Received: 23 September 2019 | Accepted: 27 January 2020 | Published online: 29 May 2020

DOI: 10.1002/saj2.20049

Soil Science Society of America Journal

SOIL BIOLOGY & BIOCHEMISTRY

Influence of environmental conditions on extracellular and intracellular antibiotic resistance genes in manure-amended soil: A microcosm study

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Abstract

The objectives of this study were to modify the protocol of a commercial DNA extraction kit to sequentially extract extracellular DNA (eDNA) and intracellular DNA (iDNA) from the same soil sample and to determine the effects of temperature (5, 20, and 35 C), water holding capacity (25, 50, and 75% of maximum water holding capacity), and freeze-thaw (−20/20 C) on t he abundance of two antibiotic resistance genes [sul1 and tet(M)], a class 1 integron-integrase gene (intI1), and a 16S rRNA gene in soils treated with dairy manure. To assess the efficiency of the eDNA/iDNA method, we performed spiking and recovery experiments with a gene that codes for a green fluorescent protein (gfp). When soils were spiked with a whole-cell preparation of gfp-containing Escherichia coli, the recovery of the gfp gene was on average 0.2 and 1.2% for eDNA and iDNA, respectively. Soils were also spiked with t he gfp gene itself, which showed that nearly 80% of the DNA could not be recovered. Results from the microcosm experiments indicate that extracellular and intracellular sul1, tet(X), intI1, and 16S rRNA genes are resilient and not readily affected by changes in soil moisture and temperature. The intracellular gene levels decreased only slightly during the 56-d incubation period, whereas the extracellular gene levels decreased dramatically by Day 7 and leveled off thereafter. Absolute gene abundances were one to two orders of magnitude higher in iDNA than in eDNA fractions but were similar when normalized to the 16S rRNA gene throughout the incubation period, indicating that enrichment of extracellular and intracellular antibiotic resistance genes did not occur.

1 | INTRODUCTION

Antimicrobial resistance (AMR) is a growing threat to the health, welfare, and economies of the world. If the rise of AMR is not mitigated, it is estimated that 10 million people will die annually by 2050 (compared with 700,000 now) and that a reduction of 2.0 to 3.5% in the gross domestic product will occur (O'Neill, 2016). Although no one can predict future outcomes, especially when other unknown human and

Abbreviations: AMR, antimicrobial resistance; ANCOVA, analysis of covariance; ARB, antibiotic-resistant bacteria; ARG, antibiotic resistance gene; eARG, extracellular antibiotic resistance gene; eDNA, extracellular DNA; iARG, intracellular antibiotic resistance gene; iDNA, intracellular DNA; PCR, polymerase chain reaction; PPS, protein precipitation solution; qPCR, quantitative real-time polymerase chain reaction; SPB, sodium phosphate buffer; tDNA, total DNA; WHC, water holding capacity; WHC_{max}, maximum water holding capacity.

environmental factors could influence the problem, current projections indicate that an AMR crisis is looming. At the microbial level, AMR is exacerbated by conditions that enrich antibiotic-resistant bacteria (ARB) as well as the horizontal gene transfer of antibiotic resistance genes (ARGs) from environmental to pathogenic species (von Wintersdorff et al., 2016). The use of antibiotic drugs can selectively enrich ARB and ARGs; however, the extent of t his contribution is poorly understood in environmental settings given that AMR is a naturally occurring event (Franklin et al., 2016). Although much attention is given to livestock production as a major source of environmental AMR, research has shown that ARB are also present in native soils not grazed by animals (Durso, Wedin, Gilley, Miller, & Marx, 2016), can be found in antibiotic-free poultry farms (Rothrock, Hiett, Guard, & Jackson, 2016), and are shed at similar levels between beef cattle raised with and without antibiotics (Agga, Schmidt, & Arthur, 2016). Given that ARGs are the underlying mechanism for AMR and are widespread in the environment, it has been suggested that they are emerging contaminants that should be controlled (Pruden, Pei, Storteboom, & Carlson, 2006; Rysz & Alvarez, 2004).

The total DNA (tDNA) of soil and other environmental matrices (e.g., sediment, water, manure) consists of extracellular DNA (eDNA) and intracellular DNA (iDNA). Once DNA becomes extracellular through cell lysis or excretion, it can be degraded by nucleases or heterotrophic bacteria, persist through transformation by competent bacteria (Pietramellara et al., 2009), adsorb to soil colloids and sand particles (Cai, Huang, & Zhang, 2006; Ogram, Sayler, Gustin, & Lewis, 1988), and be used in biofilm formation and maintenance (Bockelmann et al., 2006; Wu & Xi, 2009). Extracellular DNA can also move through the soil profile by water capillarity or leaching (Ceccherini, Ascher, Pietramellara, Vogel, & Nannipieri, 2007). Antibiotic resistance genes can be present as eDNA and iDNA; thus, it is important to know their distribution in these fractions if mitigation strategies are to be developed to prevent their dissemination. Intracellular ARGs (iARGs) can only be spread through cell replication, conjugation, and transduction (Alekshun & Levy, 2007), whereas extracellular ARGs (eARGs) can be spread through transformation and degraded/absorbed. Based on information about the presence and persistence of eDNA in soils and sediments (Levy-Booth et al., 2007; Pietramellara et al., 2009), eARG-based transformation could be playing an important role in the proliferation of AMR in these environments (Zhang, Snow, Parker, Zhou, & Li, 2013). Because eARGs and iARGs likely possess significant differences with respect to their mobility and availability to indigenous bacteria, it is important to understand their partitioning, persistence, and fate in the environment (Mao et al., 2014).

Starting in the late 1980s, procedures were developed or modified for the isolation of eDNA and iDNA from environmental samples, such as sediments and soils (Agnelli et al., 2004; Ascher et al., 2009; Corinaldesi, Danovaro, & Dell'Anno, 2005; Lever et al., 2015; Ogram, Sayler, & Barkay, 1987). Early extraction methods relied on the use of customized reagents and extraction protocols, but then there was a shift toward the use of commercially available DNA extraction kits (Ascher et al., 2009; Zhang, Niu, Zhang, & Zhang, 2018). Although t here is no universally accepted "best extraction method" for eDNA and iDNA for all sample types, the use of kits has many advantages, such as prepared reagents and streamlined procedures (Lever et al., 2015). Molecular biology results could then be cross-compared among facilities that used the same kit, assuming similar samples are targeted for investigation, given that DNA extraction efficiencies will vary based upon the sample type and microbial community characteristics (Zhou, Bruns, & Tiedje, 1996). To improve our understanding of the persistence and fate of ARGs within eDNA and iDNA pools, we conducted a soil microcosm study to investigate the influence of environmental factors. The objectives of this study were (a) to modify the protocol of a commercial DNA extraction kit to sequentially extract eDNA and iDNA from the same soil sample and (b) to determine the effect of temperature, water holding capacity (WHC), and freeze-thaw on the abundance of selected ARGs in soil with dairy manure treatment.

Core Ideas
- Antibiotic resistance genes can be extracellular and intracellular in the environment.
- A soil DNA extraction kit was used to sequentially extract extracellular and intracellular DNA.
- Extracellular and intracellular gene levels were resilient under changing soil conditions.
- Enrichment of extracellular and intracellular antibiotic resistance genes did not occur.

2 | MATERIALS AND METHODS

2.1 | Sequential extraction of extracellular and intracellular DNA

Extracellular and intracellular DNA were sequentially extracted from soil using a modified version of the FastDNA Spin Kit for Soil (MP Biomedicals) protocol based on the method developed by Ascher et al. (2009). The kit components, such as the lysing matrix, sodium phosphate buffer (SPB), MT buffer, and protein precipitation solution (PPS), are proprietary materials; thus, we could not obtain details about their chemical composition. Prior to following the manufacturer's protocol, a crude eDNA extraction was

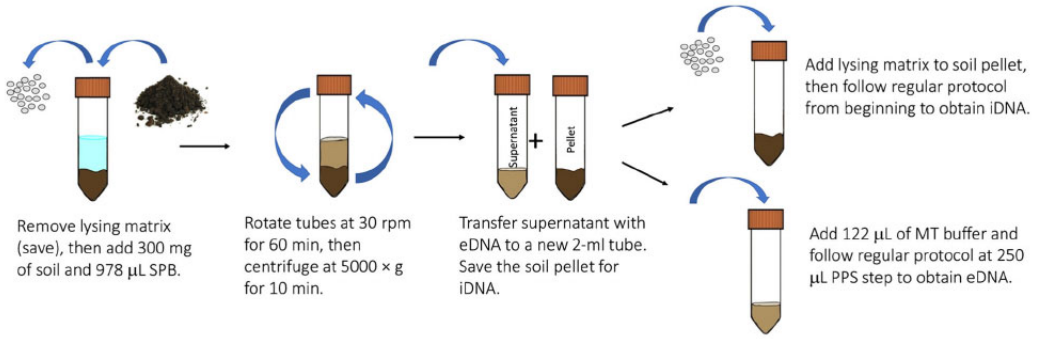

FIGURE 1 Modifications to the initial steps of FastDNA Spin Kit for Soil protocol to obtain extracellular DNA (eDNA) and intracellular (iDNA) from soil. PPS, protein precipitation solution; SPB, sodium phosphate buffer.

performed (Figure 1). This was accomplished by first removing (and saving) the lysing matrix from the tube, followed by the addition of 300 mg of field moist soil and 978 l of SPB to the empty lysing matrix tube. Next, the tubes with soil and SPB were rotated at 30 r pm for 60 min, followed by centrifugation at 5000 × g for 10 min to pellet the solids, and then the supernatant was transferred to a new 2-ml microcentrifuge tube. At this point, the supernatant and pelleted solid contain the eDNA and iDNA, respectively. The crude eDNA (supernatant) was purified by first adding 122 l of MT buffer and then following the manufacturer's protocol starting with the addition of 250 l of protein precipit ation solution. The iDNA was extracted by placing the lysing matrix back into the tube with the pelleted solids and performing the extraction following the manufacturer's protocol. Tot al DNA extractions were performed using the same kit following the manufacturer's protocol as stated without modification.

2.2 | Soil spiking and recovery experiments

Frozen (−75 C) field moist soil samples from a long-term field study (McKinney, Dungan, Moore, & Leytem, 2018) were used in spiking experiments to determine the recovery of a gene that codes for a green fluorescent protein (gfp) following extracellular, intracellular, and total DNA extractions. The soil was a Portneuf silt loam (coarse-silty, mixed, superactive, mesic Durinodic Xeric Haplocalcids), and samples were from plots that had received dairy manure at rates of 0, 17, 35, and 52 Mg ha^{-1} in fall 2012 but were collected the following spring from the 0- to 30-cm layer. Soils were spiked with either DNA or whole cells of Escherichia coli O157:H7 strain B6-914 gfp, which does not produce Shiga-like toxins I or II (Fratamico, Deng, Strobaugh, & Palumbo, 1997). The gfp genes harbored in this E. coli strain were used because they are not found in soil microorganisms. Escherichia coli gfp was grown overnight at room temperature in Tryptic soy broth, followed by centrifugation at 5000 × g for 10 min at 5 C to pellet the cells, then washed twice and resuspended in 50 mM potassium phosphate buffer (PPB) (KH_2PO_4 and K_2HPO_4, pH 7.2) to an optical density of 2.0 at 600 nm. Colony-forming units of the whole cell suspensions were determined by plating 10-fold serial dilutions on Tryptic soy agar plates and only counting plates with 20–300 colonies. A value of 250 gfp gene copies per E. coli cell, as determined via the method of Lee, Kim, Shin, and Hwang (2006), was used to estimate the number of gfp copies in the whole cell spikes. DNA spikes were prepared by performing DNA extractions on E. coli gfp grown overnight in Tryptic soy broth using the DNeasy Blood and Tissue Kit (Qiagen) following the gram-negative bacteria protocol. Quantitative real-time PCR (qPCR) was used to determine the abundance of gfp genes in the DNA extracts. Using the above information, 100 l of whole cells or 50 l of DNA were spiked into the soil samples. A 15-min acclimation period at room temperature was used between the spiking of DNA or whole cells into soil and the start of DNA extractions. All experiments were performed in triplicate.

2.3 | Soil incubation experiments

The Portneuf silt loam (pH 8.2; electrical conductivity, 0.85 dS m^{-1}; total C, 15.8 g kg^{-1}; total N, 0.8 g kg^{-1}; bulk density, 1.4 g cm^{-3}) used in this study was obtained from a field wit hout prior manure application. Manure (total C, 174 g kg^{-1}; total N, 12.5 g kg^{-1}) was obtained as lot scrapings from a local dairy. Prior to their use, the soil and manure were air dried and then sieved (2 mm) or ground, respectively. Soil incubation experiments were conducted to determine the effect of temperature, moisture, and freeze-thaw on

two ARGs [sul1 and tet(M)], the class 1 integron-integrase gene (intI1), and the 16S rRNA gene. All experiments were performed in triplicate with 10 g of soil amended with 3% manure (w/w, dry wt. basis) in 21-ml glass headspace vials, which were capped with polytetrafluoroethylene/butyl septa and aluminum seals (Millipore Sigma). Triplicate vials were created for all sampling dates, so each set could be destructively sampled for eDNA/iDNA without having to disturb vials incubated for longer time periods. The temperature experiment was conducted at 5, 20, and 35 C in the dark with the soil mixtures adjusted to 50% of their maximum WHC (WHC_{max} = 0.34 kg kg $^{-1}$) using sterile deionized water. For the moisture experiment, the soils were adjusted to 25, 50, and 75% of the WHC_{max} and incubated at 20 C in the dark. After adjustment of the moisture, the soil mixtures were sieved (2–4 mm) prior to their addition to the vials. To keep the conditions within the vials aerobic, 27-gauge hypodermic needles wit h cotton plugs were inserted into the septa. Sample vials were removed from their respective incubator once a week for 8 wk (including Week 0) and frozen at −20 C until processed for DNA. For the freeze-thaw experiments, all samples were allowed to equilibrate for 7 d at 50% WHC_{max} and 20 C, after which a set of sample vials was removed for DNA extraction. The remaining sample vials were immediately frozen at −20 C for 3 d, followed by i ncubation at 20 C for 4 d, after which a set of samples was removed for DNA extraction. The freeze-thaw cycle was repeated weekly until all eight sample sets had been processed.

2.4 | Quantification of genes

Quantitative real-time PCR was used to quantify the antibiotic resistance, gfp, intI1, and 16S rRNA genes on a CFX96 Touch Real-Time P CR Detection System (Bio-Rad). Each individual reaction consisted of 12.5 l of 2× SsoAdvance Universal Probes Supermix (Bio-Rad), 250 nM of forward and reverse primers and probes (Integrated DNA Technologies), 2 l of DNA template (10-fold diluted in tris–ethylenediaminetetraacetic acid buffer solution), and ster ile DNase/pyrogen-free water to create a final volume of 25 l. Primer and probe sequences, annealing temperatures, and amplicon lengths for all target genes (except the gfp gene) can be found in Dungan, McKinney, and Leytem (2018). Primer and probe sequences and amplicon length for the gfp gene can be found in Klerks, Zijlstra, and Van Bruggen (2004); an annealing temperature of 55 C was used. Probes include a 6-FAM 5 fluorophore, a ZEN internal quencher, and a 3 Iowa Black FQ quencher (Integrated DNA Technologies). The thermocycler conditions consisted of one cycle at 95 C for 3 min, 40 cycles at 95 C for 15 s, and annealing temperature for 30 s. The qPCR runs included a standard curve covering seven orders of magnitude (10^2–10^8 gene copies $^{-1}$), and each sample was analyzed in duplicate. Standards were created using gBlocks gene fragments (Integrated DNA Technologies). All standard curve r^2 values were consistently .99, and amplification efficiencies ranged from 71 to 109%. Absolute abundance refers to gene copies per gram dry soil; relative abundance refers to gene copies per 16S rRNA gene copies.

2.5 | Statistical analysis

The statistical analyses were performed in SAS (SAS Institute, 2004). All gene data were $\log_{10}$ transformed prior to analysis to improve the homogeneity of variance and normality. The REG (linear regression) Procedure was used to generate slope estimates and P values to determine if the slopes were significantly different from zero. Analysis of covariance (ANCOVA) in the GLM (generalized linear model) Procedure was used to generate P values to determine whether slopes were significantly different from each other. Statistical significance was determined using an = .05.

3 | RESULTS AND DISCUSSION

3.1 | Sequential extraction of extracellular and intracellular DNA

Most studies using the simultaneous or sequential extraction of eDNA and iDNA from soil, sediment, or sludge are time consuming and laborious due to making reagents, performing the extractions from scratch (as opposed to using a kit), and lengthy protocols (Alawi, Schneider, & Kallmeyer, 2014; Corinaldesi et al., 2005; Mao et al., 2014; Zhang et al., 2013). Our study used a slightly modified protocol of the FastDNA Spin Kit for Soil (MP Biomedicals) using only the reagents and supplies that come wit h the kit, except that additional 2-ml microcentrifuge tubes are needed. This eDNA/iDNA sequential extraction is similar to that by Ascher et al. (2009), except they deviated f rom the manufacturer's protocol during the purification of crude eDNA and iDNA extracts, whereas we did not. All five of the previously mentioned eDNA/iDNA extraction publications perform three washing steps (shaking or rotating) on the environmental matrix, followed by centrifugation to separate the matrix from the supernatant with the crude eDNA. In the present study, however, only one washing step was implemented because the soil would form a very dense pellet after centrifugation that would not resuspend during the second washing. This likely occurred because our test soil is a silt loam and contains a high percentage of silt (71%) and some clay (9%). During preliminary tests, increasing the rotational speed to 60 rpm during washing and reducing the centrifugal force by an order of magnitude (i.e., 500 × g) did not alleviate the pelleting issue (data not

shown). The only way to resuspend the soil into solution was to aggressively vortex, which in turn could lyse cells and add iDNA to the eDNA fraction. Therefore, we decided to perform only one washing step but compensated by increasing the volume of SPB from 300 l (per washing step) to 978 l (to match the volume added for iDNA extraction) and increasing the rotation time from 30 to 60 min. In addition, four eDNA/iDNA extraction papers (Alawi et al., 2014; Corinaldesi et al., 2005; Mao et al., 2014; Zhang et al., 2013) used membrane filters (0.02–0.22 m pore size) to remove contaminating microbial cells and viruses from the supernatant containing the eDNA. We did not filter our supernatant because DNA can become trapped on membrane filters, resulting in a underestimation of the eDNA fraction (Liang & Keeley, 2013). In addition, microbial cells will be pelleted during centrifugation and viral DNA will not be amplified because we used bacterial-specific primers and probes during PCR.

Experiments were conducted to determine the effectiveness of the DNA extraction methods (i.e., extracellular, intracellular, and total) to recover a plasmid-encoded gfp gene that was spiked into soil as a DNA preparation or whole cells. When DNA was spiked into the soils, 7.4–9.4% of the gfp genes were recovered during eDNA extraction, and 8.6–11.0% were recovered in iDNA extracts (Table 1). In this case, recovery of the gfp gene in the iDNA fraction means that about 10% of the DNA from the spike was probably adsorbed to soil particles and/or physically trapped within microsites, but it was released during the iDNA extraction procedure. With about 8% of the spike DNA being contained in the eDNA fraction, the remaining 82% of the gfp DNA was likely still adsorbed to soil particles and/or degraded/sheared to some extent, making it ineffective for PCR amplification. When the soils were spiked with whole cells, the mean recovery of the gfp gene ranged from 0.1 to 0.2% for eDNA and from 1.1 to 1.3% for iDNA (Table 1). The presence of gfp genes in the eDNA fraction is probably due to the excretion of DNA from viable and dead (lysed) cells, which could have occurred during the sequential extraction procedure. In addition, extracellular gfp genes were also detected in the supernatant of whole-cell suspensions at 3.0×10^{9} gfp copies ml^{-1} using qPCR, compared with 1.0×10^{13} copies ml^{-1} based on counts of viable whole cells.

Overall, the mean recovery of eDNA+iDNA was relatively low at 1.3% in soil spiked with whole cells (Table 1). The low recovery of the gfp gene could be due to a variety of factors, such as DNA adsorbing to soil particles and plastic microcentrifuge tubes, DNA shearing during bead beating, DNA degradation due to endonucleases and heterotrophic bacteria, and/or incomplete elution of the DNA from the spin filter. Another interesting result from the spiking study was that the amount of eDNA+iDNA recovered was higher than that from tDNA extractions for both DNA and whole-cell tests. This likely occurred because pre-lysis washing of soil improves DNA extraction recovery by helping desorb eDNA from soil particles through physical agitation (Ascher et al., 2009; He, Xu, & Hughes, 2005).

Comparing DNA versus whole-cell spiking, the mean recovery was 7–10% higher for soil s piked with DNA and then with whole cells, no matter which of the three DNA extraction techniques was performed (Table 1). When whole cells lyse, they release cellular components (proteins, enzymes, cell walls, etc.) that could adsorb DNA and cause it to pellet during DNA extraction. A DNA solution extracted from a pure culture should not contain (or contain much less of) these cellular components. Because the same soil samples were used for both whole-cell and DNA spiking studies, they should contain the same microbial biomass prior to spiking. Spiking whole cells into the soil will increase the cellular components much higher after lysing then spiking with DNA, which could be a reason the DNA extraction efficiency is higher in DNA spiked soil samples compared with whole cell spiked soil samples. Also, qPCR was used to determine the number of gfp copies in the DNA spikes, and serial dilutions, plating, and estimated gfp copies per cell were used to determine the number of gfp copies in the whole-cell spikes. Because both methods contain their own biases, it could also be partially responsible for the gfp recovery differences we found between the soils spiked with DNA or whole cells. Finally, manure application did not have an effect on eDNA, iDNA, or tDNA extraction efficiencies because the recoveries were all within 2% of one another and no clear trends emerged (Table 1).

3.2 | Effect of temperature, water holding capacity, and freeze-thaw on extracellular and intracellular DNA

Soil amended with dairy manure (3% w/w, dry wt. basis) was used to compare the effect of temperature, WHC, and freeze-thaw on the abundance of extracellular and intracellular sul1, tet(M), intI1, and 16S rRNA genes. Even though intI1 does not promote resistance to any pollutants, its linkage to antibiotic, metal, and disinfectant resistance genes and its ability to readily transfer between diverse groups of bacteria make it a good proxy for ARG pollution (Gillings et al., 2015). Antibiotic resistance gene detection in soils without manure was very infrequent and usually below the limit of quantification (data not shown), whereas this was not the situation in manure-amended soil (Figures 2–5). It is well known that the incorporation of manure into soil increases the abundance and diversity of ARGs in soil (Heuer, Schmitt, & Smalla, 2011; Jechalke et al., 2013; McKinney et al., 2018; Ruuskanen et al., 2016; Udikovic-Kolic, Wichmann, Broderick, & Handelsman, 2014). In addition to sul1 and tet(M), we

TABLE 1 Recovery of the gfp gene from spiked field soils as determined by quantitative polymerase chain reaction after extracellular DNA (eDNA), intracellular DNA (iDNA), and total DNA extractions

Manure application rate	Spike	Extraction type	Mean spiked gfp copies	Mean extracted gfp copies	Mean recovered	Mean recovered eDNA + iDNA
Mg ha^{-1}			g dry soil^{-1}		%	
0	DNA	extracellular	2.16×10^{10}	1.99×10^{9}	9.2	17.8
		intracellular		1.87×10^{9}	8.6	
		total	2.19×10^{10}	2.22×10^{9}	10.2	10.2
	whole cells	extracellular		6.30×10^{9}	0.2	
		intracellular	3.95×10^{12}	4.68×10^{10}	1.2	1.3
		total	3.95×10^{12}	3.39×10^{10}	0.9	0.9
17	DNA	extracellular	2.20×10^{10}	2.06×10^{9}	9.4	19.1
		intracellular		2.14×10^{9}	9.7	
		total	2.21×10^{10}	2.06×10^{9}	9.3	9.3
	whole cells	extracellular		6.35×10^{9}	0.2	
		intracellular	3.98×10^{12}	4.36×10^{10}	1.1	1.3
		total	3.99×10^{12}	3.85×10^{10}	1.0	1.0
35	DNA	extracellular	2.19×10^{10}	1.63×10^{9}	7.4	18.0
		intracellular		2.31×10^{9}	10.5	
		total	2.21×10^{10}	2.25×10^{9}	10.2	10.2
	whole cells	extracellular		6.25×10^{9}	0.2	
		intracellular	3.98×10^{12}	5.08×10^{10}	1.3	1.4
		total	3.96×10^{12}	3.34×10^{10}	0.8	0.8
52	DNA	extracellular	2.20×10^{10}	1.80×10^{9}	8.2	19.2
		intracellular		2.42×10^{9}	11.0	
		total	2.19×10^{10}	2.23×10^{9}	10.2	10.2
	whole cells	extracellular		4.86×10^{9}	0.1	
		intracellular	3.97×10^{12}	4.77×10^{10}	1.2	1.3
		total	4.00×10^{12}	3.68×10^{10}	0.9	0.9

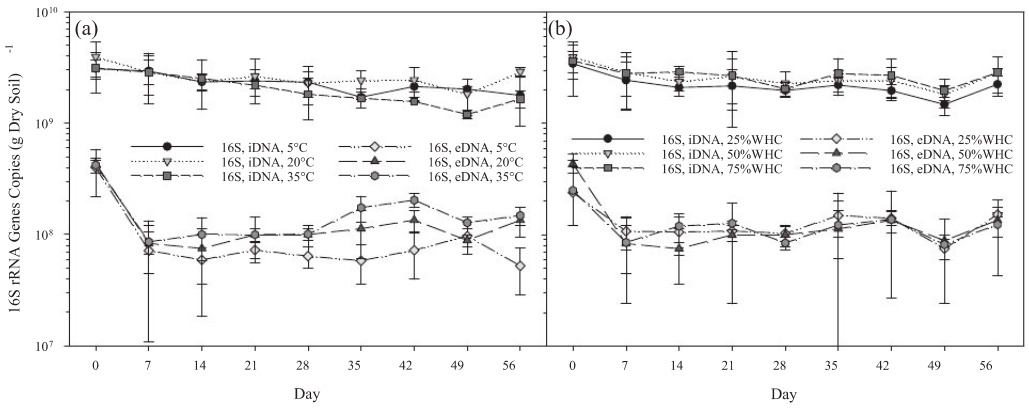

FIGURE 2 Effect of (a) temperature and (b) water holding capacity (WHC) on extracellular and intracellular 16S rRNA gene (g dry soil) $^{-1}$ abundances over time in soil amended with 3% dairy manure (w/w, dry wt. basis). The temperature experiments were conducted at 50% WHC_{max}, and the WHC experiments were conducted at 20 C. Error bars are 95% confidence intervals.

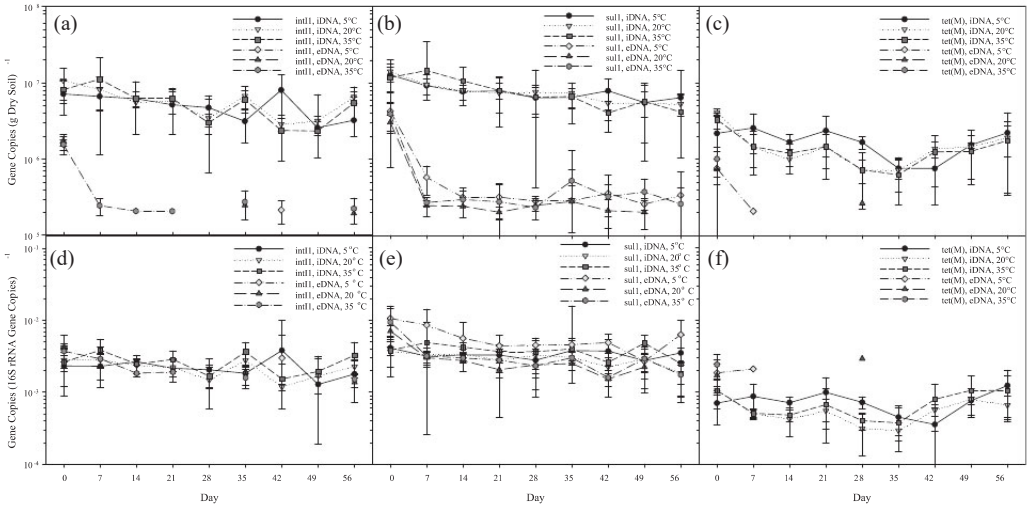

FIGURE 3 Effect of temperature (5, 20, and 35 C) on extracellular and intracellular gene abundances over time in soil amended with 3% dairy manure (w/w, dry wt. basis). Absolute abundance [gene copies (g dry soil)$^{-1}$] of (a) intI1, (b) sul1, and (c) tet(M). Relative abundance [gene copies (16S rRNA gene copies)$^{-1}$] of (d) intI1, (e) sul1, and (f) tet(M). The temperature experiments were conducted at 50% WHC_{max}. Error bars are 95% confidence intervals.

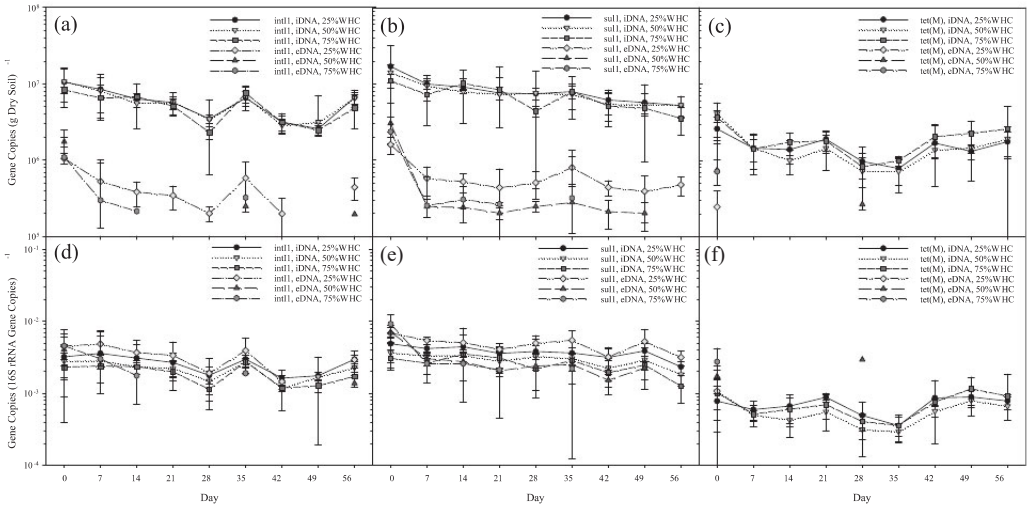

FIGURE 4 Effect of maximum water holding capacity (25, 50, and 75% WHC_{max}) on extracellular and intracellular gene abundances over time in soil amended with 3% dairy manure (w/w, dry weight). Absolute abundance [gene copies (g dry soil)$^{-1}$] of (a) intI1, (b) sul1, and (c) tet(M). Relative abundance [gene copies (16S rRNA gene copies)$^{-1}$] of (d) intI1, (e) sul1, and (f) tet(M). The WHC experiments were conducted at 20 C. Error bars are 95% confidence intervals.

attempted to quantify erm(B); however, it was not detected at a high enough frequency and level in manure-amended soil to draw conclusions (data not shown). This is a surprising result because erm(B) is routinely detected in agricultural soils that have a history of manure treatment (Dungan, Strausbaugh, & Leytem, 2019).

Figure 2 shows the effect of temperature and WHC on the absolute abundance of the 16S rRNA gene over time in eDNA

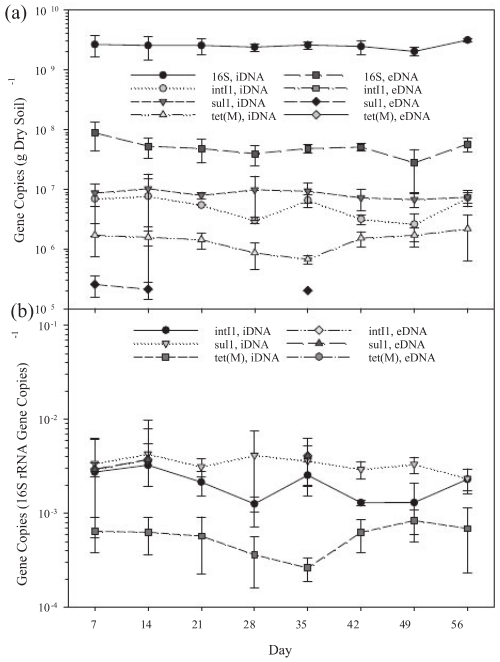

FIGURE 5 Effect of freeze-thaw conditions (20/−20 C) on extracellular and intracellular gene abundances over time in soil amended with 3% dairy manure (w/w, dry wt. basis). (a) Absolute abundance [gene copies (g dry soil)$^{-1}$] and (b) relative abundance [gene copies (16S rRNA gene copies)$^{-1}$]. The freeze-thaw experiments were conducted at 50% WHC_{max}. Error bars are 95% confidence intervals.

and iDNA fractions from manure-amended soil. Overall, there was little effect of these parameters, at least within t he ranges tested, on 16S rRNA gene levels in the two DNA fractions. This is particularly surprising because both soil temperature and moisture have a large influence over the growth characteristics of microorganisms, and therefore one would expect to see more dramatic differences. There was a shar p decline in the extracellular 16S rRNA gene level between 0 and 7 d, which was probably due to the development of strong bonds with soil colloids, degradation by endonucleases and heterotrophic bacteria, and/or transformation into competent bacteria. Regarding intracellular 16S rRNA gene levels, only a slight decrease occurred by Day 56.

The effect of temperature and WHC on the absolute and relative abundance of intI1, sul1, and tet(M) in eDNA and iDNA fractions from manure-amended soil are presented in Figures 3 and 4, respectively. Similar to the 16S rRNA gene results, these dat a demonstrate that temperature and WHC had little effect on the ARG levels, regardless of the DNA fraction. When calculated on an absolute basis, extracellular intI1 and sul1 levels displayed a sharp decline between 0 and 7 d, which then leveled off thereaf ter (Figures 3A,B and 4A,B). In contrast, extracellular tet(M) was rarely detected past Day 0 (Figures 3C and 4C). The rapid initial dissipation of the eARGs could be a result of sorption, degradation, and/or transformation, similar to what we believe occurred with extracellular 16S rRNA. Except for Day 0, the eARG absolute abundances were approximately one to two orders of magnitude lower than the iARG abundances (Figures 3 and 4). This is in agreement with Zhang et al. (2013), in which eARGs were found to be two to three orders of magnitude lower then iARGs (on an absolute basis) in sludge samples from cattle manure storage ponds and swine waste treatment lagoons but is in contrast with Mao et al. (2014), who found that eARG levels were significantly greater than iARGs by approximately 0.5 orders of magnitude in river sediments. To our knowledge, no studies have been conducted to address eARG and iARG abundances in soil. When the ARG levels from the present study were calculated on a relative basis, there was little difference between the eARG and iARG levels, as well as little change in the trend throughout the 56-d incubation period (Figures 3D,E and 4D,F). These consistent trends indicate that enrichment of extracellular and intracellular genes (i.e., intI1 and sul1) did not occur at any time during the study period.

Freeze-thaw events have the potential to influence the function and structure of soil microbial communities (Sharma, Szele, Schilling, Munch, & Schloter, 2006). However, aside from studies that have addressed cold storage and pretreatment conditions on soil microbial properties (Lee, Lorenz, Dick, & Dick, 2007), there is no information on freeze-thaw effects on the abundance of soil ARGs, including those in extracellular and intracellular f ractions. The present study was not designed to mimic natural freeze-thaw conditions as discussed by Henry (2007) but to determine the stability of ARGs under more extreme temperature changes. Figure 5 shows the effect of seven freeze-thaw (−20 C/20 C) cycles on extracellular and intracellular ARGs and16S rRNA gene. Every gene that had enough data points to perform a linear regression had slopes that are not significantly different from zero, except for extracellular 16S rRNA and intracellular sul1 absolute abundances (Table 2). This indicates that there were no freeze-thaw effects on eDNA and iDNA levels over the 7-wk experiment. Because freeze-thaw is a nondiscriminatory physical degradation process, it is not surprising that all genes were affected in a similar way. Because the ARG levels did not decrease with time, it demonstrates their ability to resist degradation and the potential ability to be horizontally transferred once soils are thawed after long freezing events.

Plotting the time-course data on semi-log plots (Figures 2–5) produced fairly flat linear responses over time. To determine if the slope of the line is significantly different from zero

TABLE 2 Slope and P values of linear regressions showing the effect of temperature, water holding capacity, and freeze-thaw on extracellular DNA (eDNA) and intracellular DNA (iDNA) in soil incubation experiments

			Temperature			Water holding capacity			Freeze-thaw (20/−20 C)	
Abundance	Gene	e/iDNA	C	Slope	P Value	%	Slope	P Value	Slope	P Value
Absolute (per g dry soil)	16S rRNA	eDNA	5	−0.00717	.0092	25	−0.00198	.2236	−0.00429	.0197
			20	−0.00328	.1757	50	−0.00328	.1757		
			35	−0.00102	.6609	75	−0.00263	.1622		
		iDNA	5	−0.00399	<.0001	25	−0.00334	.0007	0.00009	.9201
			20	−0.00269	.0077	50	−0.00269	.0077		
			35	−0.00654	<.0001	75	−0.00219	.0287		
	intI1	eDNA	5	–	–	25	−0.00556	.0376	–	–
			20	–	–	50	–	–		
			35	−0.00948	.0277	75	–	–		
		iDNA	5	−0.00635	.0005	25	−0.00679	.0006	−0.00414	.0960
			20	−0.00636	.0026	50	−0.00636	.0026		
			35	−0.00854	.0002	75	−0.00645	.0017		
	sul1	eDNA	5	−0.01290	.0005	25	− 0.00597	.0023	–	–
			20	−0.01623	.0039	50	−0.01623	.0039		
			35	−0.00947	.0140	75	−0.02107	.0232		
		iDNA	5	−0.00491	.0002	25	−0.00721	<.0001	−0.00251	.0220
			20	−0.00671	<.0001	50	−0.00671	<.0001		
			35	−0.00921	<.0001	75	−0.00739	<.0001		
	tet(M)	eDNA	5	–	–	25	–	–	–	–
			20	–	–	50	–	–		
			35	–	–	75	–	–		
		iDNA	5	−0.00439	.0436	25	−0.00245	.1610	0.00143	.5240
			20	−0.00326	.1967	50	−0.00326	.1967		
			35	−0.00402	.0827	75	−0.00045	.8392		
Relative (per 16S rRNA gene)	intI1	eDNA	5	–	–	25	−0.00523	.0060	–	–
			20	–	–	50	–	–		
			35	−0.00678	<.0001	75	–	–		
		iDNA	5	−0.00236	.1058	25	−0.00345	.0184	−0.00423	.0622
			20	−0.00368	.0155	50	−0.00368	.0155		
			35	−0.00200	.2265	75	−0.00426	.0044		
	sul1	eDNA	5	−0.00586	.0012	25	− 0.00400	.0004	–	–
			20	−0.00927	.0002	50	−0.00927	.0002		
			35	−0.00845	<.0001	75	−0.01664	.0030		
		iDNA	5	−0.00092	.3303	25	−0.00387	<.0001	−0.00259	.0774
			20	−0.00402	<.0001	50	−0.00402	<.0001		
			35	−0.00267	.0373	75	−0.00520	<.0001		
	tet(M)	eDNA	5	–	–	25	–	–	–	–
			20	–	–	50	–	–		
			35	–	–	75	–	–		
		iDNA	5	−0.00040	.8358	25	0.00089	.5731	0.00135	.5516
			20	−0.00057	.7752	50	−0.00057	.7752		
			35	0.00251	.2173	75	0.00173	.3734		

Note. Bold values indicate s lopes that are significantly different from zero and have a $P < .05$. Slope values shown are estimates derived from simple linear regression of $\log_{10}$(gene copies) vs day. Dashes indicate that there were not enough data points to estimate the slope.

TABLE 3 Statistical comparison of linear regression slopes for extracellular DNA (eDNA) and intracellular DNA (iDNA) between the different temperatures and water holding capacities in the soil incubation experiments

			Temperature			Water holding capacity		
Abundance	Gene	e/iDNA	5 vs. 20 C	5 vs. 35 C	20 vs. 35 C	25 vs. 50%	25 vs. 75%	50 vs. 75%
Absolute (per g dry soil)	16S rRNA	eDNA	0.2557	0.0738	0.5065	0.6366	0.8129	0.8134
		iDNA	0.2292	0.0206	0.0006	0.6122	0.3720	0.6984
	intI1	eDNA	–	–	–	–	–	–
		iDNA	0.9966	0.4009	0.4033	0.8682	0.8946	0.9733
	sul1	eDNA	0.5654	0.4853	0.2438	0.0355	0.0379	0.5220
		iDNA	0.2623	0.0087	0.1219	0.7408	0.9084	0.6558
	tet(M)	eDNA	–	–	–	–	–	–
		iDNA	0.7238	0.9094	0.8106	0.7911	0.5114	0.3576
Relative (per 16S rRNA gene)	intI1	eDNA	–	–	–	–	–	–
		iDNA	0.5322	0.8652	0.4275	0.9082	0.6786	0.7647
	sul1	eDNA	0.1989	0.2514	0.7549	0.0248	0.0005	0.0461
		iDNA	0.0324	0.2224	0.3454	0.9096	0.3155	0.3728
	tet(M)	eDNA	–	–	–	–	–	–
		iDNA	0.9498	0.2947	0.2670	0.5731	0.7448	0.3749

Note. Bold values indicate slopes that are significantly different from each other ($P < .05$). Dashes indicate that there were not enough data points to estimate a slope for at least one of the lines.

(P .05), linear regression was performed in SAS, and the results can be seen in Table 2. The slope estimates [$\log_{10}$(gene copies) d^{-1}] given were produced using $\log_{10}$ transformed gene copies to compare the magnitude and direction (positive or negative) of the slopes with one another. Also, a slope of 1, 0, and −1 means an increase, no change, or decrease, respectively, of gene copies by an order of magnitude per day. Thus, a slope value can give you the percent change in gene copies. For example, a slope of −0.004 $\log_{10}$ (gene copies) d^{-1} would be equal to a 0.4% reduction in gene copies per day. Looking at Table 2, the majority (48%) of the slopes are slightly negative and significantly different from zero, whereas 26% were not significantly different from zero. For 27% of slopes there were not enough data points to estimate a slope. The slope estimates that are not significantly different from zero indicate no response to the specific treatment.

An ANCOVA model was used to determine if the slopes within the specified treatment comparisons were significantly different from one another (Table 3). The absolute abundance of intracellular 16S rRNA at 35 C has a significantly greater negative slope [−0.00654 $\log_{10}$ (gene copies) d^{-1}] than at 5 C [−0.00399 $\log_{10}$ (gene copies) d^{-1}] and 20 C [−0.00269 $\log_{10}$ (gene copies) d^{-1}] (Tables 2 and 3). Looking at Figure 2A (iDNA), this difference is probably caused by the lower abundance for 35 C at 49 d of incubation, causing the slope to be more negative than the other two temperatures. Thus, the significant difference between the slope at 35 C and the other two temperatures is probably caused by variance in 16S rRNA gene copies between vials and not due to temperature effects. According to the ANCOVA modeling results (Table 3), there was a significant difference in slope between 5 and 35 C ($P = .0087$) for absolute abundance of intracellular sul1, in which 35 C [−0.00921 $\log_{10}$ (gene copies) d^{-1}] has a significantly steeper slope than 5 C [−0.00491 $\log_{10}$ (gene copies) d^{-1}] (Table 2). There is also a significant difference between the 5 and 20 C slopes ($P = .0324$) for the relative abundance of intracellular sul1, in which 20 C [−0.00402 $\log_{10}$ (gene copies) d^{-1}] has a significantly steeper slope than 5 C [−0.00092 $\log_{10}$ (gene copies) d^{-1}] (Table 2). Comparing these results with Figure 3B (absolute abundance) and Figure 3E (relative abundance), the 95% confidence intervals are overlapping for each sampling date, indicating that the significant difference in slope is likely due to variance in the data from separate incubation vials, as opposed to a temperature effect.

Regarding WHC effects, extracellular sul1 had significant differences in slope between 25 versus 50% WHC_{max} ($P = .0355$) and 25 versus 75% WHC_{max} ($P = .0379$) for absolute abundances (Table 3). Twenty-five percent WHC_{max} [−0.00597 $\log_{10}$ (gene copies) d^{-1}] had a significantly shallower slope than 50% [−0.01623 $\log_{10}$(gene copies) d^{-1} and 75% [−0.02107 $\log_{10}$ (gene copies) d^{-1}] (Table 2) WHC_{max}. Comparing these results with Figure 4B, 25% WHC_{max} does not have as steep a decline in absolute gene copies from 0 to 7 d of incubation and has higher absolute gene copies for the rest of the sampling dates compared with the other

two WHCs. This is why 25% WHC_{max} has a significantly shallower slope than the other two, but with overlapping 95% confidence intervals for almost every sampling date, missing data that do not allow us to see if the same trends occur with other eARG, and no significant differences between extracellular 16S rRNA absolute abundances for the three WHCs, it is difficult to say there was an effect of WHC on absolute abundances of extracellular sul1. The relative abundances for extracellular sul1 have significantly different slopes for all three WHCs (Table 3), but comparing this with Figure 4E, all the relative gene abundances are bunched together, with overlapping 95% confidence intervals for most sampling points, clouding the ability to determine if soil moisture had an effect on eDNA abundances.

4 | CONCLUSIONS

Understanding of the partitioning, persistence, and fate of eARGs and iARGs in soils is necessary for the scientific community to make more informed decisions about their horizontal gene transfer and risk to public health. In this study, we have modified the protocol of a commercially available DNA extraction kit for the purpose of sequentially extracting eDNA and iDNA from the same soil sample without the need for additional chemical reagents and supplies. The spiking experiments showed that about 1% of the gfp gene from whole cells was recovered using eit her the eDNA/iDNA or tDNA methods. Spiking the gfp gene into soil showed that 7–9% of the eDNA was recovered, but it also demonstrated that much of the DNA (about 80%) was unavailable for extraction and analysis. We speculate that this occurred as a result of DNA adsorbing to soil particles, becoming trapped in microsites, and/or being deg raded by endonucleases and heterotrophic bacteria. Overall, the sequential extraction method was helpful in understanding the abundance and persistence of extracellular and intracellular genes in manure-amended soil. Absolute gene abundances were one to two orders of magnitude higher in iDNA than in eDNA fractions but were similar when normalized to the 16S rRNA gene throughout the incubation period, indicating that enrichment of eARGs and iARGs did not occur. The initial decline in absolute gene levels in eDNA during the first week of the study was likely caused by adsorption to soil particles and/or degradation. Our results demonstrate that extracellular and intracellular sul1, tet(M), and intI1 are quite resilient and not readily affected by soil temperature, moisture content changes, and freeze-thaw cycles. Researchers should consider using this method to better understand the distribution of ARGs in eDNA and iDNA fractions in soils. A common eDNA/iDNA extraction method should be used among researchers to ensure results generated from different ARG studies can be more easily compared by reducing the bias associated with the use of various DNA extraction procedures.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

The authors thank Sheryl Verwey for assistance. Mention of trade names or commercial products in the publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. The USDA is an equal opportunity provider and employer.

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How to cite this ar t icle: McKinney CW, Dungan RS. Influence of environmental conditions on extracellular and intracellular antibiotic resistance genes in manure-amended soil: A microcosm study. Soil Sci. Soc. Am. J. 2020;84:747–759. https://doi.org/10.1002/saj2.20049