



Occurrence of antimicrobials and antimicrobial resistance genes in beef cattle storage ponds and swine treatment lagoons



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HIGHLIGHTS

- Partitioning of antimicrobials between water and sludge is compound specific.
- Antimicrobial resistance genes occurred in both water and sludge.
- The ARG abundance varied more substantially in swine lagoons than in cattle ponds.
- Correlations between ARGs and antimicrobials are system dependent.

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ABSTRACT

Livestock manure treatment and storage structures are potential environmental sources of antimicrobials and antimicrobial resistance genes (ARGs). In this study, the occurrence of antimicrobials and ARGs was investigated in the water and the sludge compartments of beef cattle storage ponds and swine lagoons. Analysis was focused on two families of antimicrobials (sulfonamide and tetracycline) and the corresponding ARGs (*sul1*, *sul2*, *tetO*, *tetQ* and *tetX*). Results showed that the pseudo-partitioning coefficients of tetracyclines were higher than those of sulfonamides, suggesting different distributions of these two classes of antimicrobials between water and sludge. The ARGs tested were detected in nearly all ponds and lagoons, with the highest relative abundance in *sul2* at 6.3×10^{-1} copies per 16S rRNA gene. A positive correlation was observed between total *sul* genes and total sulfonamides in water while the correlation was negative in sludge. No significant correlation was found between total *tet* genes and total tetracyclines in either water or sludge, but significant correlations were observed for certain individual *tet* genes. Ammonia concentrations strongly correlated with all ARGs except *tetX*. This study provided quantitative information on the occurrence of antimicrobials and ARGs in the liquid and solid compartments of typical manure treatment and storage structures.

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1. Introduction

Antimicrobials are broadly used in the livestock industry to prevent and treat diseases and to promote growth. However, 17–80% of the antimicrobials administered to animals are not adsorbed and are excreted through urine and feces (Halling-Sorensen et al., 1998; Montforts et al., 1999). Bacteria exposed to antimicrobials in the gut of animals may develop antimicrobial resistance (Sarmah et al., 2006). Antimicrobial

resistance is conferred by antimicrobial resistance genes (ARGs), which usually reside on mobile genetic elements such as plasmids, integrons, and transposons (Allen et al., 2010). Because ARGs may be transferred to human pathogens, they are considered a class of contaminants with emerging concerns (Pruden et al., 2006; Shah et al., 2012).

Livestock facilities have been recognized as potential sources of antimicrobials and ARGs in the environment (Koike et al., 2007; Peak et al., 2007). Multiple classes of antimicrobials, including tetracyclines and sulfonamides, were detected in groundwater and surface water adjacent to swine and poultry farms (Campagnolo et al., 2002). In the river sediment downstream from livestock facilities, the concentrations of sulfonamide and tetracycline compounds were 3.5 µg/L and 86.4 µg/L during low-flow sampling events (Pei et al., 2006). In the same study, sulfonamide and tetracycline resistance genes (*sul2* and *tetW*) were detected at the levels of 10^{-7} – 10^{-5} copies per copy of the 16S rRNA gene. Tetracycline resistance genes and erythromycin resistance genes

Abbreviation: ARG, Antibiotic resistance gene.

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were also repeatedly detected over a 3-year period in groundwater downstream of swine lagoons (Koike et al., 2007, 2010).

Storage ponds and treatment lagoons are commonly-used manure management structures at beef cattle and swine facilities. Similar to municipal wastewater treatment plants (Zhang et al., 2009), these manure management structures often contain high levels of antimicrobials and ARGs (Campagnolo et al., 2002; McKinney et al., 2010). In one study, nine antimicrobials were detected at concentrations that ranged between 0.62 and 32.67 µg/L in a swine lagoon in China (Ben et al., 2008). In another study, 1000 µg/L chlortetracycline, 400 µg/L sulfamethazine and 240 µg/L lincomycin, were reported in swine lagoon wastewaters in the U.S. (Campagnolo et al., 2002). Efforts were made to correlate the level of antimicrobials and that of the corresponding ARGs. Smith and colleagues detected an average of 1.95 µg/L tetracycline and approximately 10^4 – 10^7 copies/mL of total tetracycline resistance genes (*tetO*, *tetW*, and *tetQ*) in cattle feedlot lagoons (Smith et al., 2004). Peak and colleagues reported 0.45–16.40 µg/L tetracycline and 10^3 – 10^6 copies/mL of total tetracycline resistance genes (*tetO*, *tetQ*, *tetW*, *tetM*, *tetB* and *tetL*) in cattle feedlot lagoons (Peak et al., 2007).

Most of the published studies were focused on either the water or the sludge compartment of waste management structures. However, it is important to investigate both compartments simultaneously because different antimicrobials behave differently in liquid–solid systems. For example, some antimicrobials tend to adsorb to solids, while others tend to occur in water (Sarmah et al., 2006). Furthermore, water and sludge in storage ponds and treatment lagoons are managed differently. Lagoon wastewater is often pumped out for irrigation at least once a year (Bodman, 1996), while lagoon sludge is removed every 5 to 20 years and land-applied to soil (Hamilton et al., 2006). Contents in storage ponds are removed every six months or more (Cooperative Extension System, 2008). Studies that investigate antimicrobials and ARGs in both the water and sludge of waste management structures are lacking in the literature.

The objective of this study was to investigate the occurrence of antimicrobials and ARGs in both the water and the sludge compartments of typical manure management structures and identify bulk water quality parameters that are potentially linked to the occurrence of ARGs. To achieve the objective, water and sludge samples were collected from four beef cattle storage ponds and three swine treatment lagoons. Sixteen antimicrobials, including members of the tetracycline, sulfonamide, and macrolide families, were quantified using liquid tandem mass spectrometry (LC–MS/MS). Multiple ARGs (i.e., *sul1*, *sul2*, *tetO*, *tetQ* and *tetX*) were measured using quantitative PCR (qPCR). Several routinely-used wastewater quality parameters were also measured and statistically analyzed to assess their correlation with ARGs.

2. Materials and methods

2.1. Sampling and on site measurements

Four sequential storage ponds holding manure wastes and runoff from a beef cattle feedlot (C1–C4) and three sequential swine wastewater treatment lagoons (S1–S3) at the USDA Meat Animal Research Center (Clay Center, NE) were included in this study (detailed description on the facilities can be found in Supporting information). Samples were collected in September 2010 and June 2011. Surface water samples were collected by submerging 1-L amber glass bottles (for chemical analyses) and 1-L polyethylene bottles (for microbial analyses) ~10 cm below the water surface. If water was deeper than 1.2 m (Table S-1), a bottom water sample was collected ~10 cm above the sediment to access the effect of stratification. Sludge samples were collected using an Ekman dredge sampler and then transferred to sterile plastic bags and amber jars. All water ($n = 22$) and sludge ($n = 14$) samples were collected from the center of the ponds and lagoons. Water temperature, pH, dissolved oxygen (DO), oxidation–reduction potential (ORP) and electrical conductivity were measured on site using field probes (YSI

Professional Plus Multiparameter, Yellow Springs, OH). Samples were transported to the laboratory on ice within 6 h of collection. Subsamples were stored at 4 °C for routine chemical analyses and at –20 °C for antimicrobial and DNA analyses.

2.2. Water quality analysis

The chemical oxygen demand (COD) of water samples was measured using high range COD digestion vials (Hach, Loveland, CO) according to the reactor digestion method (Jirka and Carter, 1975) which is approved by the US Environmental Protection Agency (EPA). Ammonia-nitrogen ($\text{NH}_3\text{-N}$), nitrate/nitrite-nitrogen ($\text{NO}_2^-/\text{NO}_3^- \text{-N}$), and total phosphorus (TP) were measured using an AQ2 Automated Discrete Analyzer (Seal Analytical, Mequon, WI) according to EPA Method 350.1 (EPA, 1993b) and 365.1 (EPA, 1993c), respectively. Dissolved copper (Cu) and zinc (Zn) were measured using inductively coupled plasma-mass spectrometry (ICP-MS) (GV Instruments Ltd., Manchester, UK) according to EPA Method 6020A (EPA, 2007). Orthophosphate-phosphorous (ortho-P) and sulfate were measured using ion chromatography according to EPA Method 300.0 (EPA, 1993a). The removal efficiency of a contaminant in the sequential ponds or the sequential lagoons was defined based on the concentration difference between the first and the last pond/lagoon.

2.3. Antimicrobial analysis

Antimicrobial concentrations in water and sludge samples were analyzed using on-line LC–MS/MS with electrospray ionization (Bartelt-Hunt et al., 2011; Snow et al., 2003). 0.5–5 mL of water sample was syringe-filtered (0.45 µm Whatman glass fiber GDX), weighed directly into a 40-mL vial, spiked with internal standards and surrogates, and thoroughly mixed with 20 mL reagent water and 500 µL 2.4 M citric acid. Analytes, internal standards and surrogates are listed in Table S-2. Calibration standards were prepared by fortifying 2.4 M citric acid with analytes (10 to 1000 ng/L) and treated in an identical manner as samples. During analysis each solution was extracted with a Spark Holland Symbiosis on-line solid extraction system using a Waters (1 × 2 mm) HLB solid-phase extraction (SPE) cartridge and then eluted with mobile phase for subsequent separation and detection. Target analytes were detected in the mass spectrometer using SRM (selected reaction monitoring) MS/MS analysis on a Waters Quattro Micro triple quadrupole mass spectrometer. A Thermo HyPurity C18 5 µm 2 × 250 mm column provided separation with a mobile phase comprised of 97:3 water/methanol and 3:97 methanol/water each containing 0.1% (v/v) formic acid. LC–MS/MS conditions and transitions were determined and optimized by infusing with concentrated standards. A capillary voltage of 4.0 kV, an extractor of 3 V and an RF lens of 0.1 V were used. The source temperature was 120 °C and the desolvation temperature was 500 °C. The nebulizer flow rate was 700 L/h in the desolvator and 30 L/h in the cone.

Solid samples (1–5 gr) were weighed in 50 mL Teflon centrifuge tubes and mixed with 20 mL of 5 mmol ammonium citrate/methanol (pH = 6) loaded onto a Burrell Wrist-Action Shaker and equilibrated for 30 min. The mixture was centrifuged for 10 min and the supernate decanted into a Labconco RapidVap™ evaporation tube. The process was repeated with 20 mL of citrate/methanol mixture followed by extraction with acetone. All extracts were combined, evaporated at 25 °C to approximately 20 mL, mixed with reagent water to a final volume of 100 mL, and cleaned using a 200 mg Waters Oasis HLB SPE cartridge followed by elution with 2.5 mL 0.5% (v/v) formic acid in methanol. Purified extracts were evaporated under nitrogen to a final volume of 200 µL, transferred to an autosampler insert, and then analyzed using similar conditions as the water samples using a 10 µL injection volume. Chlortetracycline concentration includes isochlortetracycline, epichlortetracycline and chlortetracycline, while the tetracycline transition includes tetracycline and epitetracycline.

To quantify the distribution of the antimicrobials between water and sludge, partitioning coefficient K_d was used in this study and was calculated using the following equation (Midwood et al., 1998).

$$K_d = \frac{\text{Concentration in sediment } (\mu\text{g/kg})}{\text{Concentration in water } (\mu\text{g/L})}$$

Since adsorption is not the only process that determined the antimicrobial distribution in these waste management structures, the calculated partitioning coefficient is termed pseudo-partitioning coefficient (Kim and Carlson, 2007).

2.4. qPCR standards

Escherichia coli cultures containing various *tet* genes were obtained from Dr. Lisa Durso of the USDA Agricultural Research Service. Standards for *sul* genes were PCR-amplified from the activated sludge of a local wastewater treatment plant. Primer sequences and annealing temperatures for all regular PCR reactions are listed in Table S-3. PCR products were then cloned using an Invitrogen TOPO TA Cloning® Kit (Carlsbad, CA), and the plasmids were extracted and purified using a QIAGEN® Plasmid Kit (Valencia, CA). The plasmids containing *sul* genes were further confirmed by sequencing at Eurofins MWG Operon (Huntsville, AL). The copy number of genes in purified plasmid DNA was calculated as previously described (Pei et al., 2006), and the qPCR standards covered 10^1 to 10^9 copies/ μL .

2.5. DNA extraction and qPCR assays

Cells in water samples were collected by centrifuging 50 mL of lagoon water at 5000 $\times g$ for 10 min at 4 °C. DNA was then extracted from cell pellets or sludge samples using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH) and was quantified using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). qPCR was performed on a Mastercycler ep realplex thermocycler (Eppendorf, Hamburg, Germany). Each 20- μL qPCR reaction contained 9 μL of 2.5 \times RealMasterMix SYBR ROX (5 Prime; Gaithersburg, MD) and 4 ng DNA. The final concentrations were 0.2 μM of each primer for *sul1*, *sul2*, *tetO* and *tetX*, 0.25 μM for *tetD*, 0.5 μM for *tetE* and 0.4 μM for *tetQ*. Primer sequences, annealing temperatures and references for qPCR on ARGs and the 16S rRNA gene are listed in Table S-4. All samples were quantified in duplicates. Sulfonamide ARGs *sul1*, *sul2* and *sul3* encode alternative variants of the DHPS enzymes, which are targets of sulfonamides (Skold, 2000). *sul3* was not included in this study because it does not occur often in livestock wastes (Guerra et al., 2004; Sharma et al., 2008). Tetracycline resistance genes are categorized into three groups according to the resistance mechanism: efflux pump, ribosomal protection and enzymatic inactivation (Roberts, 2005). Representative *tet* genes from each category (*tetD* and *tetE* for efflux pumps, *tetO* and *tetQ* for ribosomal protection, and *tetX* for enzymatic inactivation) were selected in this study because of their occurrence in animal-related environments (Aminov et al., 2002; Patterson et al., 2007; Peak et al., 2007; Storteboom et al., 2010).

2.6. Validation of DNA extraction and qPCR

To assess the efficiencies of DNA extraction and PCR amplification, known amounts of *E. coli* cells containing individual ARGs were spiked into various types of samples, i.e., surface water in C1 (C1-S), bottom water in S2 (S2-B), sludge of B1 and S2 from first sampling event, following procedures reported by Koike et al. (2007). The DNA of pure *E. coli*, non-spiked samples and spiked samples was extracted as described above. qPCR was conducted and recovery was calculated as (ARG copy number in spiked sample – ARG copy number in non-spiked sample)/ARG copy number in the *E. coli* spiked.

2.7. Data analysis

All statistical tests were performed using R2.13.1 (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was defined as $p \leq 0.05$. Two tailed *t*-test was used to compare the difference between two groups, and Fisher's protected least significant difference (LSD) test was used to compare the differences among three or more groups. Correlation analysis was conducted using Pearson's correlation coefficient (*r*), with $|r| \geq 0.5$ being considered strong, $0.5 > |r| > 0.3$ moderate and $|r| \leq 0.1$ weak (Cohen, 1988). Principal component analysis (PCA) was applied to ARG data.

3. Results and discussion

3.1. Water quality analyses

Water quality varied substantially between the cattle ponds and swine lagoons. The COD of the surface water in cattle ponds was generally higher than 1000 mg/L, while that of the surface water in swine lagoons was usually less than 400 mg/L (Tables S-5 and S-6). The COD of the bottom water in swine lagoons was generally much higher than that of the surface water. The total phosphorus (TP) in cattle ponds was relatively constant in the two sampling events (22.5 ± 4.1 mg/L in Sep 2010 and 32.7 ± 10.3 mg/L in Jun 2011). In contrast, the TP in swine lagoons increased from 14.0 ± 5.4 mg/L in Sep 2010 to 33.8 ± 18.8 mg/L in Jun 2011. The removal efficiencies for COD and TP were less than 15% in both the cattle pond series and the swine lagoon series in Sep 2010. The removal efficiencies for COD and TP were 13% and 31% in cattle ponds, and 59% and 65% in swine lagoons in Jun 2011. Ammonia-N was <50 mg/L in cattle ponds but was generally >70 mg/L in swine lagoons. Reduction in $\text{NH}_3\text{-N}$ was substantial in the sequential cattle ponds: 99% removal in 2010 and 76% in 2011.

3.2. Antimicrobial concentrations

The occurrence of antimicrobials in the storage ponds and treatment lagoons was dependent on sample matrix (water vs. sludge) and animal type (cattle vs. swine). Samples were divided into four categories: cattle-water, cattle-sludge, swine-water, and swine-sludge. Among the 16 antimicrobials tested, all were detected in at least one water sample (Table S-7) and only 11 were detected in sludge samples (Table S-8). The five antimicrobials absent in sludge were erythromycin, sulfamethoxazole, sulfamethizole, sulfathiazole and virginiamycin. Furthermore, sulfamethoxazole, lincomycin and virginiamycin were not detected in either water or sludge of cattle ponds. Detection frequencies in water and sludge were similar for cattle ponds and swine lagoons (Table 1). Hence, results from cattle and swine were also combined to generate the detection frequencies in all water and all sludge samples (Table 1). With the exception of sulfamethazine, most sulfonamide compounds were detected more frequently in water than in sludge. Members of the tetracycline family (i.e., chlortetracycline, oxytetracycline, and tetracycline) were detected in all sludge samples, but only in ~40% of water samples. Among macrolides, erythromycin and tiamulin were detected more frequently in water, while tylosin was more likely to occur in the sludge. Among other antimicrobials tested, lincomycin had similar detection frequencies in water and sludge, while virginiamycin and monensin had higher detection frequency in water than in sludge. In the water compartment, the concentration of most antimicrobials in water was ≤ 4.9 $\mu\text{g/L}$ except monensin, which ranged between 45.3 and 307.8 $\mu\text{g/L}$ in cattle storage ponds (Table 1). In the sludge compartment, the concentration of sulfonamides was low (≤ 6.3 $\mu\text{g/kg}$), while tetracyclines in both cattle ponds and swine lagoons were high (6.2 to 7218.4 $\mu\text{g/kg}$, Table 1).

Water and sludge in waste management treatment and storage structures are often handled differently. Irrigation with wastewater could potentially transport low- K_d antimicrobials into soil and crops,

Table 1
The detection frequency and ranges of antimicrobials in cattle storage ponds and swine treatment lagoons.

	Antimicrobials	Cattle-water (n = 10)	Cattle-sludge (n = 8)	Swine-water (n = 12) ^a	Swine-sludge (n = 6)	Water (n = 22) ^b	Sludge (n = 14)	
Tetracycline	Chlortetracycline	40% ^c (0.303–2.005) ^d	100% (34.4–1045.5)	42% (0.343–2.743)	100% (1089.9–7218.4)	41%	100%	
	Oxytetracycline	10% (1.471)	100% (1.9–24.6)	33% (0.010–3.138)	100% (7.7–65.6)	23%	100%	
	Tetracycline	20% (0.569–1.843)	100% (14.3–545.8)	50% (0.147–2.860)	100% (90.2–1623.8)	36%	100%	
Sulfonamide	Sulfamethoxazole	0%	0%	42% (0.104–0.342)	0%	23%	0%	
	Sulfamethazine	20% (0.026–0.342)	100% (0.7–6.3)	58% (0.092–0.574)	33% (0.4)	41%	71%	
	Sulfachloropyridazine	10% (0.431)	38% (1.2–1.3)	58% (0.062–0.443)	0%	36%	21%	
	Sulfadimethoxine	90% (0.027–0.586)	88% (0.5–3.4)	50% (0.179–0.548)	0%	68%	50%	
	Sulfamerazine	0%	13% (0.6)	42% (0.335–0.548)	17% (0.9)	23%	14%	
	Sulfamethizole	10% (0.452)	0%	50% (0.213–0.490)	0%	32%	0%	
	Sulfathiazole	10% (0.130)	0%	50% (0.135–0.540)	0%	32%	0%	
	Erythromycin	50% (0.234–1.266)	0%	50% (0.146–1.592)	0%	50%	0%	
	Tiamulin	70% (0.012–0.455)	0%	58% (0.138–3.636)	100% (1.2–26.5)	64%	43%	
Macrolide	Tylosin	10% (0.040)	50% (0.5–0.8)	42% (0.505–4.913)	100% (4.0–170.4)	27%	71%	
	Others	Lincomycin	0%	0%	58% (0.011–0.517)	83% (1.3–4.4)	32%	36%
		Virginiamycin	0%	0%	42% (0.682–0.877)	0%	23%	0%
	Monensin	90% (45.300–307.800)	100% (6.2–297.1)	100% (0.705–0.895)	50% (2.7–17.5)	94%	79%	

^a For monensin, n = 6.

^b For monensin, n = 16.

^c Percentages are detection frequency.

^d Numbers in bracket are ranges of antimicrobial concentrations, in unit of µg/L (water) or µg/kg (sludge).

and may lead to contamination of these compounds or degradation products in surface and ground water through runoff and leaching (Sarmah et al., 2006). In comparison, the land application of sludge from storage ponds and treatment lagoons would provide a route of dissemination for highly-sorptive antimicrobials. In addition, the distribution of antimicrobials between water and sludge may also influence the degradation of these compounds. Biochemical and physiochemical degradations are often slower for compounds bound to particles than those dissolved in water due to lower availabilities (Doi and Stoskopf, 2000; Thiele-Bruhn, 2003; Watts, 1998). For instance, the half-life of oxytetracycline was 32 days in sediment, whereas it was as short as 128 h in seawater (Samuelsen, 1989). To our knowledge, this is one of the first studies to quantify antimicrobial concentrations in both water and sludge of manure management structures.

Partitioning of antimicrobials in liquid–solid systems depends on antimicrobials and adsorbents. The physicochemical properties of antimicrobials, such as molecular structure, size, shape, solubility, speciation, and hydrophobicity, are responsible for the difference (Sarmah et al., 2006). In addition, the partitioning of the same antimicrobial is affected by the properties of adsorbents. As shown in Table 2, the pseudo- K_d values from this study were in better agreement with the K_d values of soil than with the pseudo- K_d values of river sediments. The difference among different adsorbents may be attributed to different organic carbon contents and particle sizes of the adsorbents.

Records show that around the time when samples were taken chlortetracycline, oxytetracycline and tylosin were administered in both livestock facilities, sulfadimethoxine was used on cattle only, and sulfachloropyridazine and lincomycin were used on swine only. All

Table 2
Partitioning coefficients (K_d , kg/L) and pseudo partitioning coefficients (pseudo K_d , kg/L) of antimicrobials in different liquid–solid systems.

Antimicrobials	Pseudo K_d in this study (sludge as adsorbent)		K_d (soil as adsorbent) (Sarmah et al., 2006)	Pseudo K_d (river sediment as adsorbent) (Kim and Carlson, 2007)
	Median	95% CI		
Chlortetracycline	2069.0	120.7–4973.4 (n = 7)	1280–2386	305
Oxytetracycline	25	0–662.0 (n = 4)	417–1026	1267
Tetracycline	489.1	14–1018.4 (n = 6)	1147–2370	1051
Sulfamethazine	1.6	0–21.2 (n = 5)	0.6–3.2	517
Sulfadimethoxine	12.2	0–55.6 (n = 6)	2.3–4.6	402
Tiamulin	8.4	0–98.0 (n = 5)		
Tylosin	32.4	0–202.5 (n = 7)	8.3–128	91
Lincomycin	55.3	0–1757.0 (n = 4)		
Monensin	1.4	0–9.6 (n = 10)	1.09–78.6	

antimicrobials were administered through injection except that chlor-tetracycline was also added to feeds when animals were sick. In general, these antimicrobials had higher detection frequency than the other antimicrobials measured. Some antimicrobials, such as tetracycline, tiamulin, and monensin, were not administered to the animals at the time of sampling. However, they were frequently detected in the cattle ponds and/or swine lagoons (Table 1), likely as a result of accumulation from previous usage on the animals.

3.3. Validation of qPCR assays

One *sul* gene and three *tet* genes were selected to assess the efficiencies of DNA extraction and qPCR amplification in different sample types. The four selected ARGs covered various resistance mechanisms against these two classes of antimicrobials. Details of qPCR assay validation results are listed in Table S-9. The recovery was gene-specific, and within each gene the variance among samples was small. For example, the recovery rate for *sul2* was $76.6 \pm 10.5\%$ and that for *tetQ* was $87.4 \pm 16.1\%$. The recovery rates for the other two ARGs were higher than 100%: $149.8 \pm 43.7\%$ for *tetD* and $321.7 \pm 63.8\%$ for *tetX*. A wide range of qPCR recovery rates is not uncommon among lagoon samples: one study reported 17.7–166.3% for 7 *tet* genes (Koike et al., 2007) and another study reported 33–400% for *sul* and *tet* genes (suppression factor, inverse of recovery rate, was reported in the latter study) (McKinney et al., 2010).

3.4. Quantification of ARGs

All ARG values in this work were calculated by normalizing the ARG concentration to the 16S rRNA gene concentration, an approximation of the relative abundance of the antimicrobial resistant population in bacterial communities. Because *tetD* and *tetE* only occurred in a few samples (data not shown), these two ARGs were not included in further analyses. The average relative abundance of ARGs ranged from 5.5×10^{-6} to 6.3×10^{-1} copies per 16S rRNA gene (Fig. 1). Swine lagoons usually had higher relative abundance of ARGs than cattle ponds, although for *tetQ* and *tetX* the difference was not statistically significant (Figs. S-1 and S-2). The first two principal components (PC1 and PC2) in the PCA analysis accounted for 85.2% and 90.5% of the total ARG data variance in the water and sludge compartments, respectively, of livestock waste management structures (Fig. 2). Data representing ARGs in cattle ponds clustered together, showing that the relative abundance of ARGs in cattle waste storage ponds was

relatively constant at the two sampling times. In contrast, data representing ARGs in swine lagoons are more scattered, suggesting that the relative abundance of ARGs varied among lagoons and with time (Fig. 2).

Due to the highly sorptive nature, tetracyclines accumulated more in sludge than in water. This distribution could presumably lead to higher selective pressure, and consequently higher relative abundance of *tet* genes in sludge than in water. However, the relative abundance of *tet* genes was similar in the water and sludge compartments in both the cattle ponds and the swine lagoons (Fig. 3). In contrast, the sulfonamide compounds were more evenly distributed between water and sludge, and relative abundance of *sul* genes was significantly higher in water than in sludge (Fig. 3). These distributions suggest that the aqueous antimicrobials may be more biologically available than adsorbed antimicrobials.

Although overall the relative abundance of ARGs in sludge was lower than that in water, since the bacteria density in sludge was higher than that in water (according to the abundance of the 16S rRNA gene, data not shown), the absolute abundance of ARGs is calculated to be 1 to 5 orders of magnitude higher in sludge (copies/g sludge) than in water (copies/mL water). Finally, the surface water and bottom water in swine lagoons were not significantly different in terms of the relative abundance of ARGs (Fig. 3), suggesting that the distribution of antimicrobial resistant bacteria might not be affected by the vertical profiles of certain water quality factors, such as DO.

3.5. Correlations

Pearson's correlation coefficients and *p*-values between ARGs and water constituents are tabulated in Table 3. Ammonia showed a strong and positive correlation with all ARGs except *tetX*. Copper exhibited moderate negative correlation with *sul^R*, *tetO* and *tetQ*. Other correlations between ARGs and water parameters were either insignificant or involved with only one ARG.

Correlation between $\text{NH}_3\text{-N}$ and ARGs has been previously reported. McKinney and co-workers studied the ARGs in various livestock lagoon systems, and found strong correlations between $\text{NH}_3\text{-N}$ and tetracycline resistance genes (McKinney et al., 2010). The authors further suggested that lagoons capable of improving water quality could also effectively remove ARGs and recommended $\text{NH}_3\text{-N}$ as a potential indicator for the occurrence of ARGs in livestock lagoons (McKinney et al., 2010). Further studies are needed to explain the correlation between $\text{NH}_3\text{-N}$ and ARGs.

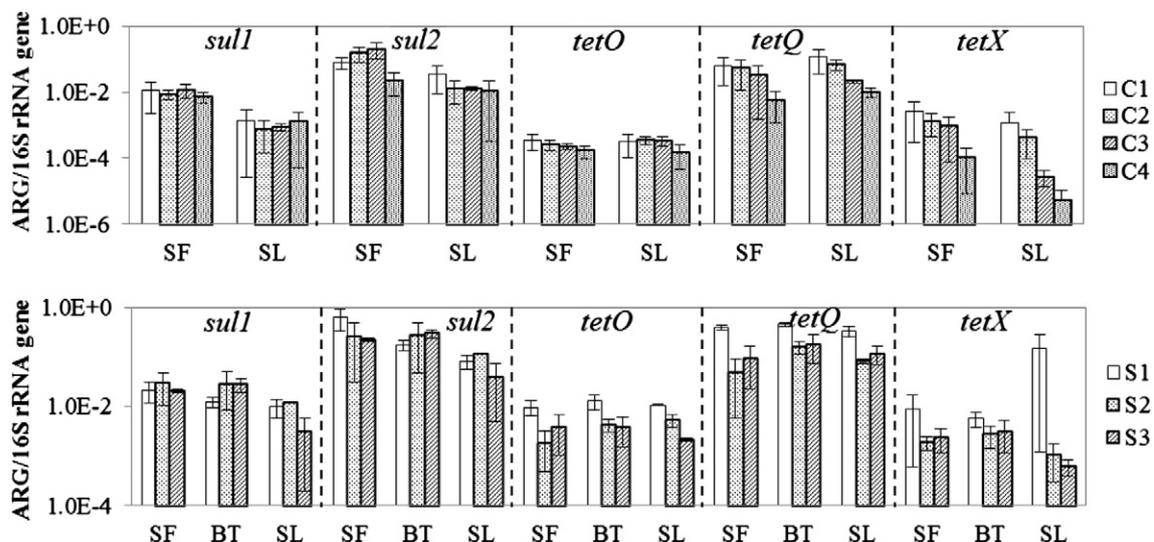


Fig. 1. The relative abundance of ARGs in cattle storage ponds (top) and swine treatment lagoons (bottom). C1–C4 and S1–S3 refer to the four cattle storage ponds and three swine treatment lagoons sampled in this study. Error bars represent the half-range of the two sampling events. SF = surface water, BT = bottom water, SL = sludge.

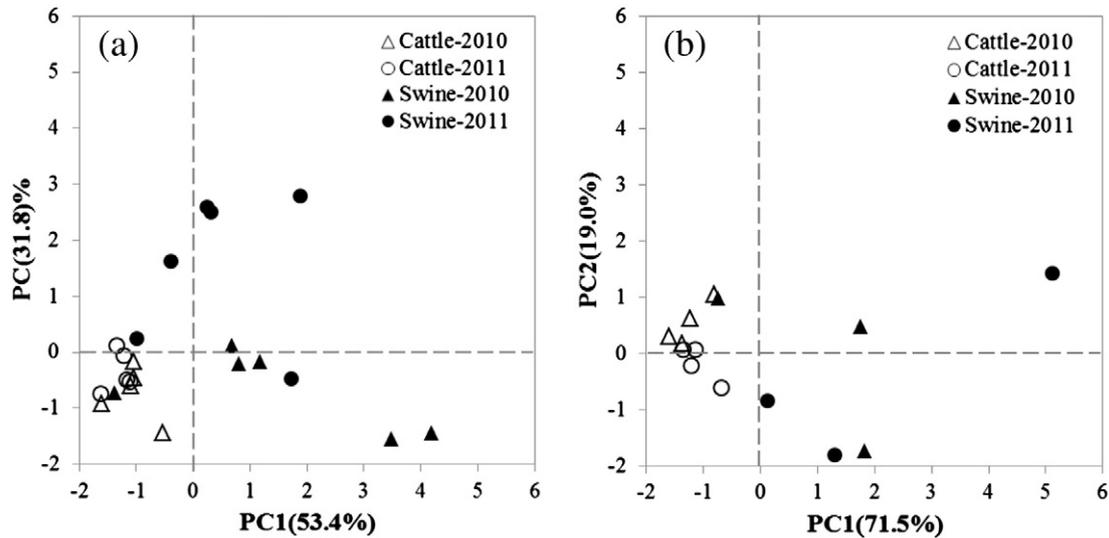


Fig. 2. Principal component analysis (PCA) of ARGs in (a) the water and (b) the sludge compartments of livestock wastewater management structures. Data are labeled according to livestock facilities and sampling time.

Copper and zinc can induce antimicrobial resistance, as the resistance to these metals and resistance to antimicrobials are often co-selected (Baker-Austin et al., 2006; Wardwell et al., 2009). Cu and Zn are often added in swine feed as growth promoters (Hill et al., 2000). In this study, Cu and Zn were detected at low levels in water (<0.065 mg/L, Tables S-5 and S-6). No significant positive correlation was observed between Zn/Cu and the ARGs tested, likely because the levels of soluble Zn and Cu were low and did not exert sufficient pressure on microorganisms. An amount of 1.04 mg/L Zn was reportedly required to induce resistance to Zn and 2.56 mg/L Cu was required to induce resistance to Cu in *E. coli* (Nies, 1992; Rouch et al., 1985). McKinney et al. found positive correlations between *tetO/tetW* and Cu when the concentration of Cu was as high as 103 mg/L (McKinney et al., 2010).

In addition to the correlation with water constituents, the correlation between ARGs and antimicrobials was also analyzed (Table 4). A positive correlation was observed between *sul2/sul^R* and total sulfonamide (including the 7 sulfonamide compounds listed in Table 1) in

water but the correlation became negative in sludge. For tetracycline resistance genes, *tetX* and *tetO* exhibited strong, positive correlations with the total tetracycline (including chlortetracycline, oxytetracycline and tetracycline) in water and sludge, respectively. Correlations between ARGs and individual tetracycline and sulfonamide compounds plus other antimicrobials tested were also analyzed and the coefficients and *p*-values were tabulated in Tables S-10 and S-11. Some significant positive correlations include the ones between lincomycin and *sul2/tetO/tetX* in water and the ones between tiamulin/tylosin and all ARGs in sludge.

Previous studies have suggested a link between the occurrence of antimicrobials and increasing antimicrobial resistance in the environment (Luo et al., 2010; Peak et al., 2007; Pei et al., 2006). However, the correlation, or sometimes the lack of correlation, between antimicrobials and ARGs is usually system dependent. In a cattle feedlot wastewater holding pond, the *tet^R* (*tetO*, *tetQ* and *tetW*) level was 4- to 8.3-fold higher in water samples containing >1.95 µg/L tetracycline than in those containing <1.95 µg/L tetracycline (Smith et al., 2004). In a wastewater

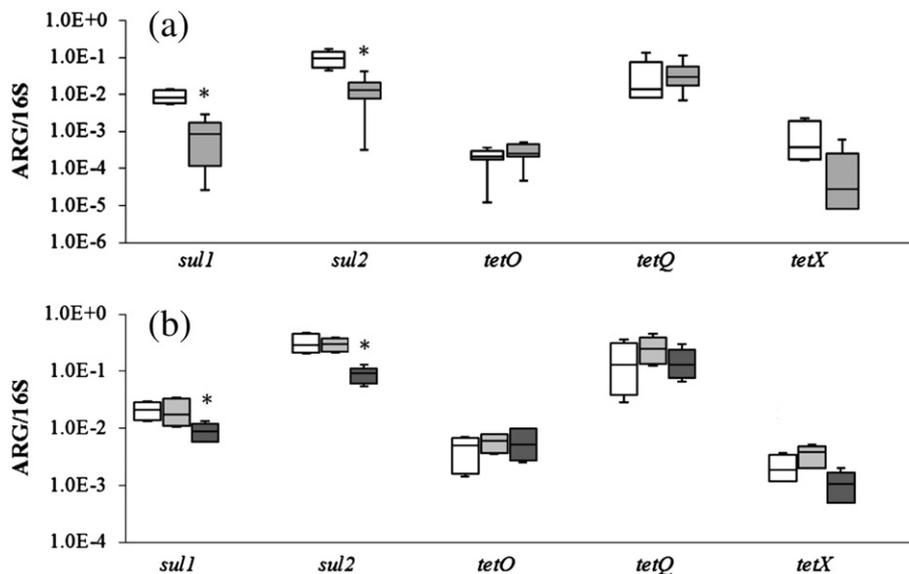


Fig. 3. (a) The relative abundance of ARGs in surface water (white) and sludge (gray) of cattle ponds. (b) The relative abundance of ARGs in the surface water (white), bottom water (light gray), and sludge (dark gray) of swine lagoons. Significant difference is marked as “*”.

Table 3

Correlations between the relative abundance of ARGs and the concentrations of water constituents. Significant correlation coefficients and their corresponding *p*-values are marked with underlines.

	<i>sul1</i>		<i>sul2</i>		<i>tetO</i>		<i>tetQ</i>		<i>tetX</i>	
	<i>r</i>	<i>p</i> -Value	<i>r</i>	<i>p</i> -Value	<i>r</i>	<i>p</i> -Value	<i>r</i>	<i>p</i> -Value	<i>r</i>	<i>p</i> -Value
COD	–0.36	<u>0.098</u>	–0.38	<u>0.083</u>	0.38	<u>0.077</u>	0.43	0.045	0.05	<u>0.813</u>
NH ₄ ⁺ -N	0.58	0.005	0.60	0.003	0.69	<0.001	0.73	<0.001	0.39	<u>0.073</u>
TP	0.15	<u>0.518</u>	0.28	<u>0.208</u>	–0.04	<u>0.847</u>	0.16	<u>0.481</u>	–0.39	<u>0.076</u>
Ortho-P	–0.35	<u>0.112</u>	–0.52	0.014	–0.37	<u>0.086</u>	–0.34	<u>0.124</u>	–0.31	<u>0.155</u>
SO ₄ ^{2–}	–0.36	<u>0.103</u>	–0.14	<u>0.520</u>	–0.15	<u>0.518</u>	–0.09	<u>0.676</u>	0.18	<u>0.410</u>
Zn	–0.36	<u>0.095</u>	–0.53	0.012	–0.24	<u>0.276</u>	–0.17	<u>0.455</u>	–0.26	<u>0.244</u>
Cu	–0.49	0.020	–0.48	0.024	–0.52	0.013	–0.43	0.047	–0.21	<u>0.343</u>

treatment plant (WWPT) effluent, the absolute abundance of *tetO* and *tetW* showed no significant correlation with the total tetracycline (i.e., oxytetracycline, doxycycline, chlortetracycline and tetracycline) concentration, which was lower than 1.1 µg/L (Gao et al., 2012). In this study, with the total tetracycline less than 7.74 µg/L in water, no significant correlation was seen between *tet^R* and tetracyclines (Table 4). One possible explanation for the inconsistency on correlation is that there is a concentration threshold for antimicrobials to effectively exert selective pressure for ARGs (Smith et al., 2004). In a laboratory experiment, it was found that at least 20 µg/L of oxytetracycline was needed to cause an increase in the relative abundance of *tet^R* (*tetB*, *tetL*, *tetM*, *tetO*, *tetQ* and *tetW*) in mesocosms derived from pristine surface water (Knapp et al., 2008).

Similar to the correlation in water, the correlation between ARGs and antimicrobials in sludge also depends on the system. For example, there was no significant correlation between *tet^R* and tetracycline (701–1150 µg/kg) or between *sul^R* and sulfonamide (76–113 µg/kg) in municipal wastewater samples (Gao et al., 2012). In contrast, the correlations between *sul^R* and sulfonamide (from <5 to 840 µg/kg) and between *tet^R* and tetracycline (from <5 to >3000 µg/kg) were significant in lagoon sediment (McKinney et al., 2010).

In addition to the concentration threshold requirement, other factors may also contribute to the lack of consistent correlation between ARGs and antimicrobials. Studies show that ARGs may persist in bacteria even after the antimicrobials in the environment have diminished, known as the “easy-to-get, hard-to-lose” phenomenon (Aminov and Mackie, 2007; Salyers and Amabile-Cuevas, 1997). Also, antimicrobials could co-select certain ARGs, as often suggested by the co-existence of several ARGs (Chung et al., 1999; Salyers et al., 1995; Speer et al., 1991b). For instance, *sul1* and *tetG* were located on the same plasmid (Ng et al., 1999), tetracycline resistance genes *tetQ* and *tetX* co-existed with tylosin resistance gene *ermF* on conjugative transposons (Chung et al., 1999; Speer et al., 1991a), and *tetM* and another tylosin resistance gene *ermB* were located on the same conjugative transposon (Clewell et al., 1995). It is noticed that in this study there was a strong positive correlation between tetracycline resistance genes and macro- lides (*tetO/tetQ/tetX* vs. tiamulin/tylosin, Table S-11).

Table 4

Correlation between the relative abundance of ARGs and the concentrations of the corresponding antimicrobials.

	Water		Sludge	
	<i>r</i>	<i>p</i> -Value	<i>r</i>	<i>p</i> -Value
<i>sul1</i> – total sulfonamide	0.22	<u>0.329</u>	–0.52	<u>0.057</u>
<i>sul2</i> – total sulfonamide	0.45	0.036	–0.56	0.035
<i>sul^{Ra}</i> – total sulfonamide	0.50	0.035	–0.56	0.037
<i>tetO</i> – total tetracycline	0.36	<u>0.104</u>	0.84	<0.001
<i>tetQ</i> – total tetracycline	0.36	<u>0.099</u>	0.50	<u>0.068</u>
<i>tetX</i> – total tetracycline	0.61	0.003	0.11	<u>0.699</u>
<i>tet^{Rb}</i> – total tetracycline	0.37	<u>0.269</u>	0.38	<u>0.183</u>

^a *sul^R* = *sul1* + *sul2*.

^b *tet^R* = *tetO* + *tetQ* + *tetX*.

4. Conclusions

In summary, antimicrobials and ARGs were quantified for the water and the sludge compartments of the waste management structures in two livestock facilities. The partitioning of the antimicrobials between water and sludge was compound specific. ARGs occurred in both water and sludge compartments, and its abundance varied more substantially in swine lagoons than in cattle ponds. The correlation between ARGs and antimicrobials is system dependent. This is one of the first studies that investigate the distributions of antimicrobials and ARGs between water and sludge in livestock waste management structures. This information is useful in developing management strategies to minimize the spread of antimicrobials and ARGs when water is discharged and sludge is land applied.

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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.scitotenv.2013.06.016>.

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