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Intracellular and Extracellular Antimicrobial Resistance Genes in the Sludge of Livestock Waste Management Structures

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Supporting Information

ABSTRACT: The sludge compartment in livestock waste management structures is a potential hotbed for the emergence and proliferation of antimicrobial resistance among bacteria. Little is known about the distribution of antimicrobial resistance genes (ARGs) between the intracellular and extracellular DNA pools in the sludge. The overall objective of this study was to assess the significance of extracellular ARGs to the total ARGs in the sludge of livestock waste management structures. In this study, sludge samples were collected from four cattle manure storage ponds and three swine waste treatment lagoons and analyzed for genetic indicators of resistance. Intracellular DNA (iDNA) and extracellular DNA (eDNA) in the sludge were separately extracted using an optimized protocol. ARGs [*sul*(I), *sul*(II), *tet*(O), *tet*(Q), and *tet*(X)] in both the iDNA and eDNA extracts were quantified using quantitative polymerase chain reaction (qPCR), and antimicrobials, including sulfonamides and tetracyclines, were measured using liquid chromatography tandem mass spectrometry. Results showed that eDNA constituted less than 1.5% of the total DNA in sludge. All ARGs tested were detected in nearly all eDNA and iDNA samples. Furthermore, every gram of dry sludge contained from 1.7×10^3 to 4.2×10^8 copies of extracellular ARG and from 3.2×10^7 to 3.2×10^{10} copies of intracellular ARG. Chlortetracycline concentrations ranged between 187 and 2674 μg/g of sludge wet weight (ww), while sulfonamide concentrations were lower than 6.3 μg/g of sludge ww. The detection of ARGs in eDNA extracts suggests that transformation is a potential mechanism in ARG proliferation in the sludge of livestock waste management structures.

INTRODUCTION

Because of extensive antimicrobial usage in animal production, livestock facilities are potential sources of antimicrobial compounds and antimicrobial-resistant bacteria to the environment. Antimicrobials and antimicrobial-resistant bacteria in the wastewater or the sludge compartments of livestock waste management structures (LWMSs), such as manure storage ponds and waste treatment lagoons, may eventually enter the soil environment through irrigation of wastewater or land application of sludge.[1] Similar to the effects of the antimicrobials on bacteria in the animal gut, when antimicrobials are concentrated in LWMSs, these compounds may also lead to the emergence and proliferation of antimicrobial-resistant bacteria.[2] The occurrence of antimicrobials and antimicrobial resistance in the wastewater compartment of LWMSs has been documented.[3−5] However, little is known about the fate of these contaminants in the sludge compartment of LWMSs, particularly how antimicrobial resistance emerges and proliferates in the presence of antimicrobials with elevated concentrations.

The proliferation of antimicrobial resistance among bacteria can occur through horizontal gene transfer (HGT) in the presence of antimicrobials.[6,7] HGT may occur via three mechanisms: conjugation from cell-to-cell contact, transduction because of infection of bacterial phages, and transformation from direct uptake of extracellular DNA (eDNA).[8] In the marine environment, conjugation and transduction frequencies in seawater were about 10^{-7} transconjugants per donor cell and 10^{-7}−10^{-9} transductants per plaque forming unit (PFU), respectively,[9,10] while transformation frequency of 10^{-6}−10^{-7} transformants per recipient cell in marine sediments was estimated.[11] Some recent studies reported the presence and

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Table 1. Information about the qPCR Primers Used in This Study

target gene	primer	sequence (5′ → 3′)	target size (bp)	annealing temperature (°C)	quantitative range (copies/μL)
sul(I)[26]	FW	CGC ACC GGA AAC ATC GCT GCA C	163	65.0	10^2–10^8
	RV	TGA AGT TCC GCC GCA AGG CTC G			
sul(II)[26]	FW	TCC GGT GGA GGC CGG TAT CTG G	191	57.5	10^2–10^8
	RV	CGG GAA TGC CAT CTG CCT TGA G			
tet(O)[26]	FW	ACG GAR AGT TTA TTG TAT ACC	171	50.3	10^2–10^9
	RV	TGG CGT ATC TAT AAT GTT GAC			
tet(Q)[50]	FW	AGA ATC TGC TGT TTG CCA GTG	169	63	10^2–10^9
	RV	CGG AGT GTC AAT GAT ATT GCA			
tet(X)[51]	FW	AGC CTT ACC AAT GGG TGT AAA	278	60	10^3–10^8
	RV	TTC TTA CCT TGG ACA TCC CG			
16S rRNA gene[25]	1369F	CGG TGA ATA CGT TCY CGG	133	56	10^3–10^9
	1492R	GGW TAC CTT GTT ACG ACT T			

persistence of eDNA in soil and sediment and suggested that eDNA-based transformation plays an important role in HGT in these environments.[12,13] Therefore, it is plausible to expect that eDNA-based transformation plays an important role in antimicrobial resistance gene (ARG) proliferation in the sludge of LWMSs.

eDNAs can originate from both the lysis of dead cells and the secretion from live cells.[14] Once released from cells, DNA may be adsorbed to particles, such as soils and sediments, in the environment. Adsorption to particles can prevent eDNA from degradation, contributing to its persistence in the environment,[15−17] and the soil adsorption capacity for eDNA can be as high as 10^3 μg/g.[18,19] Studies found that adsorbed DNA was still bioavailable for transformation and, therefore, represented a potential reservoir for HGT.[20,21] The sludge in storage ponds and treatment lagoons is nutrient-rich environments and can support a large quantity of microbes, making it an ideal place for the emergence and proliferation of antimicrobial-resistant bacteria. Despite their potential environmental impacts, little has been done to investigate the extracellular ARGs in livestock waste management structures.

The objectives of this study were 3-fold: (1) to verify the occurrence of ARGs in the eDNA in the sludge of LWMSs, (2) to quantify intracellular and extracellular ARGs in the sludge of LWMSs, and (3) to investigate the correlation between antimicrobials and intracellular ARGs. To achieve the objectives, 10 antimicrobials, including members of the tetracycline, sulfonamide, and macrolide families, as well as monensin, were analyzed using liquid chromatography−tandem mass spectrometry (LC−MS/MS). eDNA and intracellular DNA (iDNA) were simultaneously extracted, and multiple ARGs [i.e., *sul*(I), *sul*(II), *tet*(O), *tet*(Q), and *tet*(X)] were quantified using quantitative polymerase chain reaction (qPCR).

MATERIALS AND METHODS

Livestock Facilities. Sludge samples were collected from four sequential storage ponds holding manure and runoff from a beef cattle feedlot (C1−C4) and three sequential treatment lagoons for swine wastewater (S1−S3) at the USDA Meat Animal Research Center (Clay Center, NE) in June 2011. Sludge samples were collected from the center of the storage ponds and treatment lagoons using an Ekman dredge sampler and then transferred to sterile plastic bags and amber glass jars for microbial and chemical analyses. Samples were transported to the laboratory on ice within 6 h of collection and stored at −20 °C for antimicrobial analyses and −80 °C for DNA analyses.

Extraction of iDNA and eDNA. eDNA and iDNA were separately extracted from sludge samples by following a published protocol,[22] with minor modifications; that is, 0.1% sodium dodecyl sulfate (SDS) was not used because of its ability to break intact cells in our samples (see the Results and Discussion). In brief, 1 g of sediment [wet weight (ww)] was washed 3 times using 0.1 M phosphate buffer (PB; 0.093 M Na_2HPO_4 and 0.007 M NaH_2PO_4 at pH 8.0). Dissolved eDNA was separated from cells by centrifugation at 10000*g* for 20 min at 4 °C and filtered through a 0.2 μm pore membrane.[23] The eDNA in filtrate was precipitated using 1% cetyltrimethylammonium bromide (CTAB). The iDNA in the cell pellet was released using two 1 min bead beatings with 2 min of chilling on ice in between. The raw eDNA and iDNA were purified using a phenol−chloroform method[24] and quantified using NanoDrop 2000c (Thermo Scientific, Wilmington, DE).

Effects of SDS on eDNA Extraction. SDS (0.1%) was used to improve the extraction efficiency of eDNA and iDNA in the protocol by Corinaldesi et al.;[22] however, it was reported to cause cell lysis in another study.[23] Therefore, the effect of 0.1% SDS on cell lysis for the sludge samples was tested on the sludge sample from lagoon S1 with and without SDS. To obtain an eDNA-free sample, 1 g of sludge (ww) was added to 3 mL of PB, followed by the treatment of 100 units of DNase I (Sigma-Aldrich Corp., St. Louis, MO) at 37 °C for 2 h before the residual enzyme was inactivated by 50 mM ethylenediaminetetraacetic acid (EDTA). The mixture was centrifuged at 500*g* for 10 min at 4 °C; the pellet was washed twice with PB; and all supernatants were combined and centrifuged at 10000*g* at 4 °C for 20 min. The supernatant was then collected to verify the absence of eDNA. Two sets of triplicate eDNA-free sludge samples were subject to eDNA extraction with and without 0.1% SDS, respectively.[22] Furthermore, purified eDNA extracts were used as templates in PCR reactions targeting the 16S rRNA gene with the universal primers 27f and 1492r.[25] Both DNA template and PCR products were examined by gel electrophoresis.

qPCR Assays. qPCR was performed using a Mastercycler ep realplex thermocycler (Eppendorf, Hamburg, Germany). A total of 9 μL of 2.5× RealMasterMix SYBR ROX (5 Prime, Gaithersburg, MD) and 4 ng of DNA were used in a 20 μL reaction mixture. Primer sequences, annealing temperatures, and references are summarized in Table 1. Positive controls for *tet* genes were obtained by PCR amplification of DNA from *tet*-bearing *Escherichia coli* (provided by Dr. Lisa Durso of the

USDA Agricultural Research Service). Positive controls for *sul* genes were derived from activated sludge of a wastewater treatment plant through PCR. qPCR standards were constructed by cloning PCR products into vectors, transforming the vectors into competent *E. coli* cells using an Invitrogen TOPO TA Cloning Kit (Carlsbad, CA), growing *E. coli* colonies, and then extracting and purifying plasmid DNA 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 plasmids was calculated as previously described,[26] and the qPCR standards ranged from 10^1 to 10^9 copies/μL.

Antimicrobial Measurements. Antimicrobial concentrations in sludge samples were analyzed after extraction using LC−MS/MS with electrospray ionization.[27,28] Sludge samples (1−5 g) were weighed in 50 mL Teflon centrifuge tubes and mixed with 20 mL of 500 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 was 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 of 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 with a 10 μL injection volume. The chlortetracycline concentration includes isochlortetracycline, epichlortetracycline, and chlortetracycline, while the tetracycline transition includes tetracycline and epitetracycline.

Data Analyses. All statistical tests were performed using SAS 9.3 (SAS Institute, Inc., Cary, NC). Common logarithm (log) transformations of ARG concentrations and the ARG/16S rRNA ratio were used in all statistical analyses. One-way analysis of variation (ANOVA) (Proc GLMMIX) was used to generate statistical significance for comparison of means of different groups. Correlation analysis (Proc CORR) was used to calculate the Pearson correlation coefficient (r) and p values between ARG and antimicrobial levels. Statistical significance was defined as p value ≤ 0.05.

RESULTS AND DISCUSSION

Effects of 0.1% SDS on eDNA Extraction. The use of 0.1% SDS could cause cell lysis in sludge samples during eDNA extraction. The eDNA yield was 6 times higher when 0.1% SDS was used in extraction than when SDS was not used (4.9 versus 0.7 ng/g of sludge ww), evidenced by the denser band at high molecular size (lane 1 versus lane 2 in Figure 1). Both eDNA extracts could be amplified using PCR primers targeting the 16S rRNA gene (lanes 6 and 7 in Figure 1). SDS (0.1%) is often used to enhance cell lysis in total DNA extraction;[29] however, conflicting conclusions have been reached on whether 0.1% SDS can cause cell lysis during eDNA extraction.[22,23] To determine if 0.1% SDS may cause cell lysis and contaminate eDNA with iDNA in LWMS sludge, DNase I was used to remove eDNA in original sludge samples. The supernatant of a DNase-I-treated sample did not contain detectable DNA (lane 5 in Figure 1), which is further proven by the negative result from PCR targeting the 16S rRNA gene (lane 10 in Figure 1).

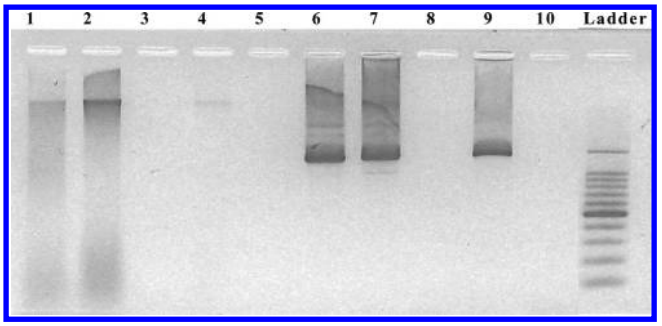

Figure 1. SDS (0.1%) caused cell lysis and resulted in contamination of iDNA in eDNA extracts. Lane 1, eDNA extracted without SDS; lane 2, eDNA extracted with 0.1% SDS; lane 3, eDNA extracted without SDS after DNase I treatment; lane 4, eDNA extracted with 0.1% SDS after DNase I treatment; lane 5, DNA purified from the supernatant of DNase-I-treated sludge; lanes 6−10, PCR products targeting the 16S rRNA gene corresponding to DNA in lanes 1−5.

eDNA-free sludge samples were then subjected to eDNA extraction with or without 0.1% SDS. No DNA was detected from the extraction without 0.1% SDS (lane 3 in Figure 1), which was verified by a negative PCR result (lane 8 in Figure 1). In contrast, a small amount of DNA was recovered from the extraction with 0.1% SDS (lane 4 in Figure 1), which was further confirmed by a positive PCR result (lane 9 in Figure 1). Taken together, a modified published eDNA extraction protocol[22] (i.e., without 0.1% SDS) was used for the eDNA extraction on all sludge samples in this study.

Characteristics of eDNA. The eDNA extracts from the sludge samples exhibited broader size ranges than the corresponding iDNA extracts. The size of the eDNA ranged between 75 and 20 000 bp, while the size of the iDNA was mostly larger than 4000 bp (Figure 2). In comparison, the size of the eDNA in seawater samples ranged from less than 100 to larger than 36 000 bp.[30] Assuming that the average bacterial gene is 1500 bp long, it was suggested that eDNA was large enough to contain multiple gene sequences.[30] In this study, the 16S rRNA gene was amplified from the eDNA extracts using the 27f and 1492r primers, indicating that the eDNA in the sludge sample tested contained largely intact 16S rRNA gene copies originating from intracellular DNA (lane 6 in Figure 1). Steinberger and Holden and Bockelmann et al. were also able to amplify the 16S rRNA gene from eDNA extracted from biofilm samples, although restriction endonuclease treatment showed different signature patterns of the PCR products from iDNA and eDNA extracts.[31,32] However, eDNA from environmental samples is not always PCR-amplifiable. For example, Corinaldesi and colleagues found that the eDNA extracted from ocean sediments could not be amplified by primers targeting the 16S rRNA gene.[22]

Yields of eDNA and iDNA. To address the large variation of the water content among sludge samples (see Table S1 of the Supporting Information), the yields of eDNA and iDNA were calculated on the basis of sludge dry weight (dw). In the sludge from the four cattle ponds, the eDNA yield was 1.0−3.9 μg/g of sludge dw and the iDNA yield was 463.2−1005.3 μg/g of sludge dw (Figure 3). In the sludge from the three swine lagoons, the eDNA yield was 0.6−9.2 μg/g of sludge dw and the iDNA yield was 190.9−584.7 μg/g of sludge dw. The yields of eDNA and iDNA showed strong correlation: Pearson's correlation coefficient r was 0.81 for cattle pond sludge and 0.69 for swine lagoon sludge ($p < 0.05$ for both). The eDNA

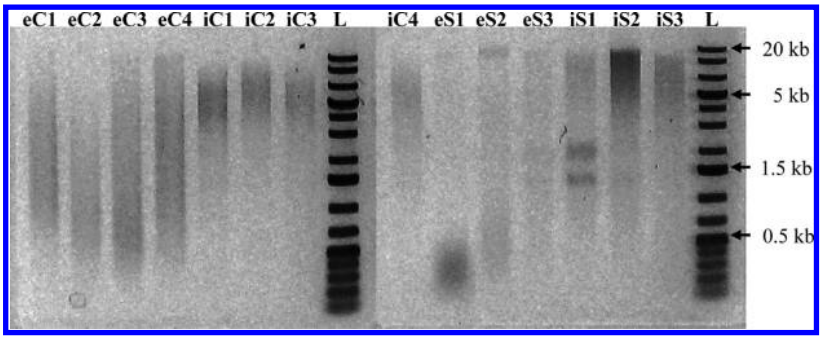

Figure 2. Gel electrophoresis of eDNA and iDNA extracted from the seven sludge samples without 0.1% SDS. L = ladder. C1−C4 are cattle manure storage ponds, and S1−S3 are swine waste treatment lagoons. Prefix "e" denotes eDNA, and prefix "i" denotes iDNA.

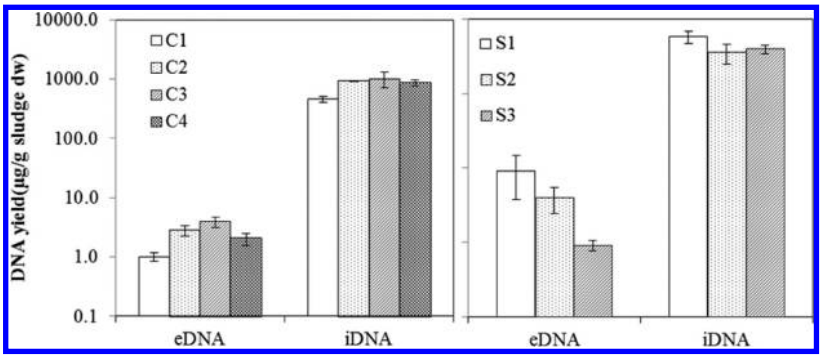

Figure 3. Yields of eDNA and iDNA from the sludge samples of the cattle manure storage ponds (C1−C4) and swine waste treatment lagoons (S1−S3). Error bars represent standard deviations of triplicate extractions.

Table 2. Comparison of eDNA Extraction Yields between This Study and Other Studies

eDNA extraction method	sample	eDNA yield (μg/g)	iDNA yield (μg/g)	reference
alkaline buffer (pH 8.0)	reservoir sediment	1 (ww)	8 (ww)	52
alkaline buffer (pH 8.0) with 0.1% SDS	marine sediment	6.7−24.3 (dw)	0.31−0.67 (dw)	22
alkaline buffer (pH 8.0)	forest soil	2.2−41.1[a]	N/A[b]	53
enzymatic	marine sediment	0.5−21.1 (dw)	N/A[b]	54
alkaline buffer (pH 8.0) with 0.1% SDS	marine sediment	0.0047−5.576 (dw)	N/A[b]	54
alkaline buffer (pH 8.0)	LWMS sediment	0.2−1.0 (ww), 0.6−9.2 (dw)	42.8−384.7 (ww), 190.9−1005.3 (dw)	this study

[a]Dry weight or wet weight not specified. [b]N/A = not available.

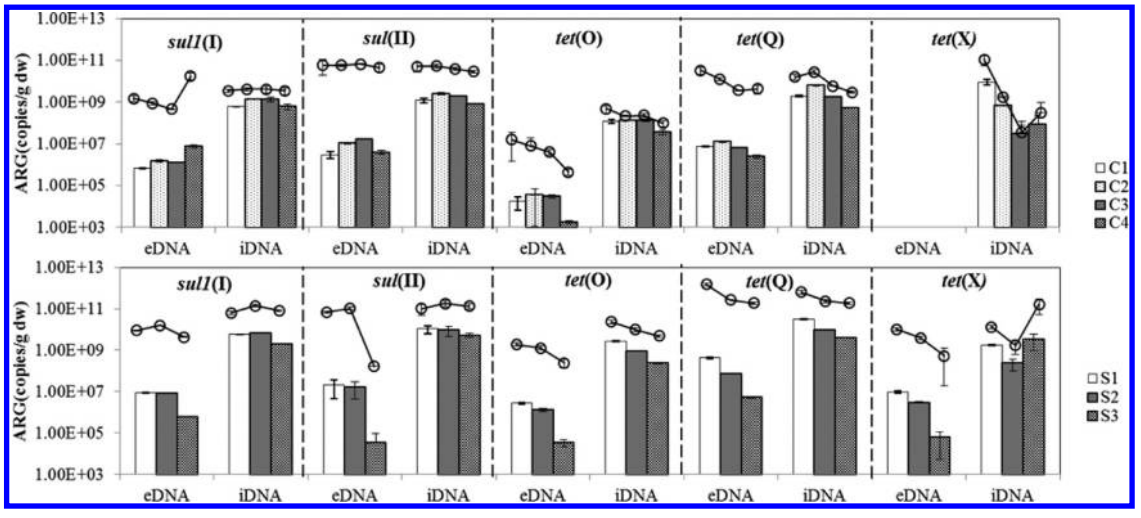

Figure 4. Absolute abundance (bar, copies/g of sludge dw) and relative abundance (open circle, ARG/16S rRNA gene) of ARGs in the eDNA and iDNA extracted from sludge samples. C1−C4 are cattle manure storage ponds, and S1−S3 are swine waste treatment lagoons. Error bar represents the half-range of duplicate PCR measurements.

Table 3. Average Relative Abundance of ARGs (i.e., ARG Copies Normalized to the 16S rRNA Gene Copies) in eDNA and iDNA and p Value for the Difference between Them

	sul(I)	*sul*(II)	*tet*(O)	*tet*(Q)	*tet*(X)
eDNA	2.3×10^{-3}	1.1×10^{-2}	7.2×10^{-5}	1.7×10^{-2}	2.8×10^{-5}
iDNA	7.3×10^{-3}	2.1×10^{-2}	1.1×10^{-3}	1.4×10^{-2}	1.8×10^{-3}
p	0.0068	0.3323	<0.0001	0.6175	0.0035

Table 4. Antimicrobials Detected in Sludge Samples (mg/kg of Sludge ww)[a]

	C1	C2	C3	C4	S1	S2	S3	usage on cattle[b]	usage on swine[b]
chlortetracycline	204.3	771.6	535.9	187.1	2674	1847.2	1089.9	feed and injection	feed and injection
oxytetracycline	2.6	24.6	9.5	2.9	16	14.6	8.4	injection	injection
tetracycline	28.7	154.6	113.4	41.1	291.8	149.6	90.2		
sulfamethazine	0.7	1.6	6.3	1.8	BDL[c]	BDL	BDL		
sulfachloropyridazine	BDL	1.3	1.2	1.2	BDL	BDL	BDL		
sulfadimethoxine	BDL	0.5	3.4	1.1	ND	ND	ND	injection	
tiamulin	BDL	BDL	BDL	BDL	24.9	26.5	8.1		
tylosin	0.8	0.5	0.6	0.6	170.4	79.5	14.1	injection	injection
lincomycin	BDL	BDL	BDL	BDL	2.5	1.4	3		injection
monensin	41.4	297.1	254	137	17.5	8.2	2.7		

[a]C1−C4 are cattle manure storage ponds, and S1−S3 are swine waste treatment lagoons. [b]Usage info at the time of sampling. [c]BDL = below detection limit.

was less than 0.5% of the total DNA in nearly all sludge samples, except those from two swine lagoons S1 and S2, where the ratio of eDNA to total DNA was 1.5 and 1.2%, respectively (see Table S2 of the Supporting Information).

The eDNA yields from the sludge samples in this study are in good agreement with other studies (Table 2). These studies included environmental samples from marine sediment, reservoir sediment, and forest soil. In contrast, the iDNA yields from the sludge samples are higher than those from marine and reservoir sediments, likely because of high biomass densities in LWMS sludge, which is rich in nutrients. The number of bacteria in lagoon sludge was estimated at 10^{10}−10^{11} copies/g of sludge ww in this study by quantifying the 16S rRNA gene in iDNA. In comparison, the abundance of bacteria in the marine sediment was estimated to be 10^{3}−10^{8} cells/g of ww sediment.[33,34]

ARGs in eDNA and iDNA. All ARGs tested were detected in both the eDNA and iDNA extracts from the sludge samples except that *tet*(X) was not detected in the eDNA extracted from cattle ponds. The ARGs in eDNA extracts were usually 2−3 orders of magnitude lower than the ARGs in the corresponding iDNA extracts (Figure 4). Every gram of sludge dw contained from 1.7×10^{3} to 4.3×10^{8} copies of extracellular ARG (eARG) and from 3.2×10^{7} to 3.2×10^{10} copies of intracellular ARG (iARG) (Figure 4). A slightly decreasing trend was seen in the absolute abundance of both eARGs and iARGs, except intracellular *tet*(X), in the sequential swine lagoons, but no consistent decline was observed in the cattle ponds (Figure 4). The relative abundance of ARGs (i.e., ARG copy numbers normalized to the 16S rRNA gene copy numbers) in eDNA and iDNA was also plotted in Figure 4. The variation in the relative abundance of ARGs within each pond/lagoon series was often less than 1 order of magnitude.

The presence of eDNA in sludge suggests that HGT through transformation could be one mechanism for ARG proliferation in LWMS sludge and in soil. Because eARGs accounted for less than 1.5% of the total ARGs in LWMS sludge (see Table S2 of the Supporting Information), transformation may not be as important as the transfer mechanisms involving iARGs (i.e., vertical gene transfer and conjugation) in antimicrobial resistance proliferation in LWMS. However, on the other hand, eARGs will likely become more important in the proliferation of antimicrobial resistance in soil after sludge land application. No information is available in the literature on what percentage or how long bacteria from LWMS sludge could survive in soil. However, studies reported that bacteria in livestock solid manure could only live weeks to months in soil after land application,[35] likely because of the failure to adjust from a nutrient-rich environment (i.e., manure) to a nutrient-limited environment (i.e., soil). Assuming that sludge-originated bacteria behave similar to manure-originated bacteria in soil, cells from sludge will die and iARGs will release to the environment. eDNA released from the dead cells along with the eDNA originally from the lagoon could persist in soil for up to several years.[12,13] Using a model soil bacterium, *Azotobacter vinelandii*, Lu and co-workers measured the transformation frequency for adsorbed eDNA to be above 10^{-5} and discovered that solution chemistry, surface type, and the conformation of adsorbed eDNA have limited influence on the transformation frequency.[21] Assuming that the transformation frequency is 10^{-5} transformants per recipient cell[21] and there are 10^{10} bacteria per gram of soil,[36] theoretically, 10^{5} indigenous bacteria per gram soil could acquire ARGs via transformation.

The relative abundance of ARGs in eDNA was compared to that in iDNA (Table 3). It was found that the relative abundance of *sul*(I), *tet*(O), and *tet*(X) in eDNA was significantly lower than that of their intracellular counterparts (p = 0.0068, <0.0001, and 0.0350, respectively). In contrast, the relative abundance of *sul*(II) and *tet*(Q) was comparable in eDNA and iDNA. The lower relative abundance of some ARGs in eDNA than in iDNA is likely due to the differential degradation of various eDNA molecules after they were released from cells. Studies found that plasmid DNA is less adsorptive than chromosomal DNA and attributed this to the lower availability of surface charges and limited flexibility of plasmid DNA.[17,18,37] Adsorbed DNA is often protected from degradation by DNase in the environment,[15−17,38] suggesting genes on chromosomal DNA may be better preserved than

Table 5. Pearson's Correlation Coefficients between ARGs in iDNA and Antimicrobials in Sludge[a]

	ARG in iDNA				
	sul(I)	*sul*(II)	*tet*(O)	*tet*(Q)	*tet*(X)
chlortetracycline	**0.8237** (0.0227)	**0.7749** (0.0407)	**0.9030** (0.0053)	**0.8999** (0.0058)	0.2494 (0.5897)
oxytetracycline	0.2424 (0.6004)	0.3170 (0.4884)	0.2511 (0.5871)	0.4424 (0.3203)	−0.1056 (0.8218)
tetracycline	0.5350 (0.2160)	0.4883 (0.2662)	0.6576 (0.1084)	0.7087 (0.0746)	0.0178 (0.9698)
tylosin	0.6813 (0.0919)	0.6049 (0.1501)	**0.8212** (0.0235)	**0.7787** (0.0391)	0.2178 (0.6390)
monensin	−0.7184 (0.0690)	−0.6939 (0.0838)	**−0.7547** (0.0499)	−0.6409 (0.1209)	−0.7097 (0.0740)

[a]The numbers in parentheses are the p values calculated for the correlation coefficients. Significant correlations are bold. The correlation analyses only included samples that had at least five pairs of data above the detection limits.

plasmid DNA. The difference of the relative abundance among various eARGs might be because some ARGs were mainly located on the chromosome, while others were mainly located on the plasmid. It is worth noting that adsorbed eDNA is still available for transformation, although the transformation frequency would be lower than free DNA under certain circumstances.[39,40]

Antimicrobial Residues in Sludge. Chlortetracycline and tetracycline were the most abundant antimicrobials in the sludge of swine lagoons (Table 4), accounting for >85% of the total mass of all antimicrobials detected. Chlortetracycline and monesin were the most abundant antimicrobials in the sludge of cattle ponds, accounting for >93% of the total mass of all antimicrobials tested. In particular, the concentration of chlortetracycline was as high as 2674 mg/kg of sludge ww, which is comparable to the range of antimicrobial concentration in some manure (i.e., 1000−10000 mg/kg).[41] Macrolides (tylosin and tiamulin) were more abundant in the sludge from swine lagoons than from cattle ponds. Sulfonamide levels were low in the sludge of both cattle ponds and swine lagoons.

Antimicrobial usage on animals affected the occurrence and abundance of antimicrobials in the sludge of LWMSs. According to the diet and vaccine records, chlortetracycline and tetracycline were administrated to both cattle and swine by feeding and injection and sulfadimethoxine was the only sulfonamide compound administered to cattle through vaccines at the time of sampling (Table 4). It is noticed that, in the three swine lagoons, the reduction of antimicrobials was significant (except lincomycin), ranging from 48% reduction for oxytetracycline to 92% for tylosin, while no reduction of antimicrobials in the cattle ponds was observed. This is likely because the swine lagoons were designed to degrade organic contaminants, while cattle ponds were mainly for manure storage prior to land application.

The presence of antimicrobials may promote the emergence and proliferation of antimicrobial resistance. For example, as little as 0.15 mg/kg of sulfadiazine in soil could exert selective pressure for *sul*(II),[42] and subinhibitory concentrations of oxytetracycline (e.g., 20 μg/L) could cause the increase of the relative abundance of *tet* genes in surface water.[43] Given the high level of antimicrobial residues in LWMS sludge, land application of the sludge could contribute to the proliferation of antimicrobial-resistant bacteria in soil.

Correlation between iARGs and Antimicrobials. Antimicrobial-resistant bacteria will proliferate to dominate bacterial populations under the selective pressure of antimicrobials.[44] The relative abundance of resistant bacteria in microbial communities can be estimated using the ARG to 16S rDNA ratio in iDNA.[4] In comparison to the total DNA, iDNA provides better estimates of living bacteria. Correlation analyses were conducted between the relative abundance of ARGs in iDNA and antimicrobial residues in sludge (Table 5). Remarkably, *sul*(I), *sul*(II), *tet*(O), and *tet*(Q) all exhibited strong, positive correlations with chlortetracycline ($r \geq 0.7749$; $p < 0.05$). Tylosin also showed strong positive correlations with *tet*(O) and *tet*(Q) ($r \geq 0.7787$; $p < 0.05$).

Because chlortetracycline was the most abundant antimicrobial in the sludge (accounted for ≥50% of the total mass of all antimicrobials detected), it is likely that chlortetracycline was responsible for a significant amount of the selective pressure exerted on the microbial communities in sludge. In addition, tylosin was reported to be correlated with the occurrence of *tet*(O) and *tet*(W), and lincomycin was reported to be correlated with the occurrence of *sul*(I) and *sul*(II).[4] Different ARGs may coexist on the same genetic elements,[45−48] and any antimicrobials that select for one ARG may co-select other ARGs on the same genetic elements.[49]

■ ASSOCIATED CONTENT

Supporting Information

Water contents of the sludge in cattle manure storage ponds (C1−C4) and swine waste treatment lagoons (S1−S3) (Table S1) and eDNA and iDNA yields in cattle manure storage ponds (C1−C4) and swine waste treatment lagoons (S1−S3) (Table S2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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Intracellular and Extracellular Antimicrobial Resistance Genes in the Sludge of Livestock Waste Management Structures

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Supporting Information:

2 pages, 2 tables.

Table S1. Water contents of the sludge in cattle manure storage ponds (C1-C4) and swine waste treatment lagoons (S1-S3).

	Water content (%)
C1	42.5
C2	78.5
C3	74.0
C4	55.6
S1	92.7
S2	86.8
S3	71.2

Table S2. eDNA and iDNA yields in cattle manure storage ponds (C1-C4) and swine waste treatment lagoons (S1-S3).

	eDNA yield (μg/g dw)	iDNA yield (μg/g dw)	eDNA/total DNA (%)
C1	1.0±0.2	463.2±57.0	0.22±0.06
C2	2.8±0.6	920.1±16.6	0.31±0.06
C3	3.9±0.7	1005.3±292.9	0.41±0.09
C4	2.1±0.5	865.8±108.1	0.24±0.05
S1	9.2±5.5	584.7±106.2	1.5±0.8
S2	3.9±1.5	354.3±105.4	1.2±0.7
S3	0.9±0.2	393.9±52.0	0.23±0.04