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Discharge of swine wastes risks water quality and food safety: Antibiotics and antibiotic resistance genes from swine sources to the receiving environments

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ABSTRACT

Swine feedlots are widely considered as a potential hotspot for promoting the dissemination of antibiotic resistance genes (ARGs) in the environment. ARGs could enter the environment via discharge of animal wastes, thus resulting in contamination of soil, water, and food. We investigated the dissemination and diversification of 22 ARGs conferring resistance to sulfonamides, tetracyclines, chloramphenicols, and macrolides as well as the occurrence of 18 corresponding antibiotics from three swine feedlots to the receiving water, soil environments and vegetables. Most ARGs and antibiotics survived the on-farm waste treatment processes in the three swine farms. Elevated diversity of ARGs was observed in the receiving environments including river water and vegetable field soils when compared with respective controls. The variation of ARGs along the vertical soil profiles of vegetable fields indicated enrichment and migration of ARGs. Detection of various ARGs and antibiotic residues in vegetables fertilized by swine wastes could be of great concern to the general public. This research demonstrated the contribution of swine wastes to the occurrence and development of antibiotic resistance determinants in the receiving environments and potential risks to food safety and human health.

1. Introduction

Antibiotic resistance has become one of the most serious clinical and public health issues in the world as indicated by the first global report of World Health Organization (WHO) on antimicrobial resistance in May 2014 (WHO, 2014). To date, investigations have been focused on antibiotic resistance in human, animal and food isolates (Levy and Marshall, 2004). The transfer of antibiotic resistance determinants from human and livestock sources to the environment has gained attention in the scientific community in the past decade (Silbergeld et al., 2008; Storteboom et al., 2010; Wright, 2010), with antibiotic resistance genes (ARGs) considered as emerging environmental contaminants (Pruden et al., 2006). Human wastes are nowadays better treated by centralized wastewater treatment plants than animal wastes with simple treatment systems such as lagoon and digester or even without any treatment before their discharge into the environment (McKinney et al., 2010; Liu et al., 2012; Zhou et al., 2013; He et al., 2014; Su et al., 2014b; Chen et al., 2015). In comparison with other livestock animals (broiler and cattle), antibiotics are commonly used as growth promoter in swine farming (Zhou et al., 2013; Durso and Cook, 2014), thus swine feedlots are of particular concern as potential hot spots for promoting the dissemination of antibiotic resistance (Gotz and Smalla, 1997; Cole et al., 2000; Binh et al., 2008).

In recent years, ARGs were frequently investigated in manure and wastewater of swine farms (McKinney et al., 2010; Wu et al., 2010; Zhu et al., 2013). Previous studies showed that the levels of ARGs such as tetracycline and macrolide-lincosamide-streptogramin B resistance genes remained high in swine wastes during composting (Wang et al., 2012), lagoon storage (Jindal et al., 2006; Brooks et al., 2014), anaerobic digestion (Chen et al., 2010; Tao et al., 2014) and constructed wetlands (Huang et al., 2014; Liu et al., 2014). These results indicate that ARGs arising from swine-feeding operations can survive typical animal waste treatment processes. However, despite the increasing knowledge of ARG variation in abundance through treatment units, there is little information on the diversification of ARGs after they are introduced into the environment. Moreover, common on-farm waste treatment systems are designed to remove nutrient, solids, organic matter and enteric bacteria (Cordero et al., 2010), but not intentionally designed for the removal of resistance determinants (Koike et al., 2007; Sapkota et al., 2007). There is a need to further investigate the fate of ARGs in animal wastes and treatment systems under different animal farming systems.

Moreover, animal wastes (including wastewater and manure) whether they are treated or not are commonly used as fertilizer in agricultural fields. Animal wastes are a reservoir for various transferable antibiotic resistance plasmids (Binh et al., 2008). Manure fertilizer has

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been reported to increase the abundance of antibiotic resistance in soils (Wu et al., 2010; Zhu et al., 2013; Heuer et al., 2011; Udikovic-Kolic et al., 2014) and in harvested vegetables (Marti et al., 2013). This may pose potential risks to food safety and human health (Becerra-Castro et al., 2015). So far, scientific knowledge on the impact of swine farming on the adjacent environments and vegetables is still limited; therefore, further research is essential to investigate the dissemination of ARGs associated with disposal of animal wastes.

The objectives of this study were to investigate the occurrence of various ARGs in manure and wastewaters of three swine feedlots with different waste management systems, and to assess their impacts on the receiving water, soil environments and vegetables due to discharge of wastewater into rivers and application of wastes on land. Since sulfonamides, tetracyclines, macrolides and florfenicol are commonly used in livestock production (Zhou et al., 2013), and such use is perceived to contribute to the development of antibiotic resistance (van den Bogaard and Stobberingh, 2000), *sul* sulfonamide resistance genes and *tet* tetracycline resistance genes were the most frequently reported ARGs in diverse livestock operations such as swine and broiler feedlots (McKinney et al., 2010; Koike et al., 2007; Wu et al., 2010; He et al., 2014), followed by *erm* macrolide–lincosamides–streptogramin B resistance genes(Chen et al., 2007, 2010). Although chloramphenicol has been banned in food-producing animals since 1994, its alternative antibiotic florfenicol was widely used and their resistance genes (*cmlA*, *floR*, *fexA*, *cfr*, and *fexB*) were prevalent in swine feedlots and their surrounding environments (Li et al., 2013). Thus the above resistance genes were selected as the target genes to study the impact of swine farms on the receiving environment. The class 1 and class 2 integron genes (int1 and int2) were included as indicators of potential horizontal gene transfer for multiple ARGs. The results from this study facilitate better understanding of the dissemination of ARGs from swine farms to the environment and potential risks to food safety and human health.

2. Methods

2.1. Information about swine farms

Three representative swine farms with different waste management systems were selected for this study. The three commercial swine farms, representing typical swine feeding operations in South China, are located in Kaiping (swine farm 1: F1) and Heshan (swine farm 2 and 3: F2 and F3) of Guangdong Province, with detailed farm management information given in the Supplementary materials (Fig. S1 and Text S1).

Two typical models of waste treatment constituted of lagoon and anaerobic digester are applied in the three swine farms (Fig. 1). Swine farm 1 is equipped with a traditional lagoon. Part of the effluent is applied for irrigation to the nearby vegetable field which covers an area of 31,600 m^2. Spring onion vegetables are extensively cultivated all the year round in this area. In addition to conventional lagoon, anaerobic digesters are employed to treat the wastewater in swine farm 2 and farm 3. The effluents from swine farm 2 and farm 3 are discharged via ditches to Longkou River which starts from a reservoir. Detailed information on wastewater treatment systems in the three farms is given in the Supplementary materials (Text S1).

2.2. Sample collection

Various samples were collected in September 2013 from the three farms and surrounding environment (Fig. 1). On the three farms, the collected samples included manure and flush wastewater from swine houses, solid wastes and wastewater from the treatment units, sediment and surface water from fishpond. For the surrounding environment, well water, soil or irrigation water from vegetable fields, sediment and water from the receiving river were collected. Three samples of each type at each location were collected to determine average concentrations. Manure samples were taken by randomly collecting fresh feces from different swine houses and then combining into one composite sample of different groups of pigs for each farm. The flush waters from different groups of pigs were sampled only in farm 3 at the washing time, and they were not accessible at the sampling time in the other two farms. Wastewaters in the different treatment units (lagoons, biogas digesters, and ponds) were collected sequentially, while the solid wastes were also collected from the corresponding locations. Meanwhile, environmental samples from the surrounding environments of the farms were collected, including well water, irrigation water and surface soil (0–20 cm) of vegetable fields, water and sediment from the receiving river (R1 to R7, see Fig. S1). In addition, control soil samples for each farm (F1SC, F2SC, and F3SC) were collected from nearby land without application of animal wastes, and control water and sediment samples (R1W and R1S) also collected from a pristine reservoir with no swine waste contamination.

Vegetables ready for harvest were first sampled from the vegetable fields of swine farm 1 (spring onion) and swine farm 2 (spring onion and water spinach) in 2013, which have been fertilized by swine wastes. For comparison, spring onion fields fertilized by swine wastewater of farm 1 were sampled again in the middle of April, 2015. Two spring onion plots far away from the research farm and with no livestock feedlots nearby were selected as reference for the vegetable field. Spring onion and surface soils were collected in the same way as before. In addition, vertical soil cores were taken randomly from the spring onion plots irrigated with swine wastewater of farm 1. More information on vegetable field collection is given in the Supplementary materials (Text S2).

2.3. Solid and aqueous sample processing

All collected samples were transported to the laboratory in a cooler for immediate processing following the protocol previously described (He et al., 2014). Briefly, aqueous samples for chemical analysis and ARG analysis were processed within 24 h. For ARG analysis, successive filtration of aqueous samples was filtered through 0.45 μm membrane filters which were stored at −80 °C for later DNA extraction. Solid samples were freeze-dried and sieved through a 2 mm mesh, then stored at −80 °C for DNA, antibiotics and metal extraction.

2.4. Vegetable processing

Excess soil was removed from all vegetables with distilled water twice, followed by washing with sterile Milli-Q water once to achieve the visual cleanliness that a typical consumer would expect in normal food preparation, as was done by Marti et al. (2013). Then the vegetables were shaken in 500 mL of the 0.85% sterile physiological saline, and the wash water were filtered through 0.45 μm filter membranes. These filter membranes were stored at −80 °C for later DNA extraction. Detailed information on vegetable processing is given in the Supplementary materials (Text S2).

2.5. DNA extraction and ARG quantification

Total DNA extraction was conducted using PowerSoil DNA Isolation Kit (MoBio Laboratories, USA) following the manufacturer's protocol. Quantitative PCR (qPCR) was used to quantify four classes of ARGs, including three sulfonamide resistance genes (*sul* genes: *sul1*, *sul2* and *sul3*), eleven tetracycline resistance genes (*tet* genes: *tetA*, *tetG*, *tetH*, *tetM*, *tetO*, *tetQ*, *tetS*, *tetW*, *tetB*/*P*, *tetT* and *tetX*), five chloramphenicol resistance genes (*cml* genes: *cmlA*, *floR*, *fexA*, *fexB*, and *cfr*) and three erythromycin resistance genes conferring resistances to macrolide–lincosamides–streptogramin B (MLS_B genes: *ermB*, *ermC* and *ermE*). The class 1 and class 2 integron genes (*int1* and *int2*) were also quantified as an indicator of potential horizontal gene transfer for multiple ARGs. The 16S ribosomal RNA (16S rRNA) gene was quantified as a measure of total bacterial load. The specific primers, annealing temperatures

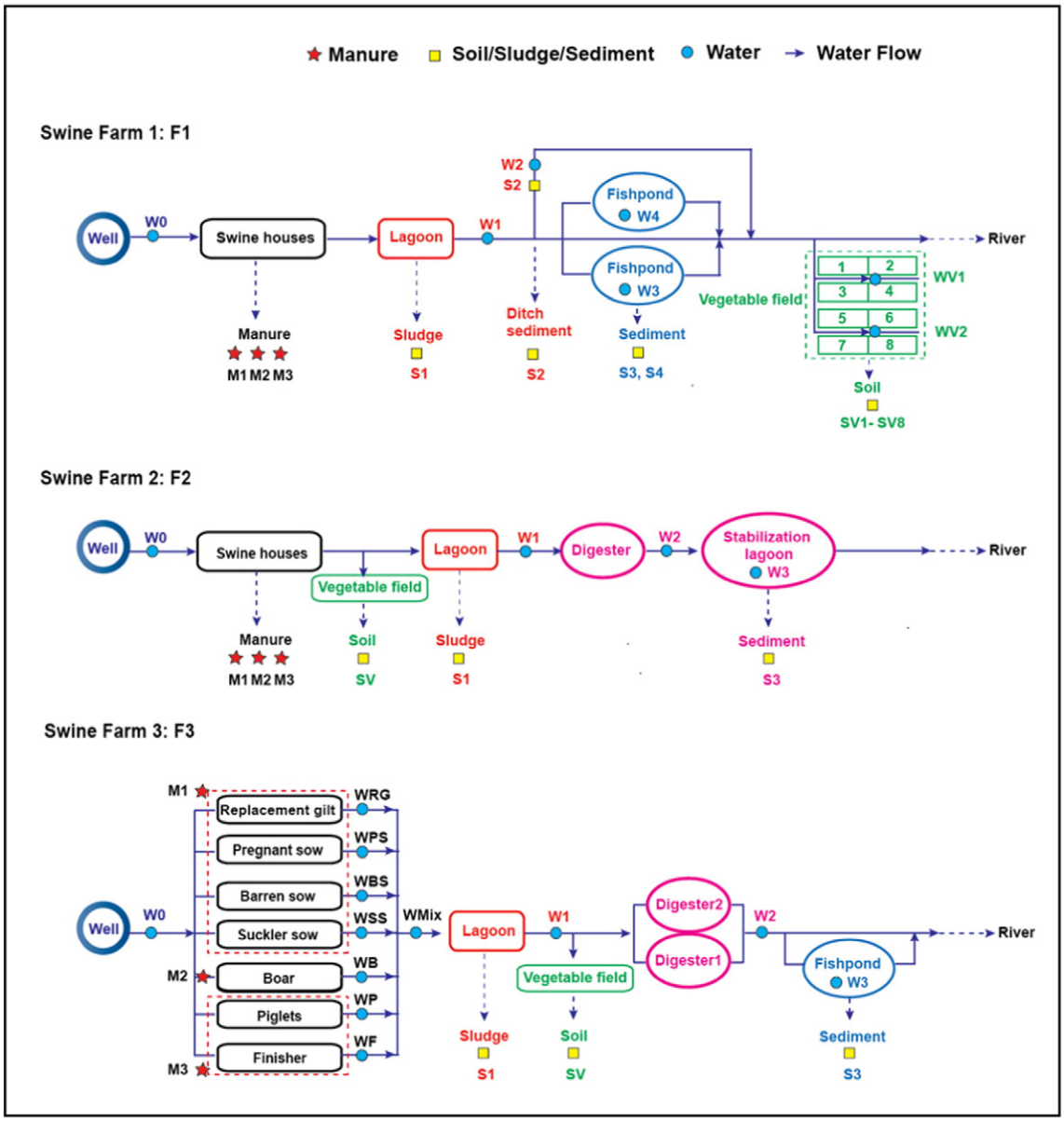

Fig. 1. Schematic illustration of sampling sites and sample types in the three swine farms. Different units of swine farm and sampling sites along the water flow are presented by different symbols in the corresponding colors. Solid arrows in dark blue indicate the water flow started from well water throughout the on-farm wastewater treatment units, and horizontal dotted arrows indicate the final effluent would eventually reach the receiving river. Collected samples are marked by symbols red star (manure), yellow square (soil, sludge and sediment), and sky blue circle (water): W, water samples; WV, irrigation water for vegetable field; WRG, flush wastewater from replace gilt houses; WPS, flush wastewater from pregnant sow houses; WBS, flush wastewater from barren sow houses; WSS, flush wastewater from suckler sow houses; WB, flush wastewater from boar houses; WP, flush wastewater from piglets houses; WF, flush wastewater from finisher houses; WMix, mixed flush wastewater from all swine houses; M, manure; S, lagoon sludge, fishpond sediment; SV, vegetable field soil.

and expected amplicon sizes for all gene targets are listed in Table S1. All qPCR assays were performed on ViiA™ 7 Real-Time PCR System (ABI, USA) using SYBR Green Real Time QPCR Kit (TOYOBO, Japan). The detailed DNA extraction, purification and ARG determination methods can be referred to our previous study (He et al., 2014; Su et al., 2014a, 2014b).

2.6. Chemical extraction and quantification

Nineteen target antibiotics, including eight sulfonamides, five tetracyclines, two chloramphenicols, one lincomycin and three macrolides were analyzed for the collected samples following our previous method (Zhou et al., 2012). Metals (Cr, Mn, Co, Ni, Zn, As, Sr and Cd) and environmental quality parameters (BOD_5, biochemical oxygen demand; COD, chemical oxygen demand; TP, total phosphorus; TN, total nitrogen; NH_3-N, ammonia nitrogen; TOC, total organic carbon) in the samples were also analyzed as we have previously described (He et al., 2014). The analytical results of general water quality parameters and metal concentrations are given in the Supporting information (Tables S2–S4).

2.7. Data analysis

Duncan's multiple range test was used to evaluate the statistical significance of difference between different samples with p-value < 0.05. Frequency of detection (FOD) of ARGs in a sample was calculated as the total number of positive ARGs divided by the total number of targeted ARGs. Pearson correlation analysis by SPSS 13.0 was performed for correlation between ARGs themselves, the correlation of ARGs and class 1 and class 2 integron genes (*int1* and *int2*) in various samples.

The Shannon-Wiener diversity index (H) (Luna et al., 2006) was calculated to evaluate the diversity of ARG composition based on their absolute concentrations.

Multivariate analysis of ARG data was conducted using CANOCO for Windows (Version 4.5) and sample classification was performed by cluster analysis using PC-ORD (Version 5) (Lepš and Šmilauer, 2003; McCune and Mefford, 1999). The objective was to identify the dissemination and diversity of ARGs and linkage to environmental parameters based on the concentration data of ARGs, antibiotics and metals. For multivariate analysis, detrended correspondence analysis (DCA) was used to calculate the length of ARG composition gradients (Lepš and Šmilauer, 2003). Redundancy analysis (RDA) was performed as recommended by DCA, with the absolute concentrations of ARGs as species and the concentrations of antibiotics/metals as environmental variables.

3. Results

3.1. ARGs in the swine wastes

Culture independent qPCR revealed that 19 ARGs were found abundant in aqueous samples, with three genes *sul3*, *cfr* and *tetB/P* being negative of the 22 ARGs analyzed in this study (Fig. 2).

The total absolute concentrations of all detected ARGs appeared to vary within all the swine wastewater samples but exhibited at least 31 times higher than those in well water and fishpond water. As expected, lagoon wastewater (e.g. F2W1 and F1W1) and flush wastewater (e.g. F3WBS, F3WSS and F3WRG) contained more abundant ARGs than most of other wastewater, suggesting they contained various ARB and ARGs from pigs (Chee-Sanford et al., 2001; Wang et al., 2012). Surprisingly, irrigation water (F1WV1) of vegetable field adjacent to swine farm1 was found to have the second highest abundance of total ARGs. The results from swine farm 2 and farm 3 showed reduction in total ARGs absolute concentrations but increased in total relative abundances following lagoon and digester treatment (Fig. S2). As part of swine waste treatment systems, the fishponds in swine farms 1 and 3 were found to have quite low ARGs concentrations (Fig. 2). The average FODs of ARGs in three well waters (F1W0, F2W0 and F3W0), three fishpond waters (F1W3, F1W4 and F3W3) and three lagoon wastewaters (F1W1, F2W1 and F3W1) were 0.91 ± 0.11, 0.84 ± 0.11, 0.96 ± 0.03, respectively (Fig. S3). Meanwhile, the total relative abundances of ARGs in these three types of aqueous samples ranged from 0.74 to 2.88, 2.34 to 4.06, 1.17 to 3.17, respectively. No significant differences of FODs and total relative abundance of ARGs were found among the well waters, lagoon wastewaters and fishpond waters ($p > 0.05$). Considering relative abundance, *tetA*, *ermE*, *tetH* and *sul2* were dominant ARGs in the well waters of the three farms (Fig. S3). *tetA*, *ermE*, *tetG* and *sul1* were dominant ARGs in the three fishpond waters. *tetA*, *tetH*, *sul1* and *sul2* were the most abundant ARGs in the three lagoon wastewaters. In general, *tetA*, *tetH*, *ermE*, *sul2*, *sul1* and *tetG* had relative higher abundances in most of the aqueous samples of the three swine farms,

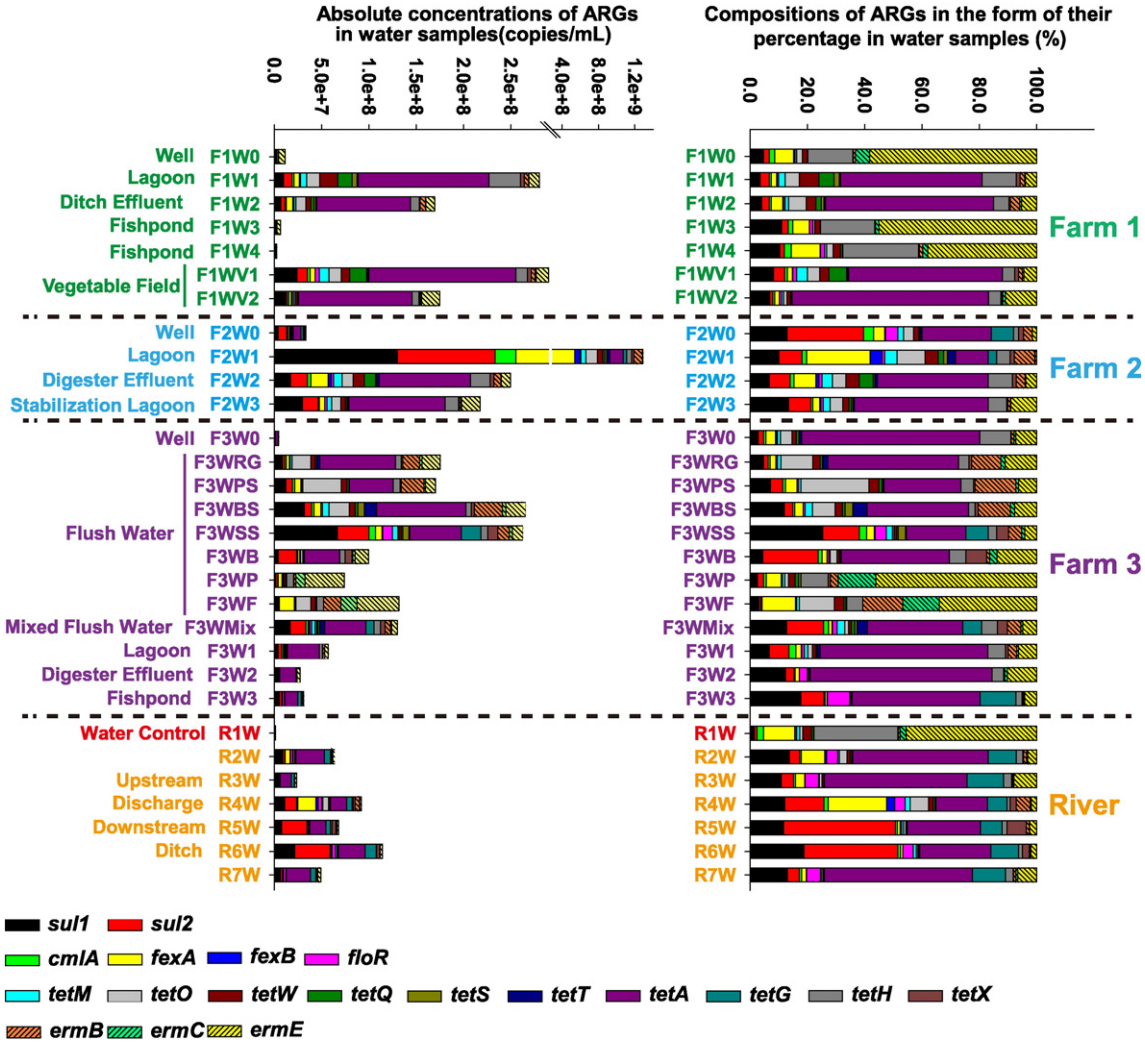

Fig. 2. Occurrence and contamination profiles of ARGs in aqueous samples from the three swine farms and receiving river. Samples are presented in different colors: green (farm 1), sky blue (farm2), purple (farm3) and yellow (the receiving river).

followed by *fexA*, *ermB*, *tetO* and *tetW*, indicating their dominance and persistence in swine feedlots. Despite the variation of total absolute concentrations of ARGs between different samples, the compositions of ARGs exhibited similar in wastewater samples within the same farm (Fig. 2).

For solid samples (manure and lagoon sludge) from the three swine farms, various ARGs were detected at high abundance with all of the 22 ARGs being positively detected in most of these samples (Fig. 3). The FODs of the 22 ARGs in manure/lagoon sludge samples (n = 14) and sediments of fishpond (n = 3) ranged from 0.77 to 1.00, 0.86 to 0.91, respectively (Fig. S4). Most FODs of the ARGs were 1.00 in manure/lagoon sludge samples, except for *cfr* (0.79), *tetB/P* (0.57), *tetG* (0.36), *tetT* (0.43) and *tetS* (0.93). Most FODs of the ARGs were 1.00 in sediments of the fishponds, except for *cfr* (negative), *tetT* (negative) and *tetB/P* (0.33) (Fig. S4). ARGs were prevalent at the highest concentrations in manure of different groups of pigs including sow, boar, piglets and finisher, and they remained at high levels in lagoon or ditch sludge. Among the detected ARGs, *tetO*, *fexA*, *ermB*, *tetW* and *cmlA* accounted for large proportions in most manure and lagoon sludge samples from the three farms, followed by *fexB*, *tetA*, *tetQ*, *cfr*, *sul1* and *sul3*. Although total concentrations and proportions of ARGs varied among these solid wastes from different swine farms, *sul* genes, *cml* genes and MLS_B genes were generally 100% detected (Fig. 3).

3.2. ARGs in the receiving environment

Due to the discharge of swine wastewaters from swine farm 2 and 3, the receiving Longkou River was found to have much higher levels of ARGs than its control water (reservoir water in the upstream, R1W) (Fig. 2 and Fig. S2). It should be noted that a higher total concentration of ARGs was observed in the effluent ditch (R6W) than in its discharge point and the downstream site. The compositions of ARGs in the affected river section (R2W, R3W, R4W, R5W, and R7W) were very different to that of the control water, but similar to those of the swine wastewaters, suggesting the influence of swine wastewater discharge and spread of ARGs from the swine farms. Moreover, the compositions of ARGs in the river sediments (R2S, R3S, R4S, R5S, R7S) were also different to that of the control, but similar to that of the effluent ditch sediment (Fig. 3). Interestingly, the FODs of ARGs in receiving river waters ranged from 0.95 to 1.00, as high as those in the lagoon wastewaters (Fig. S3). And average relative abundance of ARGs in the river waters was

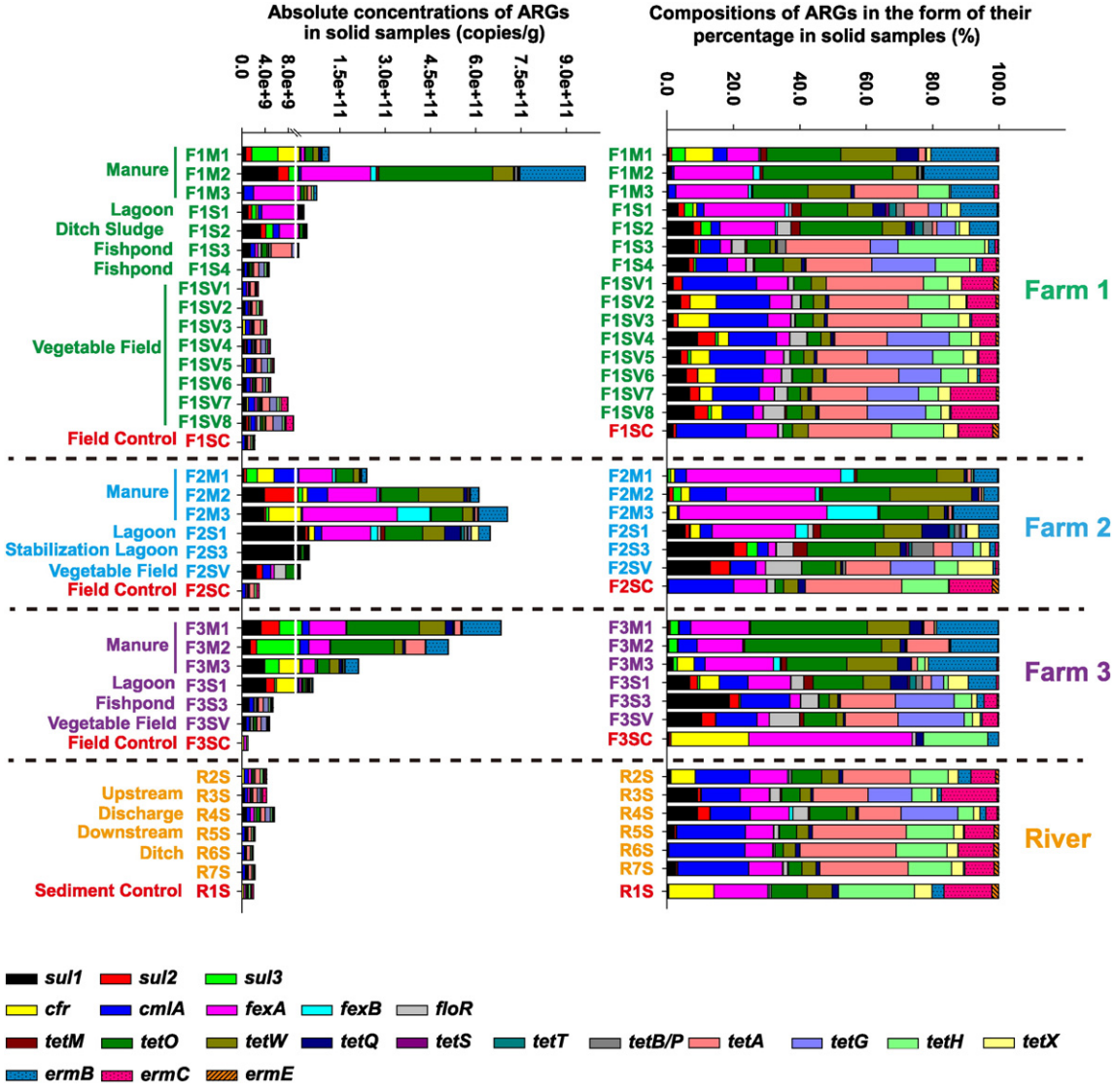

Fig. 3. Occurrence and contamination profiles of ARGs in solid samples from the three swine farms and receiving river.

3.05 ± 1.70, slightly higher than the lagoon wastewaters (2.18 ± 1.00), but not significantly ($p > 0.05$). However, FODs and the total relative abundance of ARGs in the control water were 0.84 and 1.09. The FODs of ARGs in the sediments of affected river section ranged from 0.77 to 0.86, while it was 0.68 in the control sediment (R1S). ARGs in the receiving river seemed to become more diverse in comparison with the control reservoir water, and the ARGs in the swine wastewaters were also more diverse than the well water in swine farm 1 and 3 as shown by the Shannon-Wiener diversity index (Fig. S5).

Total ARG concentrations were found slightly higher in the ten swine waste-applied soils compared to the three control soils (Fig. 3), but not significantly ($p = 0.13$). However, the FODs of the 22 ARGs and the Shannon-Wiener diversity index in the ten vegetables soils were significantly higher than those in the three control soils ($p < 0.005$) (Fig. S4), suggesting the ARG composition patterns in the swine waste-applied soils were also very different to those of the respective control soils without application of swine waste.

3.3. ARGs in the vegetables

ARGs were frequently found in the ready consumption vegetables, varied in abundance per cell of bacteria, but consistent in occurrence with their soils (Fig. 4, Fig. S9, and Fig. S10). But the reference vegetables contained less ARGs than those vegetables irrigated with wastewater (Fig. 4). In the vegetables collected in 2013 from the research fields applied with swine wastes of farm 1 and farm 2, most ARGs were positive except for *sul3*, *cfr*, *tetT* and *tetB/P* (Fig. S9). Relative abundance and composition of ARGs showed that *ermE*, *ermC*, *tetA*, *tetH*, *floR*, *fexA* and *sul1* were dominant in these vegetables. However, in the vegetables collected in 2015, *ermE*, *tetC* and *fexA* were not detected, while *sul3*, *tetT* and *tetB/P* were positive; *sul1*, *cm1A*, *floR*, *tetA* and *tetX* were dominant ARGs considering relative abundance (Fig. 4). Interestingly, these dominant ARGs were also highly detected in the vegetable field soils and the swine wastewaters, suggesting their potential origin from the swine wastewater irrigated in the vegetable fields. As is expected, when compared to those washed vegetables, the unwashed vegetables contained more abundant ARGs in terms of absolute concentration (Fig. S10).

It should be noted that the surface soil from the treated vegetable plots (SV-A, 0–20 cm depth) was found to carry more diverse and abundant ARGs when compared to those reference soils (R1SV and R2SV). Although most ARGs decreased in absolute concentrations (copies/dry weight) from surface layer to the substratum layer (three layer of soil profile from the research field: SV-A, SV-B and SV-C) (Fig. S10), their relative abundances increased in the soil profiles (Fig. 4). To the best of our knowledge, few studies have reported the variation of ARGs in vertical soil profiles of vegetable plots irrigated with swine wastewater.

3.4. Antibiotics in the swine farm environments

Eighteen antibiotics were detected in the swine manure and wastewater, and environmental samples (Fig. S6). The detected antibiotics were: eight sulfonamides and diaminopyrimidines (SG: sulfaguanidine, SDZ: sulfadiazine, SMZ: sulfamethazine, SMM: sulfamonomethoxine, SCP: sulfachlorpyridazine, SMX: sulfamethoxazole, SQX: sulfaquinoxaline and TMP: trimethoprim), five tetracyclines (OTC: oxytetracycline, TC: tetracycline, CTC: chlortetracycline, MT: methacycline and DC: doxycycline), one chloramphenicols (FF: florfenicol), three macrolides (ETM-H_2O: erythromycin-H_2O, LCM: leucomycin and TYL: tylosin) and one lincosamides (LIN: lincomycin). In comparison with those control samples, higher levels of these antibiotics were observed in the aqueous and solid samples associated with swine wastes. The antibiotics LIN, LCM, TYL, DC, CTC, TC, OTC, TMP, SMM and SMZ were found with relatively high concentrations in various swine wastewater as reported previously

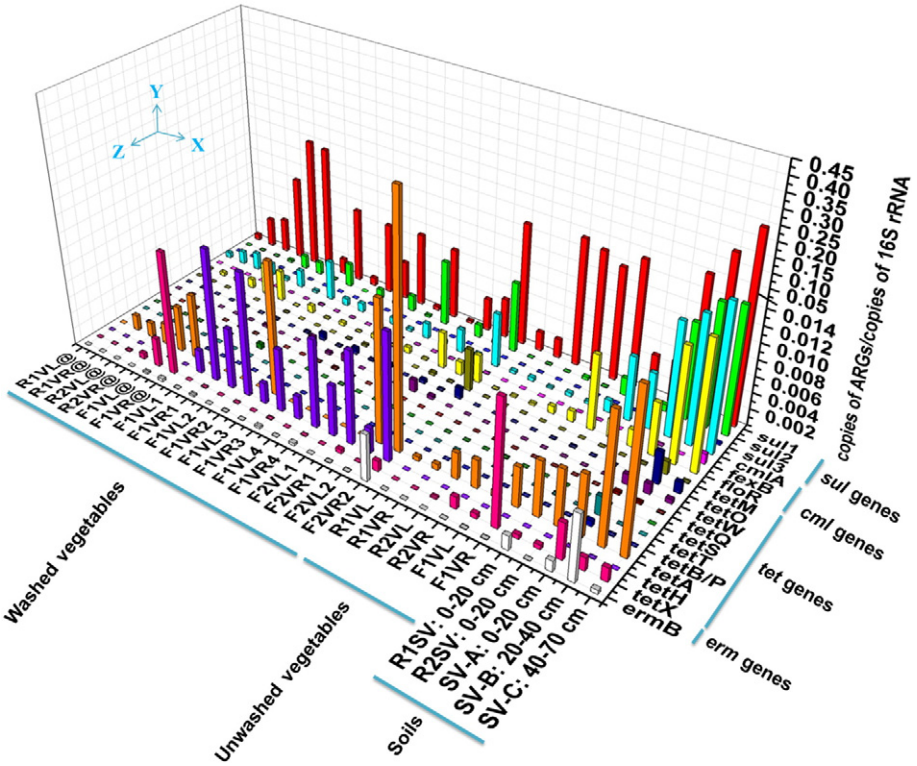

Fig. 4. ARG profiles in vegetables (wet weight) and soils (dry weight). Each panel represents one of the positively quantified ARGs. For comparison, those positives in the vegetables sampled in 2013 but negatives in those sampled in 2015 were not shown on this graph. The Y axis shows relative abundance of ARGs (Z axis) in vegetables and in soils (X axis). A break at 50% of axis length is injected on Y axis (from 0.015). R: reference spring onion field; S-A, S-B and S-C: vertical soil profiles of vegetable field. VL: vegetable leaves; VR: vegetable roots; those suffix with @ and those sampled in 2013 (F1VL1–F2VR2) were prior processing with sterile Milli-Q water to remove excess soil to achieve a visual cleanliness before shaking in 0.85% sterile physiological saline (washed vegetables).

(Zhou et al., 2012). CTC showed the highest concentrations in swine manure, lagoon sludge and fishpond sediment and vegetable field soils, followed by TC, OTC and LCM. CTC was detected at concentrations up to 1,030,000 ng/g dw in manure (F2M3) and 35,000 ng/L in swine house flush wastewater (F3WP). In the sediments of Longkou River, tetracyclines CTC, OTC, DC and TC were the dominant antibiotics, with concentrations ranging from 17.1 ng/g dw (DC) to 1170 ng/g dw (CTC).

The total concentrations of antibiotics decreased dramatically from the flush water to digester effluent in the swine farm 3, but not in swine farm 2 due to the malfunction as demonstrated by general wastewater quality parameters (Fig. S6). Antibiotics were detected in the final effluents of the three swine farms with variable concentrations. This resulted in the detection of various antibiotics in the receiving Longkou River. A higher total concentration of antibiotics was observed at the effluent discharge point (R4W) than at the other river sites.

It is worth noting that several antibiotics (SDZ, SM, SMM, TMP, CTC and LCM) were even found in vegetables from the fields applied with swine wastes (Table S5). CTC was detected at the highest concentration in leaves and roots of spring onions and water spinach at the concentration range of 16.9 ± 2.95 ng/g to 199 ± 6.08 ng/g dw, followed by SM in roots of spring onion and water spinach (2.73–5.41 ng/g dw), LCM in leaves of spring onion (3.14 ng/g dw), and TMP in leaves of water spinach (0.86 ng/g dw). SDZ was positively detected, but at concentrations blow its method quantification limit.

4. Discussion

4.1. Dissemination of ARGs from swine farms to the receiving environments

The results from this study showed the occurrence of various ARGs in swine manure and wastewater from the three swine farms. Despite some reduction in the total concentrations of ARGs from raw wastewater to final effluent in the swine waste treatment systems, the ARGs were disseminated into the receiving environments via application of manure-wastewater on land or discharge of wastewater into the receiving river.

In large swine farms, animals generate huge amounts of animal wastes, which contain high levels of organic substances, nutrients and diverse microorganisms. On-farm waste treatment systems such as lagoon and digester are designed to reduce these traditional contaminants such as nitrogen and phosphorus, but not the emerging contaminants like ARGs (Pruden et al., 2006). In the present study, *sul* genes, *cml* genes, *tet* genes and *erm* genes were partially removed mostly in aqueous phase by the on-farm lagoon and digester treatment units (Fig. 2), which is consistent with the results reported in Taiwan (Tao et al., 2014). However, specific resistance genes behaved differently in the treatment systems. Some genes *fexA*, *fexB*, *floR*, *sul1*, *tetA*, *ermB*, *ermC* and *ermE* were found to be recalcitrant to the swine waste treatment processes, consistent with previous reports in a sewage treatment plant (Yang et al., 2014), laboratory-scale treatment systems (Zhang et al., 2013; Ma et al., 2011) and in other confined animal feeding operations (Wang et al., 2012; Chen et al., 2010). This result also explains the concomitant dominance of these resistance genes in the receiving environments due to animal waste discharge.

Two-way cluster analysis on ARG data set revealed interesting grouping for the aqueous samples (Fig. S7) and solid samples (Fig. S8). As revealed in Fig. S7, irrigation water was grouped with the wastewater from the swine farm 1, while the receiving river water grouped with the wastewater from the swine farm 3, suggesting the influence from this swine farm. For the solid samples, it was found that the river sediments, fishpond sediments and vegetable field soils were grouped far away from their respective controls (Fig. S8), suggesting they were contaminated by ARGs from the swine farms.

RDA diagrams in Fig. 5 showed the positive linkage of the ARGs in both aqueous and solid samples to the environmental variables (antibiotics and metals) (correlation coefficient $R > 0.9$). This indicates that the environmental variables influenced the development and dissemination of ARGs from the swine farms to the receiving environments. In fact, our previous studies have demonstrated that soil-bound antibiotics could still exert selective pressure on soil bacteria (Peng et al., 2014, 2015). Moreover, the ARGs included in the present study are mostly located on mobile genetic elements, so genetic linkage or co-selection may facilitate their dissemination as previously indicated (He et al., 2014). Class 1 and class 2 integron genes (*int1* and *int2*), defined as an indicator of horizontal gene transfer potential, showed good correlation with various ARGs (Fig. S7 and Fig. S8). Thus, multiple factors could contribute to the spread of ARGs under the influence of swine feedlots.

The first gradient in the two diagrams of RDA explained 56.8% and 39% of the total ARG variability for the aqueous and solid samples, respectively. The aqueous samples displayed a separation along the first axis as the levels of ARGs increased (from right to left on diagram) (Fig. 5a), while manure and lagoon sludge samples were respectively grouped together on the right side of the diagram and separated along the second axis (Fig. 5b). The RDA displays clearly how the ARG diversity increase as the ARG levels increase in both aqueous and solid samples.

It should be noted that swine manure contained the highest level of ARGs but they had lower ARG diversity when compared to the swine wastes-applied soils (Fig. 5b), indicating that swine wastes may have enhanced the development of ARGs in the receiving soils from an ecological view. This is consistent with a previous study on bloom of resident antibiotic-resistant bacteria in soil following manure fertilization (Udikovic-Kolic et al., 2014). In the receiving river, higher ARG level and diversity was noted at the effluent discharge point than at other river sites, suggesting significant direct impact of swine wastewater on the receiving river at the discharge point. Thus the discharge of swine wastes could affect surface water quality and soil microbial ecology.

4.2. Environmental impact of swine feedlots

Since manure or wastewater constitutes the largest source of ARGs originating from swine feeding operations (Jindal et al., 2006), some studies attempted to evaluate on-farm waste treatment systems in reducing antibiotics, antibiotic resistance bacteria or ARGs (Wang et al., 2012; Jindal et al., 2006; Brooks et al., 2014; Chen et al., 2010; Tao et al., 2014; Huang et al., 2014; Liu et al., 2014; Chen et al., 2012). The efficiency of different treatment technologies varied in reducing different types of ARGs. However, the reduction in resistance levels worked in the range of percentages but ARGs may persist for many years without selection pressure of the corresponding antibiotics (Johnsen et al., 2009). In the present study, we observed not only the dissemination of ARGs from the source (swine farms) to the receiving environment (vegetable fields and receiving river), but also their remarkable increase in diversity. On-farm lagoon and anaerobic digester treatment could reduce the ARGs in total absolute concentration but failed to remove them. Obviously, discharge of animal manures or common on-farm treated wastes could result in contamination of ARGs as well as antibiotics in the receiving environments (especially the river water and food). In particular, ARGs and some antibiotics were abundantly quantified in the vegetables grown in swine wastes-applied fields. Moreover, this study showed that common cleanliness of vegetables with tapwater used in food preparation failed to remove ARGs to their background level (copies per cell of bacteria) as found in the reference site. Potential human exposure to antibiotic resistance determinants and antibiotic residues via eating vegetables and drinking surface water as well as other exposure pathways should not be ignored (Boonsaner and Hawker, 2015; Kim and Aga, 2007). Evidence also indicates that the use of antibiotics in food animals is associated with antibiotic resistance among bacteria isolated from humans (Angulo et al., 2004; Smith et al., 2002; van den Bogaard and Stobberingh, 2000). Further research on the

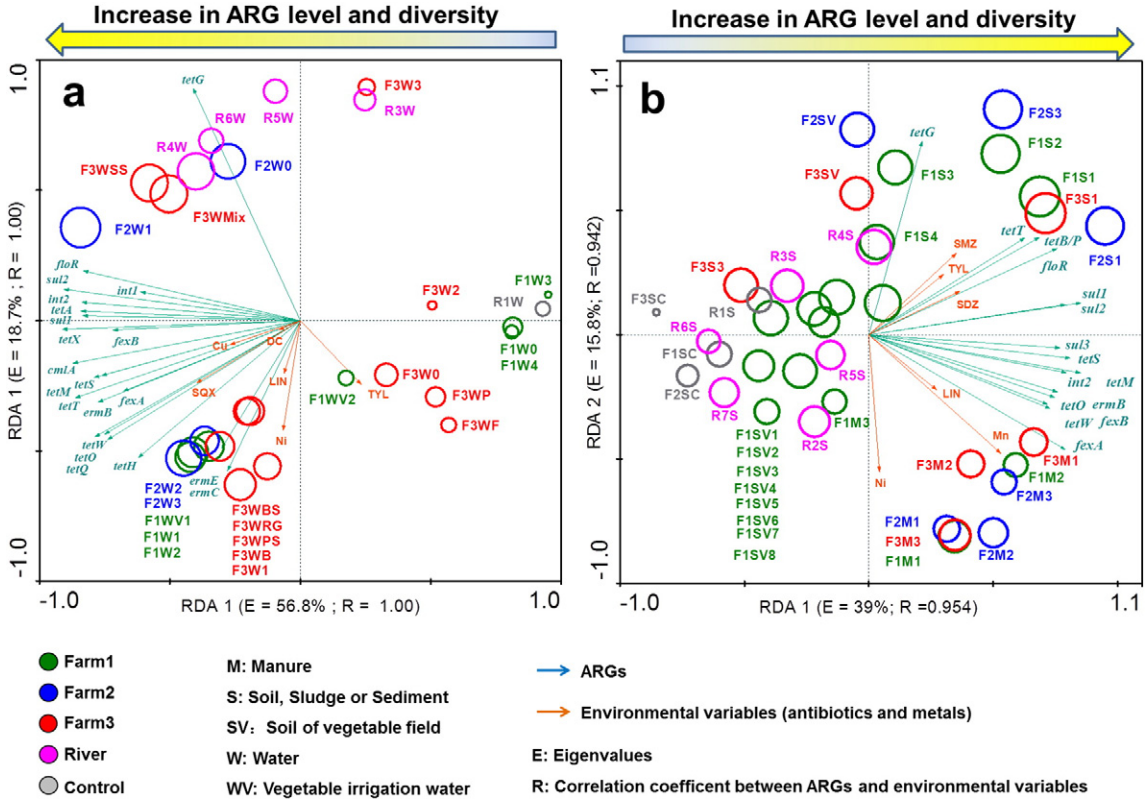

Fig. 5. Redundancy analysis (RDA) on the diversity of antibiotic resistance genes (ARGs) in the aqueous samples (a; N = 28) and solid samples (b; N = 37) based on environmental variable scores (antibiotics and metals), with the size of the site symbols corresponding to Shannon-Wiener diversity of ARGs. Environmental variables in orange arrows are chosen according to the significance ($p < 0.1$) calculated from the forward selection procedure. The lengths of the arrows reveal the strength of the relationship and the intersection angle between the arrows can express the correlation. The percentage of variation explained by each axis is shown, and the relationship is significant ($p < 0.01$).

adverse human health effects of antibiotics and ARGs in food and water are warranted.

The causes shaping the emergence and diversification of ARGs under the influence of swine feedlots could be complex. In terms of biological evolution, for one reason, soil and water habitats host an impressive bacterial diversity (Vaz-Moreira et al., 2014; Daniel, 2005). When resistant bacteria and ARGs are introduced into the receiving soils by animal wastes application, mobile genetic elements carrying ARGs or naked ARGs could transfer to indigenous bacteria or other habitats. In this way, antibiotic resistance could support their environmental dissemination independent of their original host (Gotz and Smalla, 1997; Heuer et al., 2011). For another reason, fitness cost imposed by acquiring ARGs and mobile genetic elements are likely to be ameliorated by subsequent evolution (Andersson and Levin, 1999; Andersson and Hughes, 2010). On the other hand, our observation indicates that the high input of ARGs and selective agents (antibiotics and metals) with animal wastes could well contribute to increased resistance level and diversity in the receiving environments, as previously discussed (Heuer et al., 2011).

As observed in this study, along the vertical soil profiles, ARGs were enriched per cell bacteria, suggesting the migration of ARGs. Thus, it is noteworthy to verify environmental impact of livestock feedlots at both horizontal (e.g. surface soil) and vertical levels (e.g. geographic gradients) (Baquero et al., 2015). Also, further studies are still needed to connect the diversity and variation of ARGs and the host bacteria and to shed light on the resistome of both pristine and anthropogenic impacted environments. Although antibiotic resistance is a natural phenomenon (Allen et al., 2009; D'Costa et al., 2011), the genes that make up environmental antibiotic resistome (D'Costa et al., 2007) were found to be shared between environmental and pathogenic bacteria (Forsberg et al., 2012; Spanogiannopoulos et al., 2014). Moreover, they have the potential to be transferred to pathogens (Wright, 2010). It remains a challenge to determine to what extent the ARGs should be removed before field application of animal wastes and to incorporate these to quantitative models as a basis for risk assessment and regulations.

5. Conclusions

This study showed that most ARGs and antibiotics survived the common on-farm waste treatment systems and were prevalent in the receiving environments including fishponds, rivers and vegetable fields. Discharge of swine wastewater led to increased abundance and diversity of ARGs in the receiving environments, which could affect microbial ecology in the surface water and soils. Antibiotics and metals in swine wastes may also enhance the development of bacterial resistance in the impacted environments. It is worth noting that vegetables from the plots irrigated with swine wastewater were found to contain various antibiotics and ARGs. Human exposure to antibiotic resistance determinants and antibiotic residues via eating vegetables and other exposure pathways should be considered when we assess the potential human health risks associated with swine waste discharge.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.envint.2016.03.023.

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