Title

Quantification and Correlation Analysis of Antibiotic Resistance Gene, ermF, and Class 1 Integron, intI1, in Commercially Available Fertilizers

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Quantification and Correlation Analysis of Antibiotic Resistance Gene, *erm*F, and Class 1 Integron, *intI*1, in Commercially Available Fertilizers

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Civil Engineering

by

Ileana Callejas

2019

ABSTRACT OF THE THESIS

Quantification and Correlation Analysis of Antibiotic Resistance Gene, *erm*F, and Class 1 Integron, *intI*1, in Commercially Available Fertilizers

by

Ileana Callejas

Master of Science in Civil Engineering

University of California, Los Angeles, 2019

Professor Jennifer Ayla Jay, Chair

The rising level of antibiotic resistance worldwide is a critical public health challenge. Antibiotic resistance genes (ARGs) and mobile genetic elements allow for bacteria to confer resistance to antibiotics in as little as a year. Antibiotics utilized for growth promotion in confined animal feeding operations (CAFOs) generates manure that is an anthropogenic source of ARGs into the environment. In this study, 10 potting soils, 7 garden soils, 4 food amendments, 4 lawn amendments, 6 manure-based soils, 5 natural soils, 3 community soils, and 3 compost soils were surveyed for antibiotic resistance gene *erm*F, mobile genetic element *intI*1, and proxy for bacterial content 16S rRNA. The ARGs were quantified through quantitative polymerase chain reaction (qPCR) to per gram of fertilizer and per gene copies of 16S rRNA. Most soils contained detectable levels of *erm*F and *intI*1, ranging from 9.66×10^{-7} to 8.31×10^{-2} gene copies

*erm*F/copies of 16S rRNA and 8.92 x 10^{-7} to 6.21 x 10^{-2} gene copies *intI*1/copies of 16S rRNA. Natural soils were significantly lower in *erm*F and *intI*1 than the other soil types. Natural soils ranged from 10^{-7} to 10^{-5} gene copies per copies of 16S rRNA while the other types ranged from 10^{-7} to 10^{-2} gene copies per copies of 16S rRNA. There was no strong correlation between *intI*1 and *erm*F.

The thesis of Ileana Callejas is approved.

Shaily Mahendra

Sanjay K. Mohanty

Jennifer Ayla Jay, Committee Chair

University of California, Los Angeles

2019

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List of Acronyms

Antibiotic resistant bacteria	ARB
Antibiotic resistance genes	ARGs
Confined animal feeding operations	CAFOs
Mobile genetic elements	MGEs
The Organic Materials Review Institute	OMRI

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Background

Antibiotics in the Environment

The rising frequency of antibiotic resistance is a critical worldwide public health challenge. At least 2 million infections and 23,000 deaths occur annually in the U.S. alone due to antibiotic resistant bacterial strains[1]. Various classes of antibiotics have been developed to fight infections. However, soon after introduction of each class of antibiotic, resistant strains have been observed in clinical isolate. For example, methicillin antibiotics were created to combat penicillin resistant bacteria in 1960, but methicillin-resistant bacterial strains emerged only two years later [2]. This rapid increase in resistance continues to occur with the creation and use of new antibiotics[2]. This pressing issue requires multidisciplinary solutions informed by research and implementation through policy, law, and other organizations.

Antibiotic resistant bacteria (ARB) confer resistance genetically through antibiotic resistance genes (ARGs) which code for mechanisms such as antibiotic efflux, target modification, reduced permeability, and other mechanisms outlined in **Figure 1**[3,4]. The ability for ARGs to proliferate genetically render them unique emerging contaminants of concern[5]. While the development of antibiotic resistance is a natural process, it is exacerbated due to overuse and misuse of antibiotics for agricultural and medical uses. Of these sources, use of antibiotics for livestock is estimated to be four times more than antibiotic use in human medicine[6].

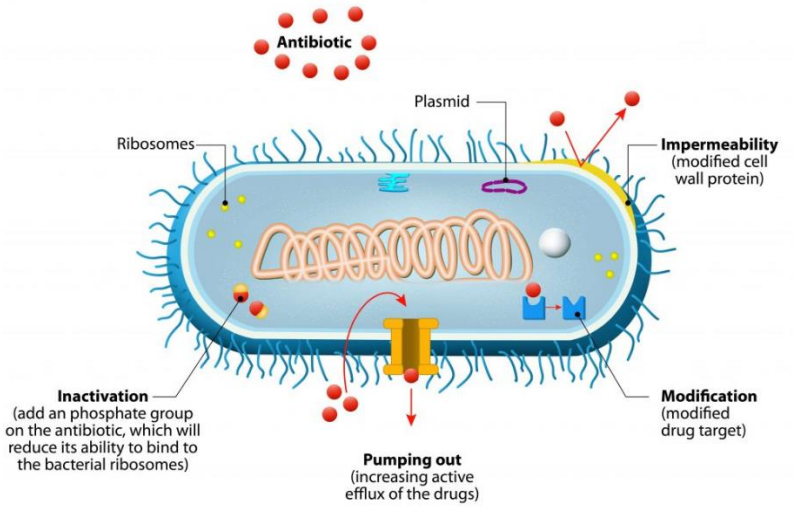

Figure 1: Resistance Mechanisms of ARB (Madigan, 2014). Red circles represent the antibiotic. Resistance mechanisms include efflux, inactivation of antibiotic, ribosome modification, modification of target, cell wall permeability, and plasmid modification.

Antibiotics for Agricultural and Animal Use

Antibiotics are used in agriculture for both crops and livestock. Antibiotics such as tetracyclines and streptomycin are sprayed on fruit trees as a pesticide[2,7]. In livestock, antibiotics are used for growth promotion and to treat or prevent disease among animals in confined animal feeding operations (CAFOs)[8]. Antibiotics used in the livestock industry include beta-lactams, macrolides, sulfonamides, tetracyclines, and other classes of antibiotics. With widespread use of antibiotics in agriculture, ARGs increase in animals and their by-products. Around 17-75% of antibiotics given to livestock are excreted through their urine and feces unchanged or as active metabolites[9]. This percentage is across multiple animals such as steer, bulls, pigs, and sheep and for different classes of antibiotics with chlortetracycline producing the biggest range of unchanged excretion in young bulls from 17-75%[9]. Multiple studies measure levels of livestock antimicrobials from agriculture in receiving streams. A Canadian study found 10 ng L^{-1} of erythromycin, a type of macrolide antibiotic, in a receiving watershed[10] from pigs, cattle, chickens, and turkeys. A

Chinese study reported 18.2 mg/day/swine and 4.24 mg/day/cattle normalized daily excretions of antibiotics in swine and dairy cattle farms[11].

Dissemination of ARGs in agricultural operations interacts with humans in multiple ways. Human-ARG interactions not only arise from proximity to agriculture, but also through meat consumption[12], water, and through manure sourced from CAFOs used as fertilizer[2] as shown in **Figure 2**. Manure from CAFOs provide valuable nutrients and organics to crops and pastures[13]. This manure can also make its way to the hands of consumers through commercially available fertilizers. The Organic Materials Review Institute (OMRI) provides independent reviews of fertilizers and livestock health care products to ensure organic production and processing[14]. However, the term "organic" utilized on products are not regulated for non-food items such as fertilizers[15] or consider levels of antibiotics, ARB, or ARGs.

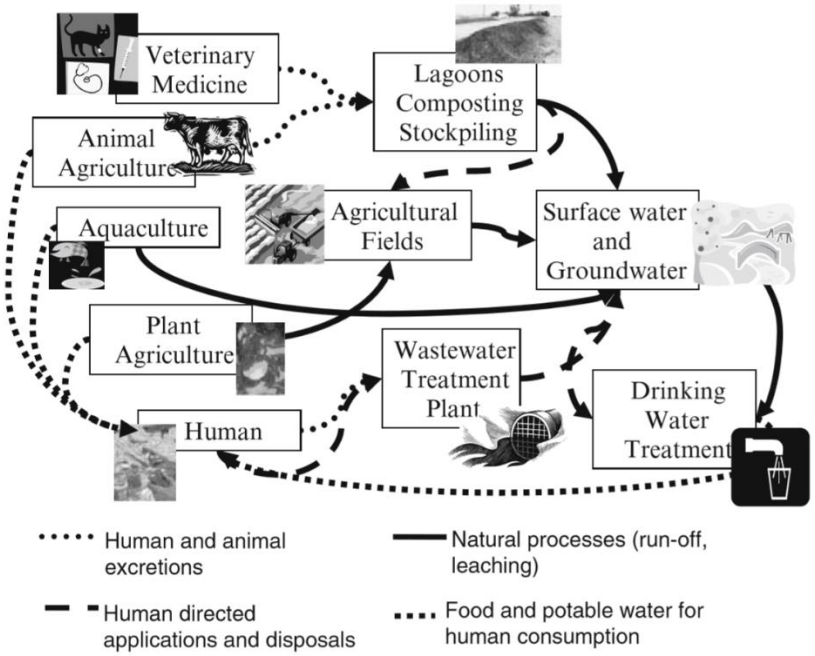

Figure 2: Major sources and pathways of antibiotics in the environment (Pruden, 2009)

Quantifying ARGs in Agricultural Settings

Swine

Multiple studies have investigated the presence and quantity of ARGs in agricultural settings with swine manure. ARGs such as *erm*F, *tet*O, and *tet*W were quantified in receiving soils and water following application of swine manure in China[16,17]. Changes in soil antibiotic resistome were found following the application of various fertilizer types, including pig manure, after a 25 year period[18]. Macrolide ARGs increased in soil and drainage water after swine manure application on agricultural fields[19]. Commercial organic fertilizers were found to increase the amount ARGs in soil through microcosm experimentation[20].

Steer

Some studies have investigated the effects of antibiotics on steer and cattle. A Canadian study found that the administration of tylosin, a type of macrolide, increases the proportion of macrolide resistant enterococci in the fecal matter of steer[21]. Resistance towards macrolides and lincosamides were detected in streptococci from cattle in a study spanning many European countries[22]. A U.S. study states that over 127 million kilograms of meat contaminated with macrolide-resistant bacteria come from cattle[23].

Poultry

ARB and ARGs were also located in poultry production. A U.S. study found a total of 142 enterococcal isolates and 144 staphylococcal isolates from flies near poultry production and poultry litter containing macrolide resistance genes[24]. An Irish study discovered *Salmonella* species resistant to at least one antibiotic in 70% of chilled chicken available for consumption in local grocery stores[25]. Over 1 billion kilograms of meat containing macrolide-resistant bacteria was found to come from poultry in the U.S.[23]

Macrolides

Macrolides were first discovered in the early 1950s[26] and marketed in 1952 as an alternative to beta-lactams[27,28] and are a class of antibiotics that are potent against many gram-positive and gram-negative microorganisms[29]. Macrolides consist of macrocyclic lactone rings, ranging from 14 to 44 rings[29] and work by binding to the 50S rRNA to prevent protein synthesis[25,28]. In the early 1960s, spiramycin was the first macrolide intended for food animal use, followed by erythromycin and tylosin in the early 1970s[22]. Macrolides can be used in animals to treat infectious diseases with macrolide-sensitive bacteria, to treat respiratory disease in poultry, and treat mastitis in lactating cows[30]. The *erm*F gene was identified in American-Type Culture Collection (ATCC) in the 1950s[31] and works through 23S rRNA modification[32]. In 2013, the U.S. Center for Disease Control (CDC), named erythromycin-resistant group A *Streptococcus,* a concerning antibiotic resistance threat in the United States[33].

Integrons

As humans created more antibiotics to fight resistance, mobile genetic elements (MGEs) called integrons began to accumulate ARGs. The class 1 integron, *intI*1 can accumulate ARGs resistant to many classes of antibiotics, and be found in many bacterial strains which can infect both plants and animals[34]. *intI*1 is deemed a proxy for anthropogenic pollution and for the resistome because it confers resistance genes for antibiotics, disinfectants, and heavy metals, it can enter commensal and pathogenic bacteria in humans and animals, and it changes rapidly under environmental pressures[35]. *intI*1 copies can be found in the natural and built environments[36], in or near hospitals[37], and in manured soils[38].

However, little is known about the quantity of ARGs in commercialized fertilizers which may have a further reach than fertilizers used on agricultural fields alone. Therefore, this survey

provides a closer look at the amounts of ARGs in fertilizers easily accessible by the public. This thesis will delve into macrolide resistance through the *erm*F, *intI*1, and 16S rRNA genes. The *erm*F gene is among the four major classes of macrolide resistance in pathogenic microrganisms.[39,40] Macrolides are among the top three classes of antibiotics used in livestock by sales in 2009 for a total of $0.6 billion U.S. dollars[41]. The class 1 integron accumulates multiple and diverse amount of ARGs[34] and is considered a proxy for anthropogenic pollution.[35] The 16S rRNA gene serves as a genetic marker due to its presence in almost all bacteria[42]. The *erm*F and *intI*1 genes were quantified in 38 gardening products, 5 "natural" soils, 3 community samples that had recently had fertilizer applied, and 3 compost-based soils via qPCR. The soils were analyzed with respect to per gram of soil and per 16S rRNA copy. The correlation coefficient between *erm*F and *intI*1 was also calculated.

Materials and Methodology

Soil and Amendment Selection

A total of 10 potting soils, 7 garden soils, 4 food amendments, 4 lawn amendments, 6 manure-based soils, 5 natural soils, 3 community soils, and 3 compost soils were characterized and quantified for antibiotic resistance gene *erm*F, and 16S rRNA and *intI*1. Gardening products spanned 16 brands and manure-based products were sourced from either poultry, steer, or bat guano. Product names, manufacturers, manure source, brand, and applicable certifications can be found in **Tables 1, 2, 3,** and **4**.

Table 1: Sample IDs, product name, and brand for potting soils, garden soils, and fruit amendments

Product Type	Sample ID	Product Name	Brand Name
Potting Soil	B1	Potting Mix	Miracle Gro
	B2	Edna's best potting soil	EB Stone
	B3	Patio Plus Premium Outdoor Potting Mix	Kellogg
	B4	Moisture Control Potting Mix	Miracle Gro
	B5	Natural and Organic Potting Mix	Jobe's Organics
	B6	Black Gold All Purpose Potting Mix	Sungro
	B7	Organic Potting Mix for all potted plants	Espoma
	B8	All Purpose Potting Mix	Vigoro
	B9	Baby Bu's Biodynamic Blend Potting Soil	Malibu Compost
	B10	Natural + Organic Potting Mix	Ecoscraps
Garden Soil	B11	All Natural Garden Soil	Kellogg
	B12	Organic Gardening Soil	Nature's Care
	B13	Premium All Purpose Planting Mix	Dr Earth
	B14	Organic Garden Soil	Ecoscraps
	B15	Flower & Vegetable Garden Soil	Sta Green
	B16	All Purpose Garden Soil	Vigoro
	B17	All Purpose Garden Soil	Miracle Gro
Fruit Amendment	B21	Organic Plus: Tomato, Vegetable	Kellogg
	B22	Garden-tone: Herb and Vegetable Food	Espoma
	B24	Vegetable and Tomato	Jobe's Organics
	B25	Starter and Transplanting Granular Plant Food	Burpee

Table 2: Sample IDs, product name, and brand for lawn amendments, manure, natural soils, community soils, and compost

Product Type	Sample ID	Product Name	Brand Name
Lawn Amendment	B31	Organic Plus: Topper Soil for Lawns, Sod, and Seed	Kellogg
	B32	Tree & Shrub Garden Soil Plus Fertilizer	Sta Green
	B33	Turf Max	Green As It Gets
	B34	Palm, Cactus, and Citrus	Kellogg
Manure	B41	Steer manure Blend	Earthgro
	B42	Steer Manure	Gardeners
	B43	Steer Manure	Wholesale
	B51	Composted Chicken Manure	G&B Organics
	B52	Chicken Manure	Earthgro
	B53	Chicken Manure	Wholesale
Natural Soils	B61	Temescal Canyon Trail	N/A
	B62	Franklin Canyon Trailhead	N/A
	B63	Los Leones Canyon Trailhead	N/A
	B64	Santa Ynez Canyon Trailhead	N/A
	B65	Tuna Canyon Park Trailhead	N/A
Community Soils	B71	Public Garden	Unknown
	B72	Housing Unit	Unknown
	B73	Public Park	Unknown
Compost	B81	Compost	Wholesale
	B82	Compost	Unknown
	B83	Bu's Blend Biodynamic Compost	Malibu Compost

Table 3: Certification, branding strategies, and manure source for potting soils, garden soils, and fruit amendments

Sample ID	Certifications		Branding			Manure Source				
	OMRI	Demeter	Organic	Natural	Premium	Poultry	Dairy Cow	Steer	Bat Guano	Not Available
B1	No	No								X
B2	No	No	X						X	
B3	Yes	No	X		X	X				
B4	No	No								X
B5	No	No	X	X						X
B6	No	No					X			
B7	No	No	X	X		X				
B8	No	No								X
B9	No	Yes	X				X			
B10	No	No	X	X						X
B11	Yes	No	X	X		X			X	
B12	Yes	No	X	X		X				
B13	Yes	No	X	X	X					X
B14	Yes	No	X							X
B15	No	No								X
B16	No	No								X
B17	No	No								X
B21	Yes	No	X			X				
B22	No	No	X			X				
B24	Yes	No	X			X				
B25	Yes	No	X	X						X

Table 4: Certification, branding strategies, and manure source for lawn amendments, manure, natural soils, community soils, and compost

Sample ID	Certifications		Branding			Manure Source				
	OMRI	Demeter	Organic	Natural	Premium	Poultry	Dairy Cow	Steer	Bat Guano	Not Available
B31	Yes	No	X			X				
B32	No	No								X
B33	No	No	X							X
B34	Yes	No	X			X				
B41	No	No		X				X		
B42	No	No						X		
B43	No	No	X					X		
B51	Yes	No	X			X				
B52	No	No				X				
B53	No	No	X			X				
B61	N/A	N/A								X
B62	N/A	N/A								X
B63	N/A	N/A								X
B64	N/A	N/A								X
B65	N/A	N/A								X
B71	N/A	N/A								X
B72	N/A	N/A								X
B73	N/A	N/A								X
B81	No	No	X							X
B82	N/A	N/A								X
B83	No	Yes					X			

Commercial Soil and Amendment Selection

Commercially available garden products chosen for the study comprised six classifications of the study: Potting Soils, Garden Soils, Food Amendments, Lawn Amendments, Manure-Based Products, and Compost-Based Amendments. This thesis study contributed an additional two manure-based fertilizers and two compost-based amendments. Soils and amendments were categorized based on product advertisement and intended use outlined by the manufacturer. Potting Soils are intended for initial planting of potted plants without the need of other soils. Garden Soils provide supplements to support plants already in garden soil. Food Amendments serve to provide supplements to plants for human consumption. Lawn Amendments consist of fertilizer-based supplements for lawn growth promotion. Manure-based products consisted of either chicken or steer manure and restore nutrients to soil. Products marketed for general use were preferred for the study. Gardening products marketed towards specific plants such as roses and orchards were not included in the survey.

All soils and amendments are easily accessible for purchase by the public through major gardening and hardware stores. All products were purchased in person from stores in Los Angeles, Ca that commonly carry gardening products: Lowe's, Home Depot, Walmart, Orchards, and Anawalt Lumber Co. Three soils were purchased from a wholesaler in Whittier, Ca. Advertised certifications were verified for possible expiration up to June 2019. Three samples were taken from each product, resulting in n=102 soil samples.

Community Soil Selection

In order to account for soils and amendments that are not accessible for purchase, but still placed in public areas, three community samples were taken from a lawn, a public garden, and a public park where fertilizer was recently applied. These samples consisted of fertilizers applied

through professional landscapers or large institutions. Three samples were taken at each site, resulting in n=9 soil samples.

Natural Soil Selection

To understand how many ARGs exist in the environment naturally, five "Natural" locations were selected in Los Angeles, Ca for soil sampling. "Natural" locations were characterized as areas with low development, areas where fertilizers and amendments are not utilized to grow vegetation, and the locations are accessible by the public. Soils were sampled from hiking trails in Temescal Canyon Trail, Los Leones Canyon Trailhead, and Santa Ynez Canyon Trailhead in Pacific Palisades, Ca, Franklin Canyon Park in Beverly Hills, Ca, and Tuna Canyon Park Trailhead in Malibu, Ca. A map of sampling sites can be viewed in **Figure 3**. Three samples were taken at each site, resulting in n=15 soil samples.

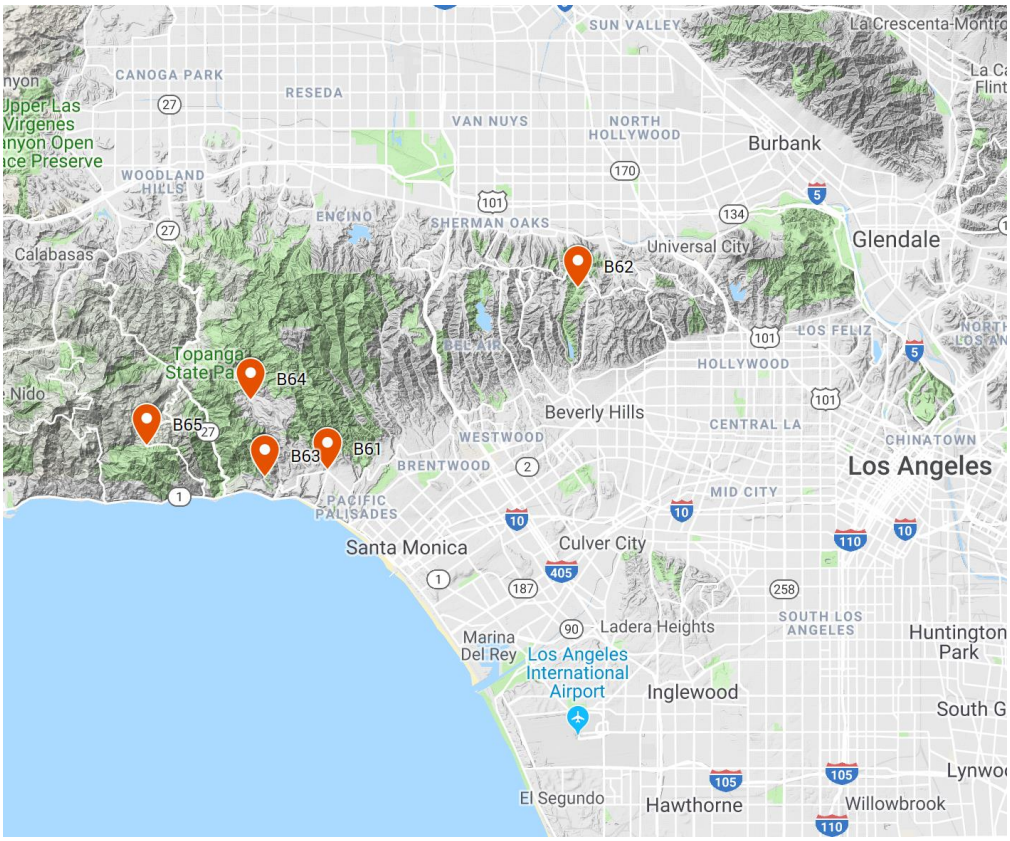

Figure 3: Map of Natural Soil Sampling Locations in Los Angeles, California Region

Sampling Methods

Fertilizer and amendment bags were measured for ARGs through weighing 0.25 ± 0.01 g of each bag into individual 2mL screw cap tubes preloaded with 1 ± 0.05 g of 0.7 mm garnet beads (Qiagen, Germantown, MD). The soils were mixed prior to sampling utilizing sterilized gloves and each bag was sampled in triplicates. Following mixing, soil from each bag was stored at 4 °C in individual 50 mL sterile Falcon tubes for soil characterization. Additionally, a quart of each type of product was stored at -20 °C for hydrometer analysis.

For the natural soils and community soils, three randomly selected areas were chosen to collect soil samples. Natural soil sampling took place in three locations within the trail heads. Each sample was taken away from the trail to avoid soils compacted by human traffic. Community soil sampling occurred by taking samples three feet apart from each other. For both types of samples, top soil (0-2.5cm) samples were placed into 50 mL sterile falcon tubes using sterilized plastic spoons. Rocks and soils were avoided during the sampling processes. All soils samples were stored at 4 °C prior to processing.

Soil Characterization

Soil samples were characterized for soil texture through hydrometer analysis and organic matter content through loss on ignition analysis for each product and natural soil.

For the hydrometer analysis, approximately 50-90 g of each soil type was weighed in triplicates and placed in a drying oven for 50 °C. Soils were re-weighed and placed in sterile beakers along with 100 mL of sodium metaphosphate solution (50 g sodium metaphosphate to 1L of deionized water for 10 samples) to add ionic charge to the sediments. Beakers were then placed on a shaker table for 125 rpm for 24 hours. The contents were transferred to another cylinder where

samples were thoroughly inverted and hydrometer measurement was taken 40 s after. Another hydrometer measurement was taken after 2 hours without disturbing the soils.

Loss on ignition analysis was performed by weighing approximately 2 g of soil and drying them at 103 °C for at least 12 hours and weighed after. Samples were then transferred to a furnace where they were ignited at 550 °C for over a two-hour period, The residues were weighed until the weight change was less than 4%, as directed by the U.S. Environmental Protection Agency (EPA). Soil characterization results are displayed in **Table 5**.

Table 5: Soil Characterization Results

Category	Brand	Soil Composition			Soil Moisture Content (%)	Loss on Ignition Analysis		
		% Sand	% Clay	% Silt		% Total Solids	%Fixed Solids	%Volatile Solids
Potting Soil	B1	88.2	1.7	10.1	5.27	42.53	24.19	75.81
	B2	89.3	1.0	9.7	3.99	58.98	23.59	76.41
	B3	93.0	1.6	5.4	4.34	58.47	45.99	54.01
	B4	86.5	0.0	13.5	5.81	40.73	26.80	73.20
	B5	93.2	0.0	6.8	4.89	53.96	27.28	72.72
	B6	85.9	1.3	12.9	2.95	70.27	45.61	54.39
	B7	86.1	0.7	13.2	5.58	40.99	21.35	78.65
	B8	95.3	2.8	1.9	3.82	63.30	32.13	67.87
	B9	97.3	0.0	2.7	4.99	43.98	54.70	45.30
	B10	91.2	2.6	6.1	4.44	55.63	50.58	49.42
Garden Soil	B11	89.1	5.3	5.6	4.20	57.64	43.52	56.48
	B12	82.6	6.8	10.6	6.16	38.10	26.61	73.39
	B13	86.2	6.4	7.4	3.74	61.30	21.12	78.88
	B14	96.1	2.8	1.1	5.57	44.44	61.17	38.83
	B15	89.0	3.2	7.9	5.33	47.26	26.46	73.54
	B16	91.5	4.3	4.2	4.58	49.25	40.14	59.86
	B17	85.1	8.5	6.4	4.85	50.50	24.09	75.91
Fruit Amendment	B21	84.3	7.7	8.1	0.95	90.41	41.68	58.32
	B22	77.4	15.7	6.9	0.78	92.04	43.24	56.76
	B24	79.6	16.6	3.8	1.05	88.70	65.17	34.83
	B25	66.4	19.4	14.1	0.58	93.85	44.42	55.58
Lawn Amendment	B31	81.2	10.0	8.8	4.16	57.54	42.70	57.30
	B32	NA	NA	NA	NA	NA	NA	NA
	B33	NA	NA	NA	NA	NA	NA	NA
	B34	NA	NA	NA	NA	NA	NA	NA
Manure	B41	90.0	8.3	1.6	3.37	66.94	66.39	33.61
	B42	NA	NA	NA	4.34	51.90	40.14	59.86
	B43	NA	NA	NA	3.87	59.51	36.62	63.38
	B51	90.5	5.7	3.8	3.91	57.31	59.70	40.30
	B52	NA	NA	NA	NA	NA	NA	NA
	B53	NA	NA	NA	3.09	64.51	42.51	57.49
Natural Soil	B61	NA	NA	NA	1.40	83.91	88.16	11.84
	B62	NA	NA	NA	1.49	83.92	94.44	5.56
	B63	NA	NA	NA	2.04	79.38	91.07	8.93
	B64	NA	NA	NA	1.51	82.41	96.22	3.78

	B65	NA	NA	NA	1.57	82.35	95.59	4.41
Community Soil	B71	NA	NA	NA	2.50	72.17	84.58	15.42
	B72	NA	NA	NA	5.22	46.00	47.09	52.91
	B73	NA	NA	NA	4.65	56.45	34.29	65.71
Compost	B81	NA	NA	NA	5.25	44.90	31.92	68.08
	B82	NA	NA	NA	5.21	48.69	68.43	31.57
	B83	96.6	1.9	1.6	2.40	73.02	75.52	24.48

DNA Extraction

All soil DNA extraction were completed utilizing the DNeasy PowerSoil Kit (Qiagen, Germantown, MD) within two weeks of collection. Extractions followed manufacturer guidelines and an additional 2mL screw cap tube preloaded with 1 ± 0.05 g of 0.7 mm garnet beads (Qiagen, Germantown, MD) without and sample (extraction blank) was also extraction to account for contamination through the extraction process should it occur. Extracted DNA was aliquoted and stored at -20 °C prior to qPCR analysis. Total DNA concentration and 260/280 absorbance ratios were quantified through UV absorption via a Nanodrop 200C spectrophotometer (Thermo Scientific, Waltham, MA).

qPCR

Samples were analyzed for ARG abundance for *erm*F as well as *intI*1 and the 16S-rRNA gene (a total bacteria surrogate measure) via qPCR. Assays utilized PowerUp SYBR Green Master Mix (Life Technologies, Grand Island, NY) entailing a 25 μL reaction volume consisting of 12.5 μL of SYBR Green MasterMix, 1.25 μL of each primer, forward and reverse primer, and 2 μL of template DNA, and 8 μL of molecular grade water for the remaining reaction volume. Primers used in the study were developed and validated previously in literature. Primer sequences, concentrations, and annealing conditions can be found in **Tables 6 and 7**. Each assay run contained a 7-point standard curve as a positive control, molecular grade water as a negative

control, and necessary extraction blanks with each sample plated in triplicate. Assays were ran in a 96-well reaction plate utilizing StepOne Plus (Applied Biosystems).

Table 6: Primer concentrations, sequences, and amplicon size

Target Gene	Primer	Concentration (nM)	Sequence (5'-3')	Amplicon size (bp)
ermF[43]	*ermF*-F	500	TCGTTTTACGGGTCAGCACTT	246
	ermF-R	500	CAACCAAAGCTGTGTCGTTT	
intI1[44]	*intI1*-F	200	CCTCCCGCACGATGATC	424
	intI1-R	200	TCCACGCATCGTCAGGC	
16S rRNA[45]	16S rRNA-F	100	CCTACGGGAGGCAGCAG	257
	16S rRNA-R	100	ATTACCGCGGCTGCTGG	

Table 7: Gene cycling conditions and times, R^2 values, and amplification efficiencies

	Holding		Denaturation		Annealing		Extension			
Target gene	Temp. (°C)	Time (min)	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)	Temp. (°C)	Time (s)	R^2	Amp. Eff.
ermF[43]	95	10	94	20	60	60	-	-	.999 ± 0.001	97 ± 1.9
intI1[44]	95	10	95	15	55	30	72	30	.999 ± 0.00	98 ± 2.2
16S rRNA[45]	95	15	95	15	60	30	72	30	.999 ± 0.005	97 ± 2.1

Samples were diluted to concentrations of 1 ng/μL for *erm*F, *intI*1, and the 16S-rRNA genes to offset inhibition effects. Selected dilution concentrations were verified to effectively correct inhibition effects through well spike and serial dilution. For serial dilutions, extracted DNA was diluted to multiple concentrations to determine inhibition occurrence in the qPCR reaction. Inhibition played a role in soil matrices and thus samples were diluted appropriately. Efficacy of selected dilution concentrations were confirmed by choosing at random and plating each sample into 6 qPCR wells, 3 of these wells being spiked with known standard quantities. qPCR results confirmed absence of inhibition effects as gene copy numbers consistently equated to the sum of the spike quantity and unspiked quantity. Respective dilution factors were back calculated during data analysis to obtain gene copies per gram.

Target-containing DNA fragments serving as positive controls were designed from sequences in the NCBI database information and ordered through IDT Technologies. Known concentrations of designed DNA fragment were run concurrently with environmental samples to comprise a 7-point standard curve and allow for gene copy quantification. Standard curve efficiencies and R^2 values are located in **Table 5**. Melt curves further verifies correct target gene amplification.

Results and Discussion

ermF

All quantities for *erm*F can be found in **Figure 4** and **Figure 5**. Quantifiable levels of *erm*F were found in six out of ten potting soils. Levels ranged from 9.63×10^3 to 1.09×10^6 copies of *erm*F per gram of soil and from 8.23×10^{-6} to 4.18×10^{-3} gene copies/copies 16S rRNA. Six out of seven garden soils had quantifiable levels of *erm*F. The minimum number of ermF was in B13

with 1.36 x 10^3 and the maximum number of *erm*F gene copies per gram of soil was in B16 with 6.79 x 10^5. Relative to 16S rRNA, gene copies ranged from 1.48 x 10^{-4} to 1.94 x 10^{-3}.

All four fruit amendments contained quantifiable levels of *erm*F. Quantifiable levels ranged from 3.56 x 10^4 to 7.69 x 10^5 gene copies per soil and 1.11 x 10^{-3} to 1.24 x 10^{-2} gene copies per copies 16S rRNA. *erm*F was detected in all 4 lawn amendments, spanning from 301 to 1.21 x10^6 gene copies per gram of soil. Gene copies per copies 16S rRNA ranged from 4.17 x 10^{-4} to 6.22 x 10^{-2}.

All six manure-based soils contained quantifiable *erm*F copies per gram of soil with a minimum of 2.01 x 10^3 and maximum of 1.38 x10^7. Manure samples contained 4.14 x 10^{-6} to 8.31 x 10^{-2} gene copies/copies 16S rRNA. Four out of five natural soils selected displayed *erm*F. Their quantities were lower compared to the other soils ranging from 7.06 x 10^1 to 9.81 x 10^3 gene copies per soil and 9.66 x 10^{-7} to 4.80 x 10^{-5} gene copies per copies 16S rRNA.

Quantifiable levels of *erm*F were detected in all three community soil samples, with a minimum of 3.17 x 10^3 and a maximum of 5.26 x 10^6 gene copies per gram of soil. Relative to 16S rRNA, gene copies ranged from 9.59 x 10^{-7} to 1.19 x 10^{-2}. Only two out of three compost-based soils contained *erm*F with ranges spanning from 1.55 x 10^3 to 6.90 x 10^5 gene copies per gram of soil, and from 3.56 x 10^{-6} to 5.67 x 10^{-4} gene copies/copies of 16S rRNA.

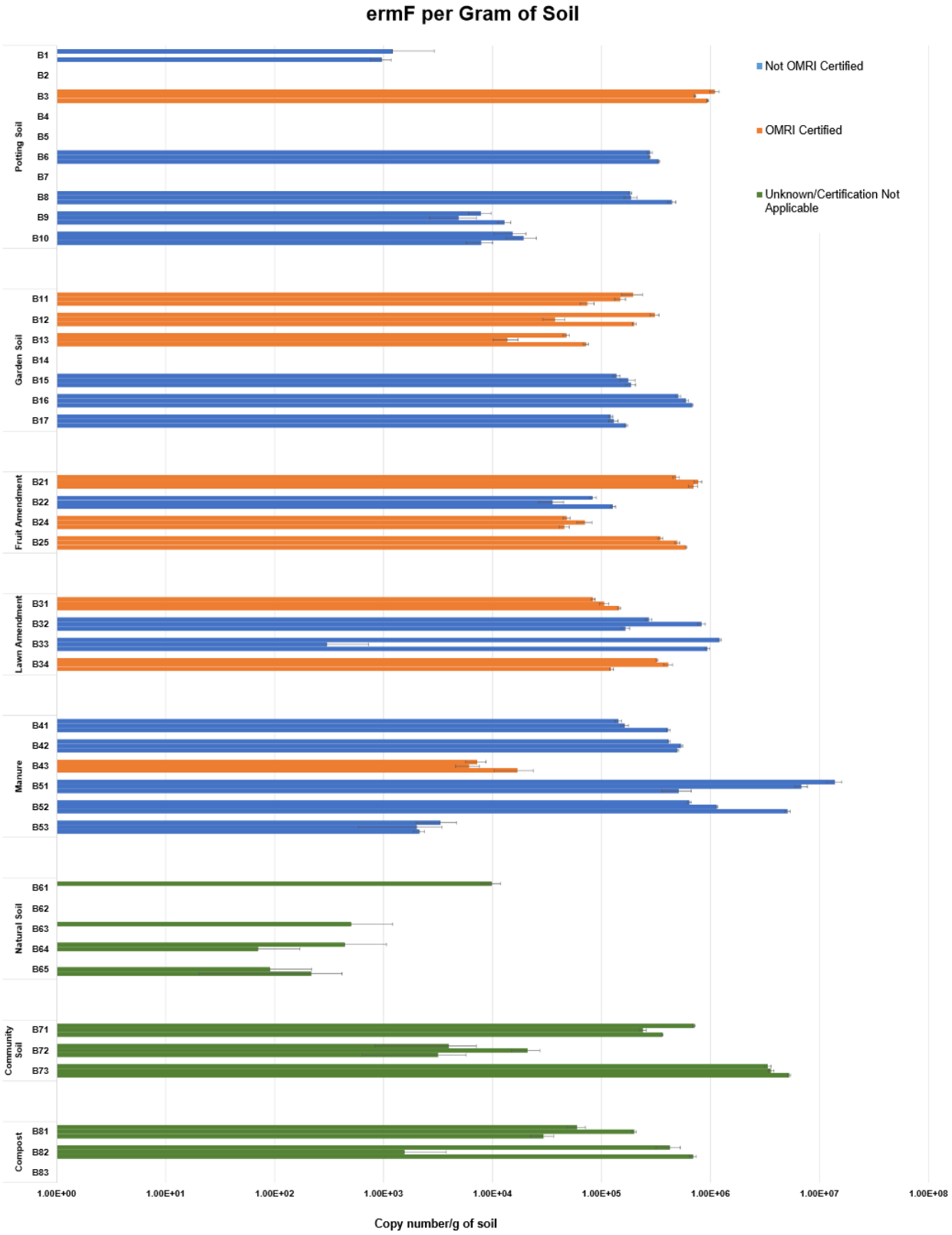

Figure 4: Copies of *erm*F per gram of soil

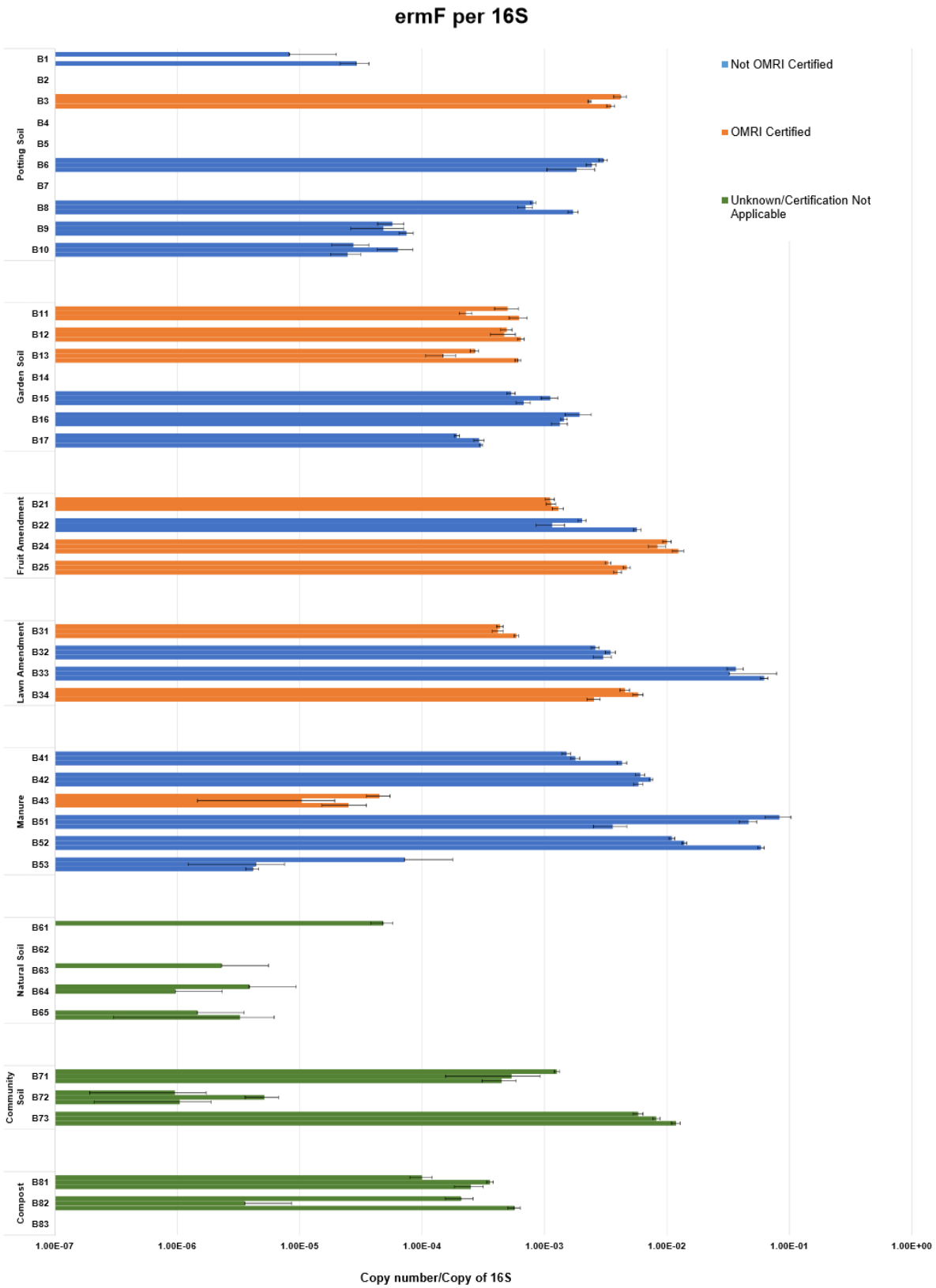

Figure 5: Copies of *ermF* per copies of 16S rRNA

*erm*F *in a Global Context*

A Canadian study found considerable copies of *erm*F in manure. The study reported 8.99 $\log_{10}$ (9.8 x 10^8), 11.43 $\log_{10}$ (2.7 x 10^{11}), 9.18 $\log_{10}$ (1.5 x 10^9), and 7.43 $\log_{10}$ (2.7 x 10^7) copies per gram of dry weight of raw manure, digested manure, dewatered manure, and composted manure, respectively[46]. A U.S. study found around 10^{-5} gene copies *erm*F/16S rRNA gene copies in slurry and 10^{-2} gene copies *erm*F/16S rRNA gene copies in dry stacks[47]. The slurry consisted of dairy manure and the dry stacks consisted of dairy, sheep, horse, and donkey manure mixed with straw and saw dust. A study in Iowa using manure from tylosin-treated swine saw *erm*F copies exceed 10^7 copies per gram of manure[48].

Within a global context, the commercialized fertilizers in the study are lower than those reported in unprocessed or slightly processed manure. This is most likely attributed to processing and the time between the animal excreting the manure and time purchased by human for application. The results from this study fall within a similar range with the U.S. study in terms of *erm*F copies per 16S rRNA copies.

***intI*1**

All quantities for *intI*1 can be found in **Figure 6** and **Figure 7**. Seven out of ten potting soils contained quantifiable levels of *intI*1 per gram of soil. Levels ranged from 2.38 x 10^3 to 9.56 x 10^5 gene copies per gram of soil, and 2.36 x 10^{-5} to 3.70 x 10^{-3} gene copies per copies 16S rRNA. All seven garden soils contain *intI*1 copies spanning from 1.25 x 10^4 to 2.58 x 10^6 gene copies per gram of soil, and 7.74 x 10^{-5} to 8.25 x 10^{-3} gene copies normalized per copies 16S rRNA.

All four fruit amendments displayed *intI*1 copies. Fruit amendment soil samples contained copies per gram of soil from 6.00 x 10^3 to 1.50 x 10^6 and 1.62 x 10^{-2} to 2.52 x 10^{-2} gene copies/copies of 16S rRNA. Similarly, all four lawn amendments had detectable levels of *intI*1

with a minimum of 5.80×10^{2} and maximum of 1.81×10^{6} gene copies per gram of soil. Relative to 16S per rRNA, gene copies range from 2.78×10^{-4} to 6.21×10^{-2}.

Every manure sample contained quantifiable levels of *intI*1 between the range 4.79×10^{4} to 9.88×10^{5} gene copies per gram of soil, and 3.35×10^{-4} to 6.73×10^{-3} genes copies normalized per 16S rRNA. Only three out of five natural soil samples contained *intI*1. Levels were generally lower than the other samples within the range of 59 to 8.46×10^{2} gene copies per gram of soil, and 8.92×10^{-7} to 1.16×10^{-5} gene copies per copies of 16S rRNA.

All three community soils contained detectable levels of *intI*1 from 2.86×10^{5} to 5.04×10^{6} gene copies per gram of soil, and 6.91×10^{-5} to 8.76×10^{-3} gene copies per copies of 16S rRNA. Every compost sample contained *intI*1 with a minimum number of gene copies per gram of soil of 3.40×10^{4} and maximum number of 2.53×10^{7}. *intI*1 relative abundances span from 3.56×10^{-6} to 5.67×10^{-4} gene copies/copies 16S rRNA.

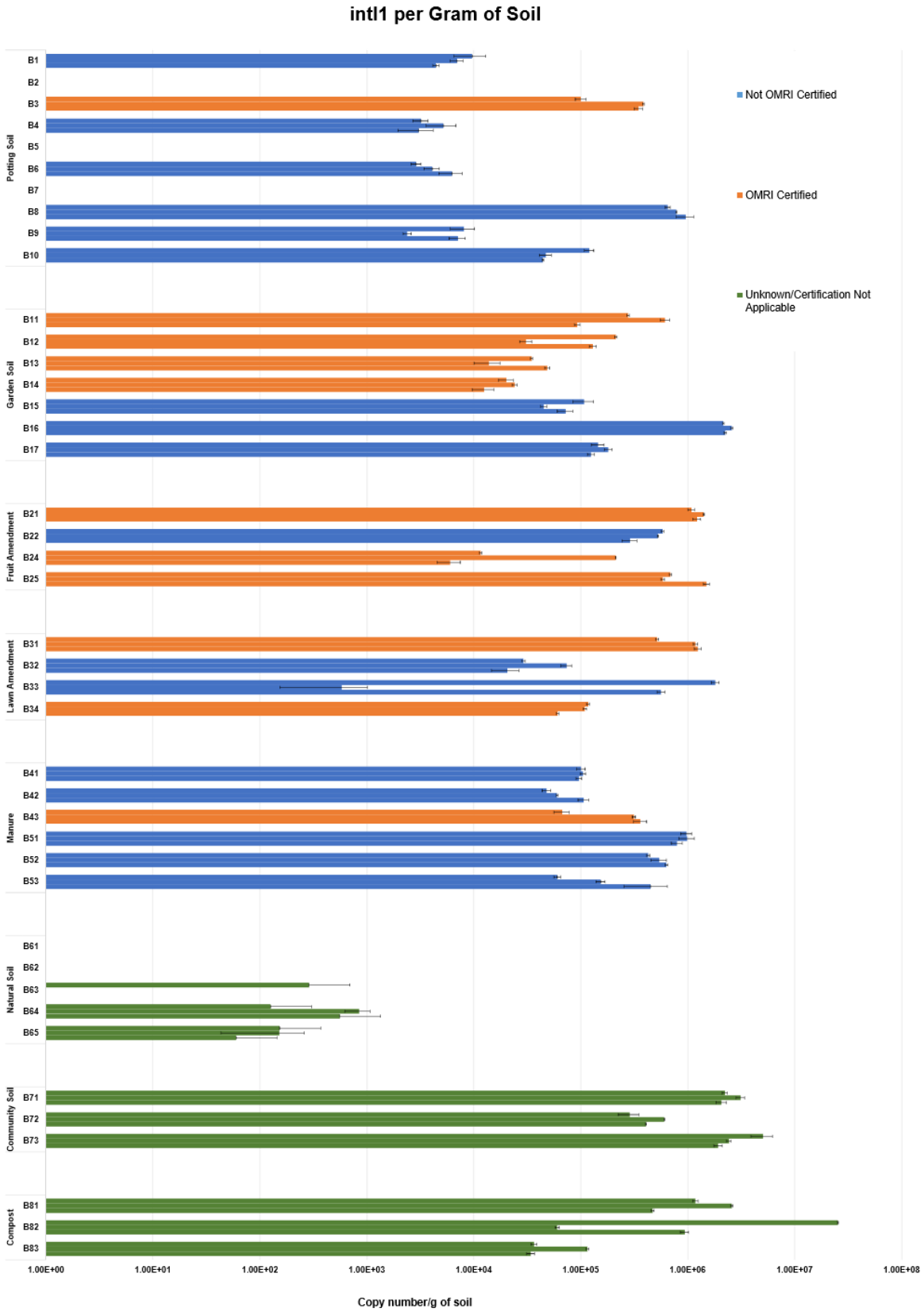

Figure 6: Copies of *intI*1 per gram of soil

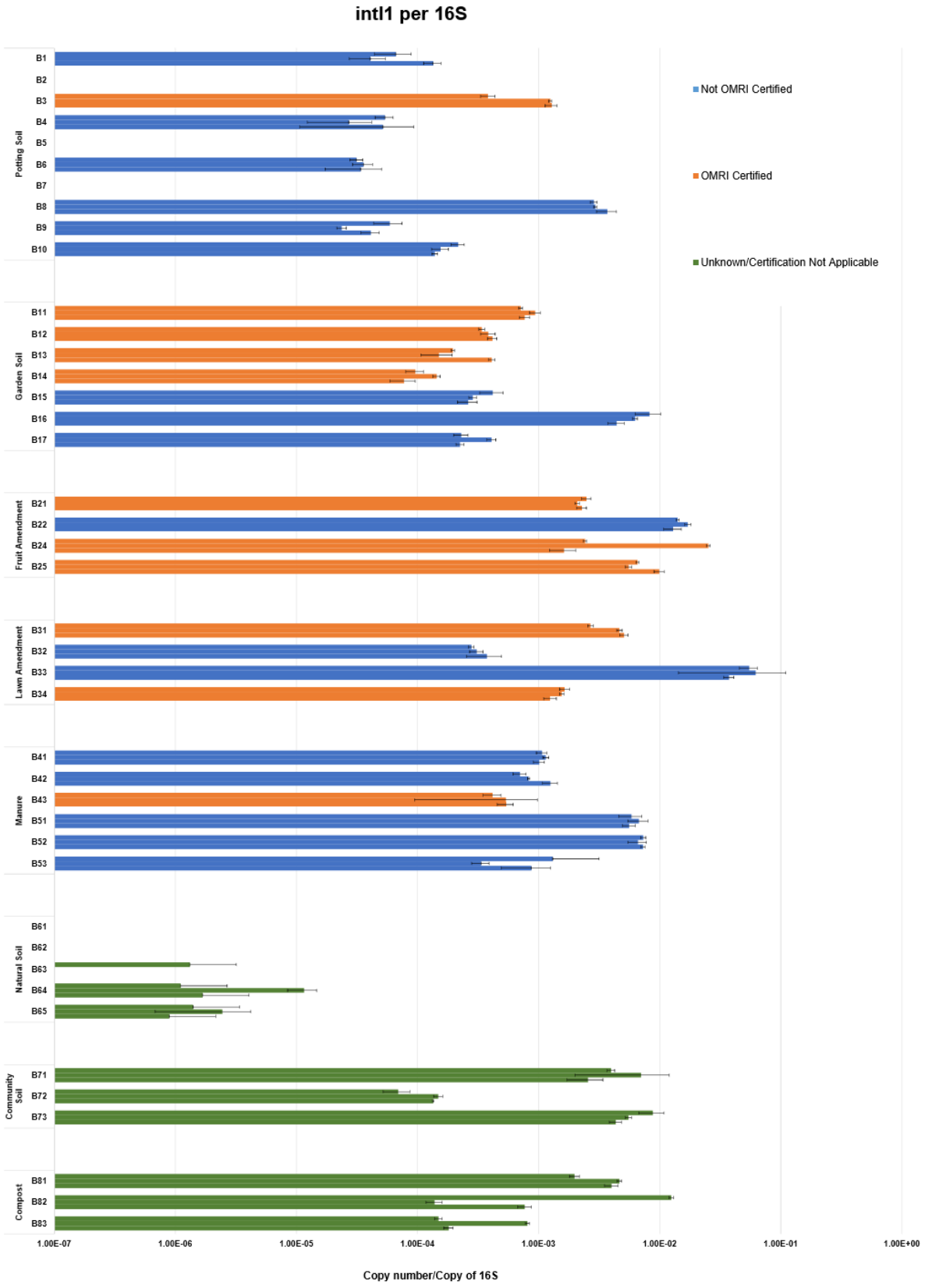

Figure 7: Copies of *intI*1 per copies of 16S rRNA

*intI*1 *in a Global Context*

One Canadian study quantified *intI*1 copies per dry weight in raw manure, digested manure, dewatered manure, and composted manure from dairy farms as 8.94 $\log_{10}$ (8.7 x 10^8) copies, 10.06 $\log_{10}$ (1.1 x 10^{10}) copies, 9.38 $\log_{10}$ (2.4 x 10^9) copies, and 8.15 $\log_{10}$ (1.4 x 10^8) copies, respectively[46]. A paper on *intI*1 states that environmental samples typically report over 1 x10^6 copies per gram, with samples near animal waste having one copy per bacteria cell[34]. An Estonian study reported levels of *intI*1 in manure made from dairy cattle and biogas plants. This study reported 7 $\log_{10}$ (1.0 x 10^7) gene copies per gram of dry weight in cattle slurry and 8.6 $\log_{10}$ (4.0 x 10^8) gene copies per dry weight of cattle slurry digestate[49].

Given this context, the values from this study are a few orders of magnitude lower than those of manure samples around the world. This may be attributed to the processing and time between when the manure is first excreted to the time the manure is available for consumers. Natural soils were indeed lower than both the commercial fertilizers and global findings in manure.

Correlation Analysis

SAS Software (SAS Institute Inc., Cary, NC, USA) was used to determine the Pearson correlation coefficient and its associated p-value. The Pearson correlation coefficient for *erm*F and *intI*1 is 0.10706 ($p < 0.2328$) per gram of soil and 0.52623 ($p < 0.0001$) per gene copies of 16S rRNA. These results indicate low to no correlation between *intI*1 and *erm*F and are depicted in correlation plots in **Figure 8** and **Figure 9**. This finding is similar to other studies that did not find high correlations between the two genes[50,51]. No significant correlations were found between soil properties and *intI*1 or *erm*F. These correlation plots can be found in **Figure 10** and **Figure 11**.

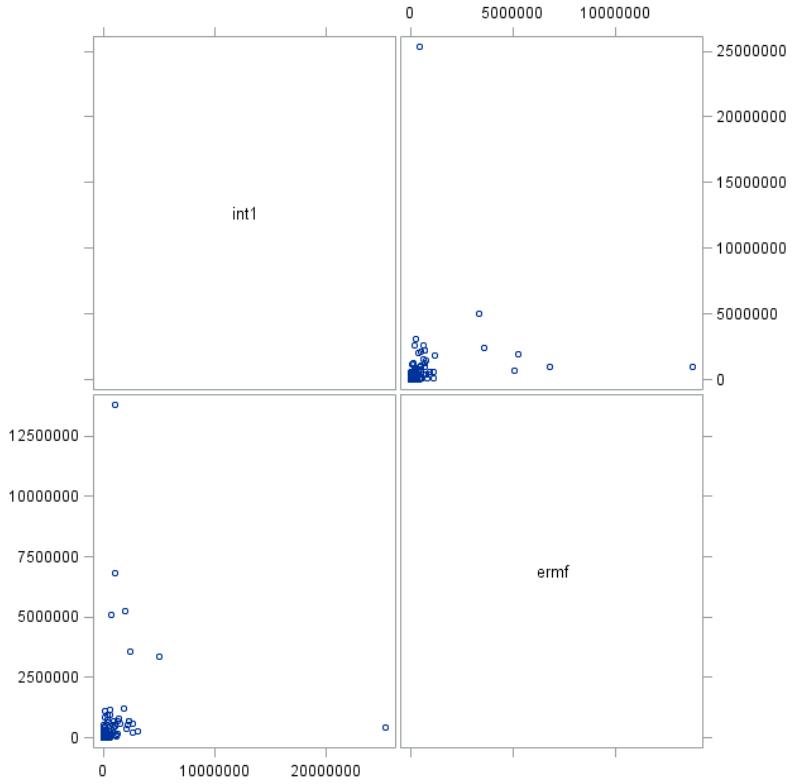

Figure 8: Correlation plot of *erm*F and *intI*1 per gram of soil

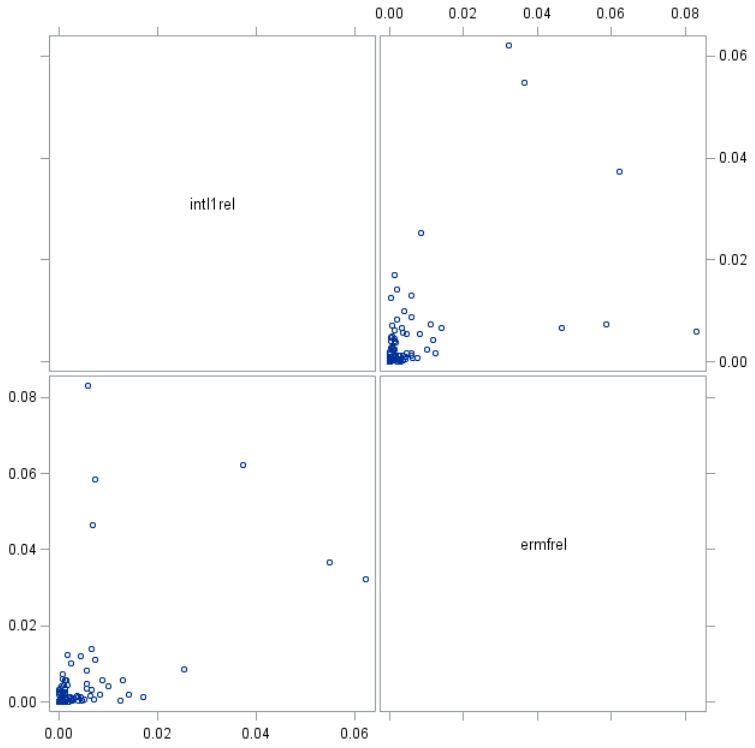

Figure 9: Correlation plot between *erm*F and *intI*1 relative to 16S rRNA

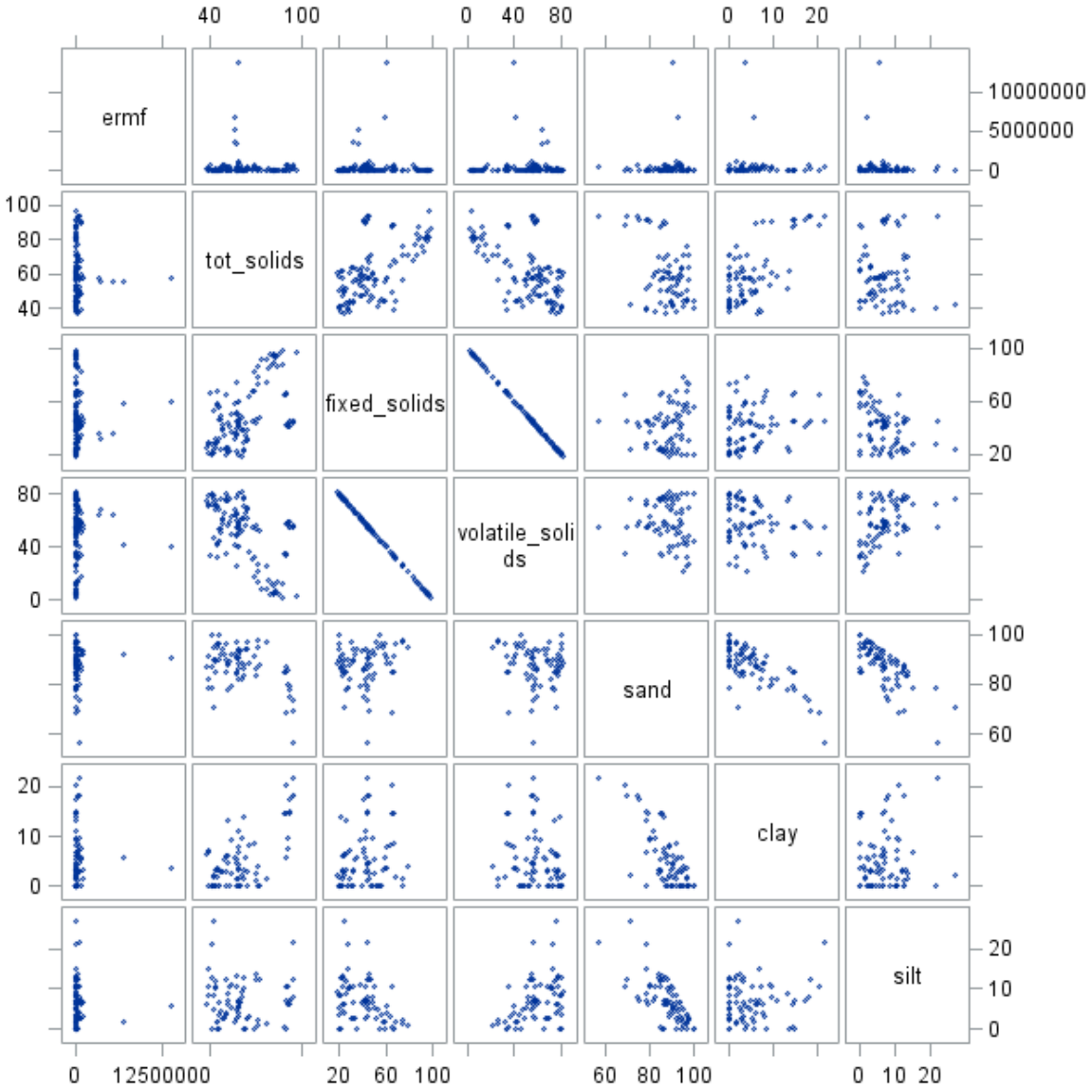

Figure 10: Correlation plot of *erm*F and soil properties

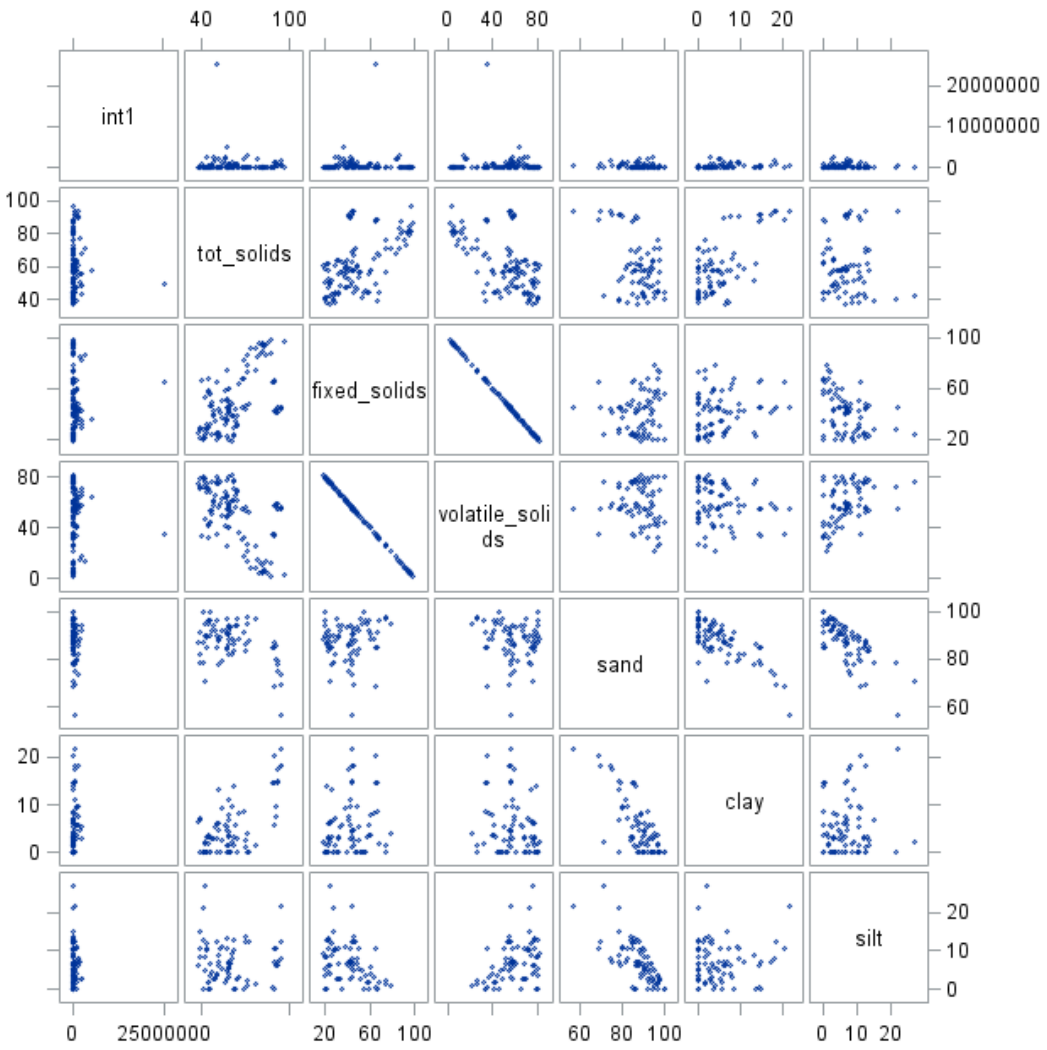

Figure 11: Correlation plots of *intI*1 and soil properties

RStudio (RStudio, Inc., Boston, MA) was used to determine if the differences between the gardening products and natural soils were statistically significant. To accomplish this, an unpaired two-sample Wilcoxon Test was performed between natural soils and each gardening type. The Wilcoxon test shows that the natural soils had significantly less *erm*F and *intI*1 levels than the other soils with a chosen alpha (alpha=0.05).

Table 8: Wilcoxon test p-values between natural soils and other soil categories

	Wilcoxon test p-values	
	*erm*F	*intI*1
Potting Soil	0.03348	0.001894
Garden Soil	4.075×10^{-5}	4.08×10^{-7}
Fruit Amendment	8.611×10^{-6}	9.678×10^{-6}
Lawn Amendment	1.706×10^{-5}	1.215×10^{-5}
Manure	2.248×10^{-6}	9.714×10^{-7}
Community Soils	6.86×10^{-5}	4.699×10^{-5}
Compost	0.02792	4.699×10^{-5}

Conclusions and Future Work

ARB and ARGs stand to threaten public health through rendering antibiotics useless. The agricultural sector involving animal husbandry plays a role in the dissemination of ARGs and MGEs in the environment. Multiple studies investigated the levels of ARB and ARGs from manure onto fields and its subsequent runoff. This study aimed to investigate the levels of *erm*F and *intI*1 in commercial fertilizers and amendments and compare them to natural environmental sources. Although the levels of *erm*F and *intI*1 are lower than those from raw manure samples, they are still higher than natural soils. This indicated that these commercially available gardening products serve as an input of ARGs and MGEs to the environment. There was also no correlation between *erm*F and *intI*1 or any of the soil characteristics. This indicates that genes are unique and there is no singular driving force determining ARG and MGE quantity. ARGs and MGEs may not be determined by physical soil properties, but perhaps may be influenced by soil geochemistry or microbial diversity.

Future work may extend this type of work to include a wider array of ARGs and MGEs. More products and brands can be tested for consistency between bags from each brand. Future work may include the fate and transport of these gardening products on residential landscapes.

Soils can be measured for ARGs and MGEs, and if food is produced, these may also be tested to see if ARGs or MGEs were acquired in the growing process.

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