calculated according to the standard curves, which were prepared by fresh PCR products of the target genes after PCR amplification. As per instructions of the manufacturer, purified PCR products were ligated into pMD19-T vector (TaKaRa, Japan) and then cloned into Escherichia coli DH5α (TaKaRa, Japan). Plasmids carrying target genes were extracted according to a QIAprep™ Spin MiniprepKit (QIAGEN, Germany) and used to generate calibration curves. The concentration and quality of the plasmid DNA were determined by agarose gel electrophoresis and spectrophotometer analysis (NanoDrop ND-2000c, Thermo, USA). The copy number of ARG genes per microliter of plasmid solution was calculated as described by Zhang et al. [28], as lengths of the pMD19-T vector and target genes were already known.

All standard curves of qPCR were generated using 10-fold serial dilutions of the plasmid carrying target genes, from 10^8 to 10^2 copy numbers. The PCR efficiencies (80.0%–110.5%) were examined to test for inhibition. R^2 values were higher than 0.991 for all standard curves (Table S2). According to the standard curves, Ct value of unknown samples was used to calculate the number of corresponding gene copies.

2.5. Analysis of antibiotics

Eight target antibiotics, namely, Tetracycline (TC), Chlortetracycline (CTC), Oxytetracycline (OTC), Sulfadiazine (SD), Sulfamethoxazole (SMX), Sulfamerazine (SM1), Sulfamethazine (SM2), and Trimethoprim (TMP) were analyzed in the collected samples. The detailed information about antibiotics chosen is shown in Table S3.

Liquid samples were filtered through 0.45 μm glass fiber filters and 0.80 g/L Na_2EDTA was added to the samples and react for 1 h. Then, 1 M HCl and NaOH were used to adjust the pH of samples to 4.8–5.0. Oasis HLB cartridges (6 mL/500 mg, Waters, USA) were successively activated with 10.0 mL methanol, 10.0 mL ultra-pure water and 5.0 mL pH 5.0 ± 0.2 ultra-pure water. Then samples were passed through cartridges at a flow rate of 5 mL/min. After that, cartridges were rinsed with 10 mL ultra-pure water and dried under gentle nitrogen gas for 30 min. Then cartridges were eluted with 10 mL mixture of methanol: acetonitrile (1:1, v/v). The elutes were concentrated to 1 mL under gentle nitrogen gas at 40 °C and later diluted to a volume of 5 mL with methanol: water (1:1, v/v). Final treated samples were stored at −20 °C in the dark and analyzed by Ultra-high Performance Liquid Chromatography tandem Mass Spectrometry (UPLC-MS/MS) within 40 days.

After the process of vacuum freeze-drying, solid samples were extracted by 10 mL extractant (5 mL methanol + 5 mL Na_2EDTA-McIlvaine) treating with ultrasonic method for 10 min, then centrifuged at 4000 rpm for 10 min. the supernatant was collected and diluted to 800 mL by ultra-pure water. The following process was the same with the liquid samples.

The final extracts were analyzed by UPLC-MS/MS system equipped with an Acquity™ UPLC and a Quattro Premier Micromass® MS (Waters/Micromass, Milford, MA). All target antibiotics were separated by a $BEHC_{18}$ column (Waters Corp., 50 mm × 2.1 mm, 1.7 μm) and then identified and quantified by the MS/MS system via the multi-reaction monitoring (MRM) mode. The MS/MS analysis was performed in the positive electrospray ionization (ESI) mode. The specific instrument conditions of eight compounds are summarized in Table S4 and Table S5.

The internal standards method was used in this study. $^{13}C_6$-sulfamethazine and $^{13}C_6$-sulfamethoxazole (Cambridge Isotope Laboratories, USA) were used to quantify the sulfonamides. Thiabendazole-d_6 and trimethoprim-d_3 (Dr. Ehrenstorfer, Germany) were used to quantify tetracyclines and trimethoprim, respectively. The recoveries of target antibiotics in water and solid samples varied from 78–140% and 71–150%. Relative standard deviations (RSDs) of target compounds ranged from 1.0% to 3.5%. They showed good recoveries and instrumental precision. The correlation coefficients (R^2) of internal calibration curves were all higher than 0.999, indicating excellent linear correlation of curves.

2.6. Statistical analysis

Statistical analyses of the data were performed using SPSS version 17.5. Paired samples *t* test was conducted to assess the significance of the differences between different systems and samples based on *P*-values. Correlations between the ARGs and other parameters monitored were analyzed using OriginPro 8.0 (Origin Lab Corporation, USA).

3. Results

3.1. Occurrence of antibiotics in eco-agricultural system

Concentrations and composition of antibiotics in EAS are shown in Table S6 and Fig. 2. In this system, total tetracycline concentrations in solid and liquid samples were as high as 139.93 mg/kg and 39.72 μg/L, respectively. Among the eight target antibiotics, OTC had the highest concentration in manure sample reaching up to 138.75 mg/kg. Of the sulfonamides analyzed in this study, SMX had the highest concentrations for all samples, ranging from 0.13 to 6.21 mg/kg in solid samples and ranging from 0.15 to 5.83 μg/L in liquid samples. SM1, SM2 and TMP were not detected in upstream component (feed, manure, biogas residue and biogas slurry), but detected in downstream component of the system (soil and fishpond).

3.2. Abundance of ARGs in eco-agricultural system

Ten *tet* genes and two *sul* genes were detected in all samples except *tetB* in swine feed sample. As shown in Table S8, the highest abundance of ARGs per sample volume was observed in manure and ADSR samples, where individual ARGs ranged from 3.94×10^7 to 6.60×10^{11} copies/g with the total ARGs at the level of 1.27×10^{12} and 4.56×10^{11} copies/g, respectively.

Relative abundance of ARGs (gene copies per 16S-rRNA gene) was used for further analyses (Fig. 3). In all samples, the average relative abundance varied greatly among the ARGs. *TetQ* had the highest relative abundance ($4.28 \times 10^{-2} \pm 6.03 \times 10^{-2}$ gene copies per 16S-rRNA gene), followed by *tetW*, *sulI* and *tetO*. Yet the relative abundance of *tetG* showed the least variation within the entire EAS, ranging from 1.08×10^{-3} to 8.30×10^{-3}. Relative abundance of ARGs in swine feed ranged from 6.30×10^{-6} to 1.38×10^{-3}. Compared to the relative abundance in manure, *tetC* and *tetM* increased in the ADSR while three ribosomal protection proteins (RPP) genes (*tetO*, *tetQ* and *tetW*) decreased ($p < 0.05$), with other genes showing no significant change after anaerobic digestion ($p > 0.05$). As for ADLR, some genes (*tetC*, *tetG* and *tetL*) increased in abundance after pipeline (made of metals) transportation ($p < 0.05$). In the downstream component, the relative abundance of *tet* genes and *sul* genes ranged from approximately 10^{-6} to 10^{-3}, and 10^{-4} to 10^{-3}, respectively, which are consistent with the previous result in agricultural soils adjacent to feedlots [29,30] and fishpond samples [31].

3.3. Comparison of ARGs in eco-agricultural system and control agricultural system

A fish farm and a vegetable soil with no deliberate input of animal waste were sampled as the control. In the control system, *tetB* was not detected in all samples and *tetC* was not detected in fishpond sample, the relative abundance of other ARGs ranged from 2.81×10^{-6} to 7.62×10^{-3} (Fig. 4). *SulI* had the highest average relative abundance ($3.18 \times 10^{-3} \pm 3.85 \times 10^{-3}$), followed by *tetG*, *tetW* and *tetO*. Most of ARGs in EAS showed significantly higher relative abundance compared with that from the control system ($p < 0.05$), except *tetG* in vegetable soil and *sulI* in fishpond, which showed no significant difference with the control system. *SulI* and *tetG* were the dominant genes in the control system, their average relative abundance were as high as 3.18×10^{-3} and 6.57×10^{-4}.

4. Discussion

Our study provides some basic information on the persistence of antibiotics and ARGs in an ESA. In the studied system, the highest concentration of antibiotics (OTC) in manure (138.75 mg/kg)

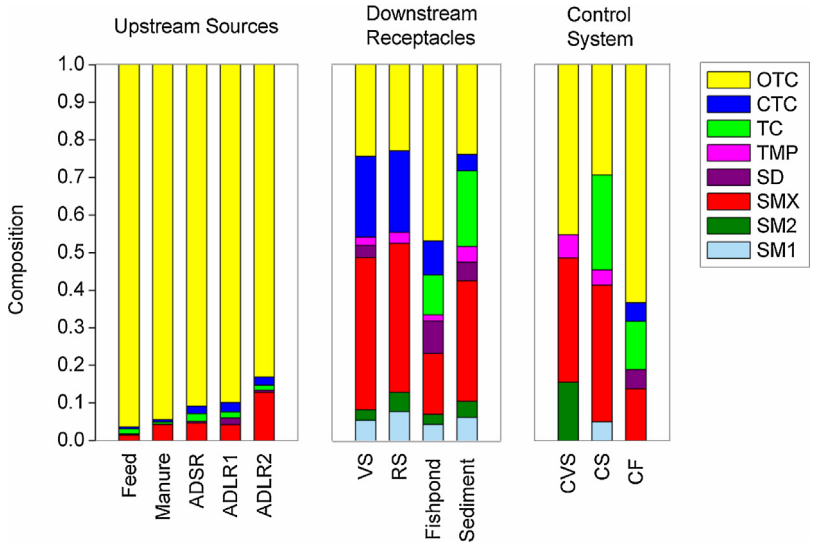

Fig. 2. Composition of antibiotics in the eco-agricultural system and control system.
ADSR = anaerobic digestion solid residue; ADLR1 = anaerobic digestion liquid residue; ADLR2 = anaerobic digestion liquid residue after the pipeline transportation; VS = vegetable soil; RS = ryegrass soil; CVS = vegetable soil in control system; CS = sediment of fishpond in control system; CF = fishpond water in control system.

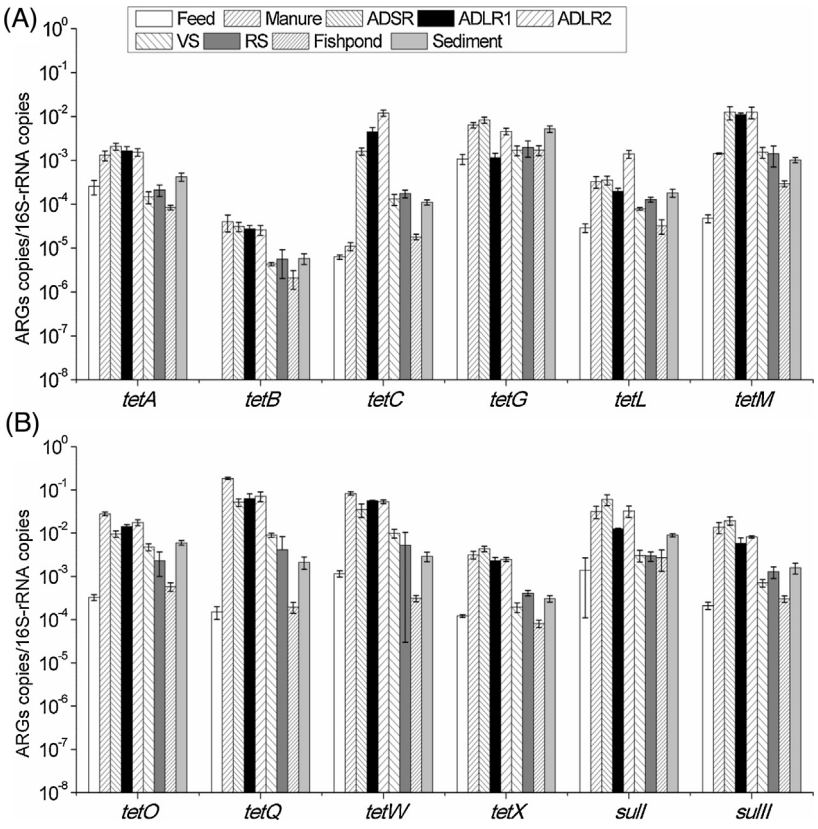

Fig. 3. Relative abundance of ARGs (gene copies per 16S-rRNA gene) in the eco- agricultural system.
ADSR = anaerobic digestion solid residue; ADLR1 = anaerobic digestion liquid residue; ADLR2 = anaerobic digestion liquid residue after the pipeline transportation; VS = vegetable soil; RS = ryegrass soil. Means ± standard deviations are shown ($n = 3$).

is largely higher than those reported for some European manures between 2002 and 2005 [7]. High concentrations of antibiotic residues in manure reflect unregulated use of veterinary antibiotics in Hangzhou area. It is noticeable that the antibiotic concentrations in solid and liquid samples of the control system ranged from not detected to 376.73 μg/kg, and not detected to 0.42 μg/L, respectively (Table S6). Although the antibiotic concentrations in control system were lower than that in eco-agricultural system, they were still high. The antibiotics in control system may come from irrigation water [32].

Our results showed that anaerobic digestion could eliminate most antibiotics effectively and the removal rate ranged from 11% to 86%. However, antibiotics contained in manure may still reach the environment as ADSR and ADLR are used on agricultural field as fertilizers. All antibiotics were detected in soil samples except TC, even including SM1, SM2 and TMP, which were not detected

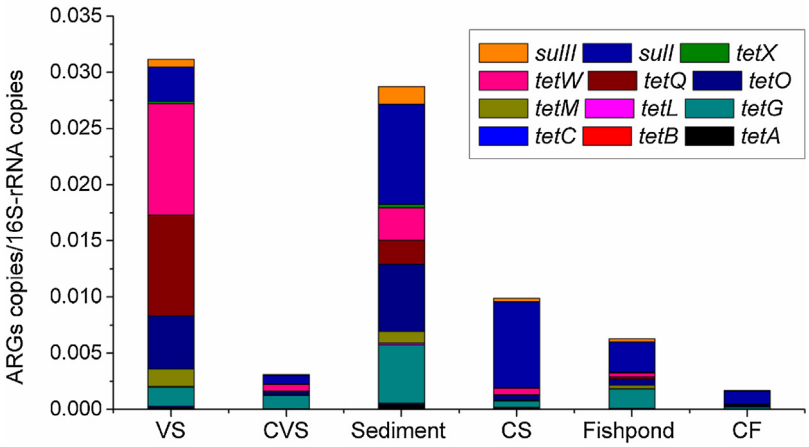

Fig. 4. Comparison of the relative abundance of ARGs in the eco-agricultural system and control system.
VS = vegetable soil in eco-agricultural system; CVS = vegetable soil in control system; CS = sediment of fishpond in control system; CF = fishpond water in control system.

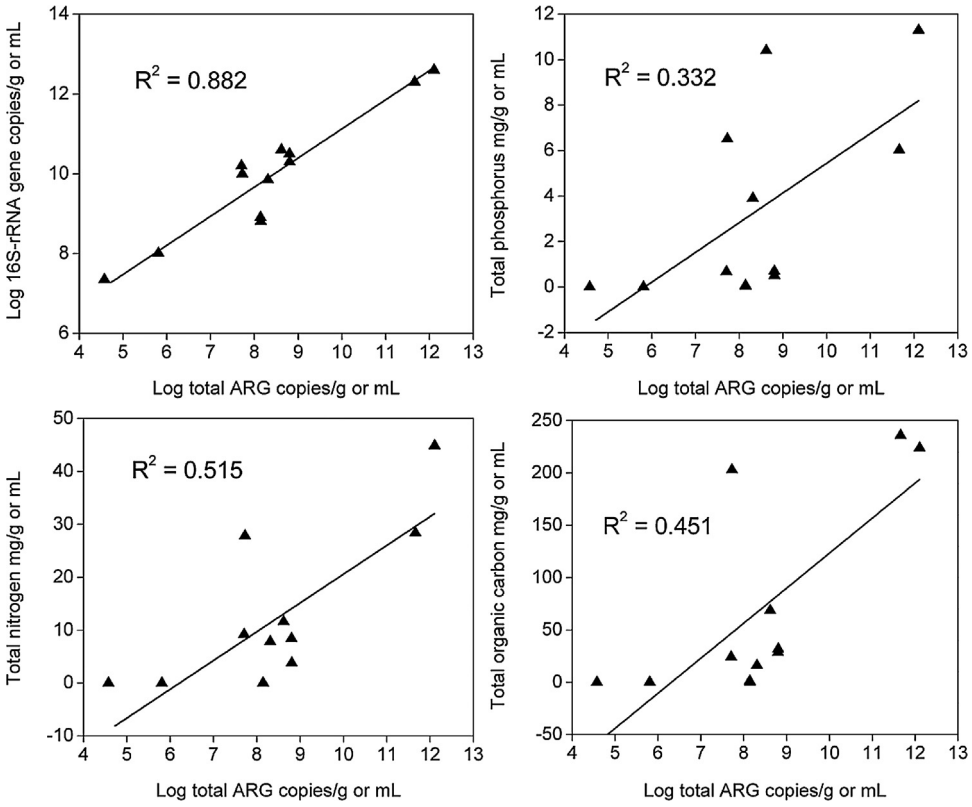

Fig. 5. Correlation between total nitrogen, total phosphorus, total organic carbon, 16S-rRNA copy numbers and ARG copy numbers in all samples.

in upstream sources. The persistence of those antibiotics could be attributed to the fact that they were used in the farm in the past and difficultly degraded in soils [33].

As for ARGs in the EAS, *tetQ*, *tetW* and *sulI* were the most abundant ones. *TetQ* had the highest relative abundance in manure (1.84×10^{-1}) ($p < 0.05$), which is consistent with our previous finding in livestock farms of Hangzhou area [34]. Zhu et al. [35] found similar results, in which *tetQ* was the most abundant *tet* genes in the swine manure. *TetQ* was first described in human colonic *Bacteroides* spp. and is normally associated with conjugative transposons [36]. Additionally, the gene has been found in anaerobe *Prevotella* [37] as well as seven Gram-positive and six Gram-negative genera respectively [36,38]. The host range of *tetW* gene was nineteen genera including Gram-positive, Gram-negative, aerobic and anaerobic bacteria [3] and it is also associated with conjugative transposons [39–41]. The wide host range and close relationship with mobile genetic elements may have contributed to the predominance of *tetQ* and *tetW*. Significant correlation between *sulI* and *intI1* ($R^2 = 0.803$, $p < 0.001$) in our study was consistent with the conclusion that *sulI* is often associated with *intI1* [20,34,42], which may be the reason for the abundance of *sulI* in the studied system. The relative abundance of *tetG* in all samples was 10^{-3}, being the least variable in the EAS. Although the host range of *tetG* was only 7 genera [3], it was found that *tetG* was abundant in activated sludge and had a high genetic diversity [43,44], which may contribute to the persistence of *tetG* in this study.

Unlike antibiotics, most of the relative abundant ARGs showed no significant removal after anaerobic digestion, some ARGs such as

tetC and *tetM* even increased in ADSR. While the highest log removal of ARGs was only 0.8 log (*tetQ*), suggesting that anaerobic digestion at mesophilic temperature may have limited ability to reduce ARGs and thermophilic anaerobic digestion may be more efficient than mesophilic anaerobic digestion in reducing ARGs because the latter effectively kills pathogens [45]. Interestingly, the relative abundance of most ARGs in ADSR showed no significant difference with that in ADLR except *tet*G, *sul*I and *sul*II, which were higher in ADSR than that in ADLR ($p < 0.05$). The similar abundance of ARGs in both ADSR and ADLR needs more attention. In ADLR, the relative abundance of *tet*C, *tet*G and *tet*L increased after pipeline transportation. The enrichment of these genes is most likely due to the aggregation of resistance genes on mobile genetic elements [3,46], as has been observed in agricultural system [47]. In addition, the presence of heavy metals in the environment (pipeline etc.) may have also provided a co-selective pressure for antibiotic resistance [48] and may exist on long-term effects on the persistence of ARGs in this system [49].

The soils for growing vegetables and ryegrass were amended with ADSR once a year and irrigated with ADLR once a week, although the relative abundance of ARGs in soils were significantly lower than that in ADSR and ADLR ($p < 0.05$), most of them were still higher than those in the control system. The accumulation of resistance genes in soils may be related to the following reasons. First, considerable amounts of antibiotics in fertilizers could pose a selective pressure on bacterial communities in soils, as it has been suggested that sulfonamide concentrations as low as 0.1 mg/kg of soil could have a selective pressure on resistant populations [50]. Additionally, the application of manure digestion products to agricultural soils may introduce bacteria carrying antibiotic resistance genes. It has been reported that the application of antibiotic-containing manure could increase the prevalence of resistance genes and isolates in a field soil [14–16]. Finally, antibiotic resistance genes on transferable plasmids introduced via manure digestion products into soils can persist regardless of the viability of introduced host cell due to horizontal gene transfer [2]. Notably, *tet* genes and *sul* genes in vegetable soil showed no significant difference with that in ryegrass soil, meaning that plants may have limited impact on the spread of *tet* genes and *sul* genes in soils.

Instead of using manures or manure digestion products as fertilizers for fishpond, farmers used ryegrass as fish feed, which could introduce antibiotic-resistant bacteria and ARGs into the fish pond and result a higher relative abundance of ARGs in fishpond of the EAS than that in the control in our study. All ARGs in sediment were significantly higher than that in fishpond water ($p < 0.05$), and the accumulation of ARGs in sediment poses a potential risk to the broader public since ARGs in aquatic environmental bacteria can be disseminated by horizontal gene transfer to other bacteria and ultimately to human pathogens.

The above discussion indicated a general profile of antibiotics and ARGs in an eco-agricultural system, and then correlation analyses were conducted to identify the relationship between ARGs and antibiotics (Table 1). All ARGs showed statistically significant correlations with the dominant antibiotics in feed (three tetracyclines and SMX) except *tetC* and *tetM*, suggesting that ARGs in this system was likely linked to the levels of residual antibiotics, which may exert a selection pressure on the microorganisms. In addition to selection from antibiotics, other factors may play a role in the observed inconsistencies in the correlations between antibiotics and ARGs. First, cross-selection may disrupt the correlations if any of the ARGs are located on mobile genetic elements that also harbor a resistance gene of a different class that is subject to a stronger selection pressure, such as heavy metals [51,52]. Additionally, antibiotic itself can be correlated, for example OTC in the system was positively correlated with both SMX and SD ($R^2 = 0.930$ and 0.360, respectively; $p < 0.05$).

Positive correlations were also observed between the total ARG copy numbers and biological and chemical parameters in this system ($p < 0.05$) (Fig. 5). The total ARG copies were correlated with 16S-rRNA gene copies ($R^2 = 0.882$, $p < 0.001$), total nitrogen, total phosphorus and total organic carbon ($R^2 = 0.515$, 0.332 and 0.451, respectively; $p < 0.05$). McKinney et al. [53] has found similar positive correlation between tetracycline resistance genes and water-quality constituents (chemical oxygen demand, total N, ammonia, nitrate, and phosphate) in livestock lagoons. This is consistent with the hypothesis that well-performing lagoons in terms of improving water quality are also effective in eliminating ARGs.

To the best of our knowledge, it is the first time that eco-agricultural system is taken as a whole to comprehensively investigate the behaviors of ARGs. The high relative abundance of ARGs reported in this study is important, and clearly indicates that unmonitored use of antibiotics in agriculture has expanded the diversity and abundance of the antibiotic resistance reservoir in the food production system. The enrichment of ARGs further exacerbates the risks of transfer of ARGs from livestock animals to human-associated bacteria, and then spread among human populations [54,55]. Considering that decreased resistance levels have been observed in Europe after the disuse of agricultural antibiotics [56], policies and management tools to facilitate prudent use of antibiotics in animal industries and animal waste management are needed.

5. Conclusions

This study assessed the abundance and persistence of antibiotics and ARGs in the EAS. Among the eight target antibiotics, OTC had the highest concentration in manure reaching up to 138.7 mg/kg. The anaerobic digestion could eliminate most antibiotics effectively and the removal rate ranged from 11% to 86%. *TetQ, tetW* and *sulI* were the most abundant ARGs in the EAS. Compared to the relative abundance in manure, *tetC* and *tetM* increased in the ADSR while three RPP genes (*tetO*, *tetQ* and *tetW*) decreased ($p < 0.05$), with other genes showing no significant change after anaerobic fermentation ($p > 0.05$). As for ADLR, *tetC*, *tetG* and *tetL* increased in abundance after pipeline transportation ($p < 0.05$). The relative abundance of most ARGs in soils and fishpond of the EAS were significantly higher than that in the control agricultural system ($p < 0.05$), except *tetG* and *sulI*.

Notes

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2015.10.037.

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