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Prevalence, Fate, and Co-selection of Heavy Metals

and Antibiotic Resistance Genes in Urban and Agricultural Soils

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Civil Engineering

by

Wei-Cheng Hung

2020

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ABSTRACT OF THE DISSERTATION

Prevalence, Fate, and Co-selection of Heavy Metals
and Antibiotic Resistance Genes in Urban and Agricultural Soils

by

Wei-Cheng Hung
Doctor of Philosophy in Civil Engineering
University of California, Los Angeles, 2020
Professor Jennifer A. Jay, Chair

The widespread occurrence of antibiotic resistance and heavy metal are critical public health issues, posing global threats to human health. Antibiotics have been widely used in medical settings and in agriculture as growth promoters in livestock. A large fraction of antibiotics are excreted unchanged and can create selective environments for the emergence and proliferation of antibiotic resistance in receiving environmental compartments such as agricultural soils. Heavy metals, known as notable environmental stressors, promote the proliferation of antibiotic resistance by exerting co-selective pressure. More research is needed to manage both issues and to address the gap in the literature that exists with respect to their synergism. Through both field and laboratory microcosm research, the present study aims to investigate the prevalence, fate, co-occurrence, and interactions of antibiotic resistance genes (ARGs) and metal(loid)s in urban and agricultural soils.

Los Angeles has a long history of lead (Pb) contamination due to automobile emissions, industrial processes, and Pb-containing paint. Although the amount of Pb coming from these sources has been significantly reduced, the legacy of Pb in Los Angeles continues to affect soil Pb concentrations in public parks. These concentrations were shown to be high, with 47 parks having one or more samples exceeding the California EPA guideline of 80 ppm. According to EPA LeadSpread model, soil Pb levels of 80–320 ppm can lead to an estimated increase of blood Pb level from 1 to 4 μg/dL. This study provides information that can be used to guide remediation decisions for authorities and for the construction of Pb-safe environments.

While bioretention systems (biofilters) have been widely and effectively used to capture chemical pollutants from surface runoff, the effect of biofilters on both heavy metals and antibiotic resistance genes (ARGs) has been relatively understudied. A comprehensive survey of seasonal and regional ARGs and metal(loid)s is presented in urban biofilters in this study. Multiple effects of soil properties, bioavailable and total metal(loid)s on ARGs within biofilters were determined using multiple linear regression analysis. This study enhances our knowledge of ARG baseline levels for risk assessment and more strategic development of urban biofilters.

Land application of biosolids is a common practice for recycling and reuse of biosolids. However, the effect of land application of biosolids on soil ARG concentrations still remains elusive. Results indicate that ARGs were higher in biosolids-amended soils than in the surrounding agricultural soils that had not received biosolids. Land-applied biosolids also create selective environments for the emergence and proliferation of ARGs in the environment. Spatial patterns of ARGs in soils also provided insight into the distribution of ARGs and their associations with types of land uses, ultimately leading to better management of soil ARGs.

The dissertation of Wei-Cheng Hung is approved.

Michael Stenstrom

Sanjay Mohanty

Richard Ambrose

Jennifer A. Jay, Committee Chair

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VITA

Education

University of California, Los Angeles, CA Apr 2017 – Present
Ph.D. Candidate in Civil and Environmental Engineering

University of California, Los Angeles, CA Sep 2015 – Apr 2017
M.S. in Civil and Environmental Engineering
Environmental and Water Resource Engineering

National Taiwan University (NTU), Taiwan Sep 2009 – Jun 2014
B.S. in Chemical Engineering and Geography
Dual degree: Chemical Engineering and Geography

Teaching and Mentoring Experience

Teaching Fellow Sep 2016 – Dec 2020
Department of Civil and Environmental Engineering, UCLA
Scientific Solutions to Environmental Inequalities: Theory and Practice (CEE 188) Spring 2020
Chemical Fate and Transport in Aquatic Environments (CEE 154) Winter 2017, 2019, and 2020
Environmental Aquatic Inorganic Chemistry (CEE 254) Fall 2016 – 2020
Climate Change, Water Quality, and Ecosystem Functioning (58SL) Spring 2018
Introduction to probability and Statistics for Engineers (CEE 110) Spring 2017

Laboratory Instructor Sep 2017 – Dec 2018
Center for Excellence in Engineering and Diversity, UCLA

Reader/Grader Mar 2016 – Jun 2016
Department of Civil and Environmental Engineering, UCLA
Introduction to Probability and Statistics for Engineers (CEE 110) Spring 2016

Presentations

Whitener, V. and **Hung, W.** (2019) Antibiotic resistance genes in reclaimed water for irrigation in the Maneadero Valley, Mexico. 258th American Chemical Society National Meeting, San Diego, CA. August 25-29, 2019

Rugh, M. and **Hung, W.** (2019) Survey of antibiotic resistance gene and heavy metal co-selection in UC campus stormwater biofilters. 258th American Chemical Society National Meeting, San Diego, CA. August 25-29, 2019

Hung, W. and Jay, J. (2019) Effect of copper on the spread of sulfonamide resistance genes and class I integrons in compost-based biofilters. UNC Water Microbiology Conference, Chapel Hill, NC. May 13-16, 2019

Publications

Hung, W., Rugh, M., Feraud, M., Avasarala, S., Kurylo, J., Gutierrez, M., Jimenez, K., Troung, N., Holden, P., Grant, S., Liu, H., Jay, J. (2021a) Prevalence of antibiotic resistance genes and co-selection of metal(loid)s in stormwater biofilters in Southern California. *Journal of Hazardous Materials*, in preparation.

Hung, W., Troung, N., Jay, J. (2021b) Tracking antibiotic resistance genes and antibiotic-resistant bacteria through the environment near a biosolid spreading ground: resistome changes, transport, and metal co-selection. *Environmental Pollution*, in preparation.

Hung, W., Horng, R. S., & Shia, R. E. (2020) Investigation of thermal insulation performance of glass/carbon fiber-reinforced silica aerogel composites. *Journal of Sol-Gel Science and Technology*, 1-8.

Parker, E., Grant, S., Cao, Y., Rippy, M., McGuire, K., Holden, P., Feraud, M., Avasarala, S., Liu, H., **Hung, W.**, Rugh, M., Jay, J., Peng, J., Shao, S., and Li, D. (2020) Predicting Unsteady Pollutant Removal in Green Stormwater Infrastructure with Transit Time Distribution Theory. *Journal of Water Resources Research*, accepted.

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Hung, W., Horng, R. S, and Hsia, R. (2020) Thermal insulation performance of silica aerogel reinforced by glass/carbon fibers. *Journal of Sol-Gel Science and Techonology* 1-8.

Hung, W., Hernandez-Cira, M., Jimenez, K., Elston, I., & Jay, J. (2018) Preliminary assessment of lead concentrations in top soil of 100 parks in Los Angeles, California. *Applied geochemistry* 99: 13-21.

Honors and Awards

NWRI Graduate Fellowship, The National Water Research Institute 2020

Southern California Scholarship, STUF and Yin-Chin Foundation of USA 2018

Taiwan Government Scholarship, Ministry of Education, Taiwan 2018

Chinese American Engineers and Scientists Association of Southern California Scholarship, Chinese American Engineers and Scientists Association 2018

Dean's Award (top 5% ranking graduate), College of Science, National Taiwan University 2013

International Exchange Scholarship, College of Science, National Taiwan University 2013

College Student Research Scholarship, Academia Sinica 2011

Presidential Award (top 5% ranking student), National Taiwan University 2010 & 2011

CHAPTER 1. BACKGROUND AND OBJECTIVES

1.1 Objectives and Scope

The overall objective of this dissertation was to assess the prevalence, fate, and co-occurrence of antibiotic resistance genes (ARGs) and metal(loid)s in urban and agricultural soils. This dissertation contains two separate main topics: (1) understanding levels of soil Pb in Los Angeles parks for the better management and legislation as shown in objective 1; (2) investigating the prevalence, fate, and co-occurrence of ARGs and metal(loid)s in urban biofilters and agricultural soils (objective 2 and 3). Studies were conducted on the soil environment in different locations to address the following objectives:

Objective 1: To assess Pb concentrations in surface park soils and provide an estimation of increased blood lead levels (BLLs) for children and to identify potential sources of Pb (using a spatial analysis tool) and their association with soil properties.

Objective 2: To investigate the levels of ARGs and metal(loid)s and the co-selective effects of metal(loid)s on ARGs in urban stormwater bioretention systems, and to compare ARG levels with those in other urban soil systems.

Objective 3: To track antibiotic resistance and heavy metals through the environment within and adjacent to the biosolid application site for resistome changes, spatial distribution, and metal co-selection.

1.2 Dissertation Overview

This dissertation is categorized into six chapters. In Chapter 1, a combination of research objectives and dissertation overview is described. Chapter 2 presents the background and current knowledge of heavy metals and antibiotic resistance. Preliminary assessment of soil Pb concentrations in 100 Los Angeles parks is shown in Chapter 3. This study has been published in

Journal of Applied Geochemistry (Hung et al., 2018). However, Pb accumulating in the urban soils may trigger co-selection on AR. Chapter 4 characterizes the prevalence of ARGs and metal(loid)s and the synergistic effect that heavy metals can pose on the proliferation of antibiotic resistance in urban stormwater biofilters. This work will be submitted to *Journal of Hazardous of Materials* for publication (Hung et al., 2021a). Geographical patterns, resistome changes, and metal(loid)s co-selection of ARGs, antibiotic resistant bacteria (ARB) in soils within and in the vicinity of biosolid application site are reported in Chapter 5. A portion of this study that is highly linked to another fertilizer study has been submitted to *Journal of Environmental Science and Pollution Research* for publication (Cira et al., 2020). Results that focus on geographical patterns of ARGs near the biosolids spreading ground will be submitted to *Environmental Pollution* for publication (Hung et al., 2021b). Last but not least, significant findings in this dissertation that fill the knowledge gaps of environmental antibiotic resistance and heavy metals are summarized in Chapter 6. Suggested future research was also discussed.

1.3 References

Hung, W., Hernandez-Cira, M., Jimenez, K., Elston, I., & Jay, J. 2018. Preliminary assessment of lead concentrations in top soil of 100 parks in Los Angeles, California. *Applied geochemistry* 99: 13-21.

Hung, W., Rugh, M., Feraud, M., Avasarala, S., Kurylo, J., Gutierrez, M., Jimenez, K., Troung, N., Holden, P., Grant, S., Liu, H., Jay, J. 2021a. Prevalence of antibiotic resistance genes and co-selection of metal(loid)s in stormwater biofilters in Southern California. *Journal of Hazardous Materials*, in preparation.

Cira, M., Echeverria-Palencia, C. M., Callejas, I., Jimenez, K., Herrera, R., **Hung, W.**, Colima, N., Schmidt, A., Jay, J. 2020. Commercially available garden products as potential sources of antibiotic resistance genes—A survey. *Environmental Science and Pollution Research*, submitted.

Hung, W., Troung, N., Jay, J. 2021b. Tracking antibiotic resistance genes and antibiotic-resistant bacteria through the environment near a biosolid spreading ground: resistome changes, transport, and metal co-selection. *Environmental Pollution*, in preparation.

CHAPTER 2. LITERATURE REVIEW

2.1 Lead in Urban Park Soils

2.1.1 Common sources of lead in the United States

Lead (Pb) is a highly stable and persistent metal that broadly exists in the environment. While the distribution and sources of Pb vary in different areas and periods during human history, the major routes of exposure to Pb currently observed include: (1) Dust/Paint: houses constructed before 1978 are likely to contain Pb-contaminated paint, which may ultimately turn into dust. Pb-based paint and Pb-contaminated dust in older houses are the major sources of Pb exposure for children in the United States (Jacobs et al., 2002); (2) Drinking water: Pb may also be found in drinking water due to leaching from Pb pipes or faucets in older communities; (3) Emission from Pb-related industries: emission from Pb-acid battery recycling plants is often associated with Pb pollution in their surrounding neighborhoods. Pb levels in soil, air, and water were shown to be high near those ongoing or historic pollution sites (Young et al., 2002); (4) Leaded aviation fuel (avgas): avgas is still currently used for over 167,000 small piston-engine aircrafts in the United States (Miranda et al., 2011). Pb emitted from aircrafts can contribute to elevated Pb levels in air; (5) Commercial products: Pb can also exist in products, such as toys, cosmetics, and jewelry as they may be processed with Pb-related ingredients (Njati and Maguta, 2019). While many authorities in the U.S. have made significant progress on reducing many Pb-based products, legacy of Pb can remain in the environments due to their high persistence (Semlali et al., 2004).

2.1.2 Lead toxicity and its major exposure pathway

Soil is considered as a major exposure pathway of Pb, particularly for children (Mielke and Reagan, 1998). In particular, Pb contaminants largely coming from anthropogenic sources such as those mentioned above tend to remain in surface soil due to atmospheric deposition and low mobility. The amount of Pb produced by human activities has been significantly reduced, lowering the frequency of soil related Pb poisoning in humans (Needleman, 2004; Thomas et al., 1999). Yet, Pb concentrations in certain areas can still be high due to the vicinity of previously Pb-contaminated sites. Exposure can result from inhalation of atmospheric soil dust and atmospheric Pb (Zahran et al., 2013) or ingestion of Pb-contaminated soil following hand to mouth contact (Abadin et al., 2007; Mielke and Reagan, 1998; Ruby and Lowney, 2012), especially for children as they are the most vulnerable to Pb poisoning. Pb has been ranked as one of the top priority toxic materials. The most vulnerable population, especially children, can be affected by relatively low levels of exposure to Pb. For example, long-term exposure to Pb can not only lead to diseases in neuro- and immune-reproductive systems but also Pb-related reduced intelligence quotient (IQ) levels and cognitive deficiencies (Kim et al., 2014).

Significant associations were found between concentrations of Pb in soil and BLLs of children in New Orleans, Syracuse, and Detroit (Bickel, 2010; Johnson and Bretsch, 2002; Mielke et al., 1997). Laidlaw et al. (2017b) analyzed the dose-response relationship in those three cities using the Integrated Exposure Uptake Biokinetic (IEUBK) model developed by the U.S. Environmental Protection Agency (USEPA). This analysis indicated that high BLLs correlated with relatively low soil Pb concentrations; as a result of this finding, the Centers for Disease Control and Prevention (CDC) has lowered the BLL threshold to 5 μg/dL. Nonetheless, no BLL concentration is considered to be safe for children as low BLLs in children have shown to affect IQ and school performance. (Centers for Disease Control and Prevention, 2012). Assumptions of safe minimal BLLs as well as other variables can lead to a wide range of differing soil Pb regulatory guidelines.

2.1.3 Regulations for lead in soils in the United States

Regulations governing safe limits of soil Pb vary among different U.S. agencies. According to USEPA guidance, a soil Pb concentration of 400 ppm in both recreational and residential areas should trigger cleanup activities. However, this regulation does not consider the full extent of health effects resulting from Pb exposure (Laidlaw et al., 2017a). The Office of Environmental Health Hazard Assessment (OEHHA) of California revised their soil Pb threshold to 80 ppm Pb in residential areas, which was determined by modeling the extent of exposure resulting from an incremental increase in BLL of 1 μg/dL, using the LeadSpread model (Office of Environmental Health Hazard Assessment, 2009). OEHHA guidelines also set the action limit where soil Pb concentrations have no more than a 2.5% probability of decreasing IQ scores by more than 1 point in a 90% of children and fetuses (Office of Environmental Health Hazard Assessment, 2009). However, soil Pb thresholds from both the USEPA and OEHHA are unenforceable since many factors may contribute to the need to cleanup soils. Currently, California Department of Toxic Substances Control (DTSC) recommends using the 95% upper confidence level (UCL) of the site's dataset to evaluate cleanup options (U.S. Environmental Protection Agency, 2002). The threshold concentrations are also intended to be used by community groups, landowners, and local agencies to estimate the degree of effort that is needed to remediate a Pb-polluted site.

2.1.4 Soil lead levels in parks and playgrounds

Previous studies have emphasized large cities where Pb contamination would be expected to be high. Residential communities (Laidlaw et al., 2018; Pouyat et al., 2007) and roadside communities (Al Obaidy and Al Mashhadi, 2013; Sun et al., 2010) in other major cities have been found to contain areas of high Pb as compared with other types of land use. Although many previous studies focused on soils from different types of land use, such as residential, industrial, and roadside areas, there are limited studies on soil Pb contamination in urban parks. Some studies across the world indicated that the average Pb concentrations in park soils were within the acceptable limits (Chen et al., 2005; Shi et al., 2008; Solgi and Konani, 2016; Zhang, 2006), while in other studies the soil Pb levels in urban parks often exceeded the local limits (Li et al., 2001; Lu et al., 2011; Madrid et al., 2002; Manta et al., 2002; Massas et al., 2010; Sun et al., 2010). However, parks within these cities appeared to have large variation that may lead to difficulties comparing with regional studies. Cities with different human activities and geography (i.e. population densities and industrial activities) may contribute to a variety of findings. In Los Angeles, both historical traffic and housing-unit related variables serve as crucial indicators of anthropogenic Pb inputs (Mielke et al., 2010). Previous investigation conducted in Los Angeles by Wu et al. (2010) showed total Pb concentrations near highways (average Pb concentration of 189 ppm; N = 299) and arterials (average Pb concentration of 224 ppm; N = 140) were higher than areas consisting mostly of residential areas (average Pb concentration of 107 ppm; N = 111) (Wu et al., 2010). The study indicated that current Pb pollution in south Los Angeles results from both household paint and traffic patterns (Wu et al., 2010). However, Pb concentrations in park soils largely remain unknown – even though the most susceptible, children, are likely to come into contact with contaminated soils in parks.

2.2 Antibiotic Resistance in the Environment

Increasing antibiotic resistance (AR) is of great concern to public health worldwide and has been exacerbated by the overuse of antibiotics for medical and agricultural purposes. Unfortunately, progress of developing new antibiotics has slowed significantly in recent decades. Certain once-life saving drugs are now considered to be less effective or “worthless” (Woolhouse and Farrar, 2014). It is essential to extend the usefulness of the antibiotics on which we currently rely. Global total antibiotic use has surged rapidly and AR prevention has even become a high priority for the World Health Organization (WHO). It is now evident that environment is the largest reservoir of AR. For the betterment of future antibiotic resources, it is urgently required to understand the prevalence, diversity, and mechanisms of environmental AR and their potential stressors.

2.2.1 Drivers and pathways of antibiotic resistance

Despite the restricted use of some key antibiotics, there are still important factors controlling emergence of AR that need to be understood. Trace levels of antibiotics (which can enter the environment after excretion in urine) can exert selective pressure on environmental microbes. In addition, ARG levels may still increase even without selective pressure and in turn cause spread dissemination of AR traits by vertical transfer as well as different horizontal gene transfer (HGT) (Dodd, 2012). HGT takes place based on three well-known genetic processes: (1) Conjugation: genes are transferred from bacteria directly to another cell; (2) Transduction: bacterial viruses move genes from one cell to another; and (3) Transformation: bacteria pick up DNA from the environment (Burmeister, 2015). These mechanisms are illustrated in Figure 1. In brief, co-selective pressure exerted by heavy metals could act as a specific driving force for the proliferation of ARGs via enhancing the co-resistant plasmid conjugative transfer.

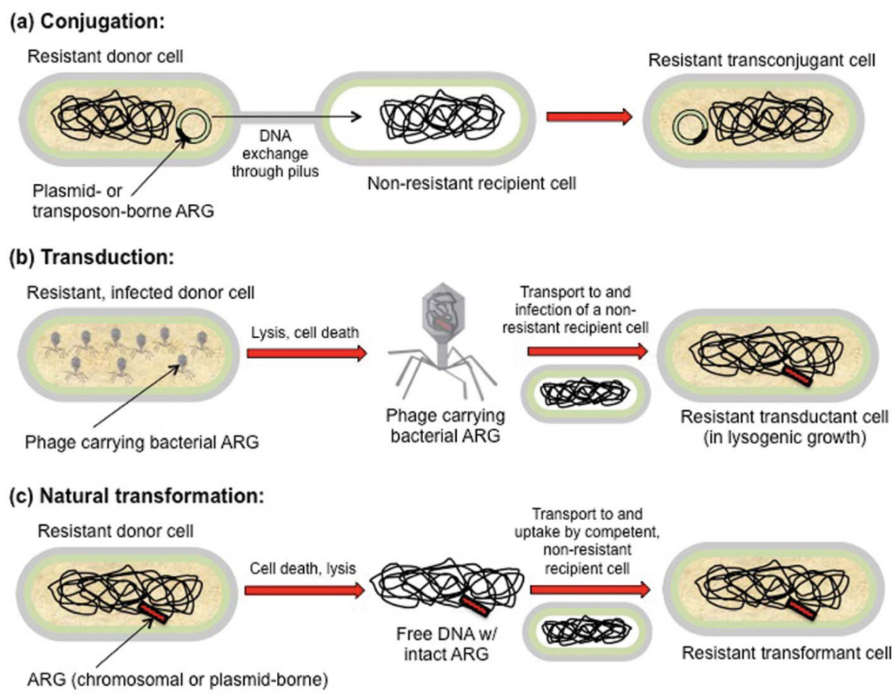

Figure 1. Mechanisms of bacterial horizontal gene transfer (HGT) by means of (a) conjugation, (b) transduction, and (c) transformation (Dodd, 2012).

Recent studies have shifted attention to the natural environment due to evidence that exchange of relevant resistance genes was observed between clinical and environmental microbes (Forsberg et al., 2012; Wright, 2010). Additionally, the majority of ARGs obtained by human pathogens was found to be originally from natural environments (Martinez, 2009). Environmental compartments can function as important reservoirs for antibiotic resistance genes (ARGs). Our knowledge of the environmental fate and transport of ARGs is rapidly growing. Certain sources of ARGs to the environment have been identified, including municipal wastewater treatment plants (Mao et al., 2015), hospital waste streams (Rodriguez-Mozaz et al., 2015), animal feeding operations (McEachran et al., 2015), manure fertilizers (Fahrenfeld et al., 2014; Tang et al., 2015), biosolids (Yang et al., 2018a), and manured fields (Heuer et al., 2011). Recent work has identified the important of environmental compartments in the movement of ARGs. Park soils (Knapp et al., 2011), airborne particulates (Ouyang et al., 2020; Sapkota et al., 2006), aquatic systems (Baquero et al., 2008; Marti et al., 2014; Martins et al., 2014; Pruden et al., 2012), biofilms (Engemann et al., 2008; Schwartz et al., 2003), and aquatic sediments (Chen et al., 2013; Cummings et al., 2011; Yang et al., 2013) can all serve as important reservoirs for ARG. In most cases, ARGs were transferred to water and soil environments as a result of agricultural and aquaculture purposes. Especially for agriculture, after manure fertilizers containing antibiotics were added to soils (Heuer et al., 2011), ARGs become mobile and may be transported via air or water.

However, there are gaps in our understanding concerning the fate of ARGs once in the environment – an extremely complex topics as ARGs can decay, persist, or grow depending on determinants in the environment. Important factors include the composition of the microbial community in the receiving environmental compartment, microbial growth rates, remaining levels of antibiotics, levels of contaminants known to exert co-selective pressure, and the geochemical parameters that would impact the bioavailability of co-contaminants. Significant remaining questions surround the importance of co-selective pressure in various environmental samples and the transport via air of ARGs and ARB, which can mobilize ARGs to other areas where selective and co-selective processes have the potential to increase the levels of these contaminants.

2.2.2 Heavy metal co-selection on antibiotic resistance

Issues of particular concern for human health are the persistence of ARGs and the synergistic effect that heavy metals can pose on the proliferation of antibiotic resistance. Metals and antibiotics are prevalent co-contaminants, and exposure to metals could potentially induce co-selection for ARGs by one of three potential mechanisms (Baker-Austin et al., 2006): (a) co-resistance occurs when resistance genes conferring resistance to metals or antibiotics are linked on the same genetic element; (b) cross-resistance occurs when a resistance gene conferring resistance to multiple metals or antibiotics; (c) co-regulation exists as more than one resistance gene conferring resistance to metals or antibiotics are manipulated by regulatory genes (Figure 2).

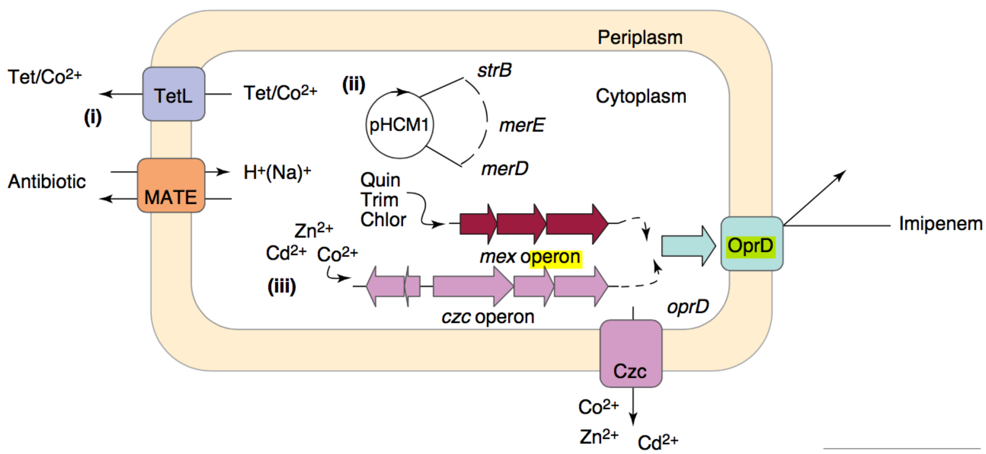

Figure 2. Examples of molecular mechanisms under metals and antibiotic co-selection. (i) Cross-resistance; (ii) Co-resistance; and (iii) Co-regulatory (Baker-Austin et al., 2006).

Numerous findings have focused on bacterial species/isolates under metal co-selection. Correlations between heavy metals and AR proliferation have been widely reported in many highly contaminated environments due to continuous and/or accidental pollution. Studies investigating co-selection in soils frequently indicate the correlation of increased metal concentrations with increased ARG levels. These environments include effluents and biosolids from waste treatment plants (Di Cesare et al., 2016; Gao et al., 2012; Jang et al., 2018; Mao et al., 2015), agricultural soils affected by land application of biosolids or manure waste (Chee-Sanford et al., 2009; Ji et al., 2012), feedlots (He et al., 2016), water bodies associated with waste discharges (Graham et al., 2011), and metal-spiked experiments in microcosms (Knapp et al., 2011; Stepanauskas et al., 2006; Wang et al., 2020) and in agricultural fields (Hu et al., 2017). For instance, it was shown in Xiangjiang River, China that significantly positive correlations existed between heavy metals and ARGs (*sul*1 gene and Cu, Zn, and Hg; *tet*A gene and Cu and Hg; Ni and *tet*W; *qnr*A, *qnr*B, and *qnr*D genes and Zn; *erm*B gene and Cu) (Xu et al., 2017). More detailed correlations are summarized in **Error! Reference source not found.** from previous studies. Although correlation doesn't represent causation, these findings suggest that heavy metals may exert selective pressure on the emergence of ARGs. Moreover, to evaluate the risk for the ARG co-selection, the concept of minimum co-selective concentrations (MCC) was first adopted and evaluated in many environmental compartments by Seiler and Berendonk (2012). The data includes minimum co-selective metal concentrations observed based on laboratory and field settings. Metals exceeding their MCCs in the environment were likely co-selective for ARGs. Yet, not many studies have focused on the ARGs in various environmental components under heavy metal driven co-selection that were caused by high degree of human and agricultural activities. Slightly contaminated soils, such as stormwater biofilters, are still poorly understood.

Table 1. The summary of the correlation between ARGs and metal(loid)s from literatures. All data shown below are significantly positive correlation (α≤ 0.05). A symbol of * and – represent correlations were found to be negative and insignificant, respectively. Abbreviations: As, arsenic; Al, aluminum; Cr, chromium; Mn, manganese; Ni, nickel; Cu, copper; Zn, zinc; Cd, cadmium; Pb, lead; Hg, mercury; Fe, iron; U, uranium; V, vanadium.

	As	Al	Cr	Mn	Ni	Cu	Zn	Cd	Pb	Hg	Fe	U	V
*sul*A	–	–	–	–	–	g	g	–	–	g	–	–	–
*sul*1	–	–	–	*e*	–	ab	ab	ab	–	–	–	–	–
*sul*2	–	–	–	*e*	–	-	a	–	–	–	–	–	e
*sul*3	–	–	–	–	–	bg	bg	b	–	abg	–	–	–
*tet*1	–	–	–	–	–	–	–	–	–	–	–	–	–
tet2	–	–	–	–	f*	–	–	–	ef*	ef	–	–	f*
tet3	–	–	–	e	–	–	–	–	–	–	–	–	e
tet4	–	–	–	–	–	–	–	–	–	–	–	–	–
*tet*A	–	–	–	–	–	ab	–	a	–	ab	–	–	–
*tet*B	–	–	–	–	–	-	–	–	ab	–	–	–	–
*tet*W	–	*e*	–	*e*	bc	c	e	–	ab	–	–	–	e
*tet*M	–	–	c	e	b	bce	be	ab	–	–	c	–	–
*tet*Q	–	–	–	–	–	–	–	–	ab	–	–	–	–
*tet*O	–	–	–	–	–	–	–	–	ab	–	–	–	–
*qep*A	–	–	–	–	–	ab	b	–	–	ab	–	–	–

	As	Al	Cr	Mn	Ni	Cu	Zn	Cd	Pb	Hg	Fe	U	V
*qnr*A	–	–	–	–	–	ab	ab	–	–	b	–	–	–
*qnr*B	–	–	–	–	–	ab	ab	–	–	b	–	–	–
*qnr*D	–	–	–	–	–	–	ab	–	–	b	–	–	–
*qnr*S	–	–	–	–	–	–	ab	–	–	–	–	–	–
*erm*B	–	–	–	–	–	bcg	–	–	–	–	–	–	–
*erm*C	–	–	–	–	–	b	–	–	–	–	–	–	–
*erm*F	–	–	–	–	–	c	–	–	–	–	–	–	–
bla_{OXA}	–	e	c	–	–	cde	e	–	def	–	–	–	e
bla_{TEM}	–	e	–	e	e	e	e	–	f	–	–	f	e
bla_{SHV}	ef	–	–	–	f	–	–	–	–	–	–	–	–
bla_{CTX}	–	–	c	e	–	–	–	–	–	–	–	ef	e

Environmental compartments: (a) water (river) by Xu et al. (2017); (b) sediment (river) by Xu et al. (2017); (c) soil by Knapp et al. (2011); (d) water + sediment (river) by Graham et al. (2011); (e) and (f) soil (unnormalized & normalized) by Knapp et al. (2017); (g) animal manure and agricultural soils by Ji et al. (2012).

2.2.3 Heavy metal co-selection on antibiotic resistance in urban stormwater runoff

Stormwater has become a significant source of pollution in the urban water environment (Müller et al., 2020). Stormwater flows over streets, through storm gutters and drains, and eventually spill into water bodies, collecting bacteria, metals, trash, and other pollutants along the way. More specifically, fecal contaminants are frequently observed to carry elevated levels of ARB and antibiotics (Karkman et al., 2019). While levels of antibiotics are relatively low in stormwater, heavy metals are distinguished by their long persistence. After infiltrating an environment, ARBs can proliferate due to long-term selective and co-selective pressure caused by heavy metals, depending on the metal's bioavailability and speciation (Isaac Najera et al., 2005). Co-selection is expected to depend on the concentrations and speciation of the antibiotics and metals, as well as on the composition of the microbial community and level of horizontal gene transfer (HGT). Mobile genetic elements (MGEs), such as plasmids and integrons, can transfer genetic materials to a variety of microorganisms (Dröge et al., 2000; Merlin et al., 2011; Schlüter et al., 2007). This process, as well as direct and indirect selection for bacteria in metal-polluted streams, may represent a critical pathway of dissemination of AR.

Stormwater and dry weather runoff are a major source of pollution in southern California, significantly impacting the region's water quality and posing risks to the health and safety of residents. Dorsey et al. (2013) reported that 54 % of isolate species of the 277 isolates collected from the mouth of Ballona Creek were pathogenic strains that can be naturally occurring, be introduced in runoff, or originate from other sources. The majority of identified isolates include species of *Enterococcus* and *Vibrio*. Another similar investigation of ARB prevalence in Los Angeles Ballona Creek showed that bacteria in streams exhibited microbial resistance against up to eight different antibiotics (Kawecki et al., 2017). Simultaneously, Ballona Creek stormwater was observed to have elevated heavy metals in dissolved phase, including barium, copper, nickel, and zinc, showing higher frequencies and magnitudes of toxicity than Malibu Creek stormwater (Bay et al., 2003). To date, total maximum daily loads (TMDLs) have been established for heavy metals, toxic organic compounds, trash, and fecal indicator bacteria by the state to control these pollutants in Ballona Creek and Estuary (Copeland, 2012). However, pollutants may still co-select for microorganisms or other pollutants simultaneously as metal concentrations lower than those typically observed in the environment can still trigger co-selection of antibiotic resistance (Seiler and Berendonk, 2012). After infiltrating an environment, ARB or ARG levels may increase after initial infiltration into biofilters due to HGT and co-selective pressure caused by heavy metals, depending on the metal's bioavailability and speciation (Isaac Najera et al., 2005). A detailed study in this area is needed considering the significant consequences of impaired urban stormwater quality.

2.2.4 Biosolids as source of ARGs and ARB

Numerous studies of ARGs in wastewater treatment plants (WWTPs) show that ARGs concentrate in the waste sludge. Several studies show levels of individual ARGs in waste solids to be greater than 10^9 copies/mL (Auerbach et al., 2007; Munir et al., 2011; Zhang et al., 2009), and in some cases the level can be even greater than 10^{10} copies per mL (Burch et al., 2016). Mao et al. (2015) showed that for twelve ARGs, even though the effluent concentration was relatively low, the total loading when both effluent and waste sludge were considered was actually higher than the loading in the influent, indicating proliferation throughout the treatment train. In a study of ARB and ARGs in influent, effluent, and biosolids in five WWTPs, Munir et al. (2011) also showed high loads of ARGs and ARB in biosolids treated by various methods, specifically conventional dewatering and gravity thickening and the advanced treatment methods anaerobic digestion (AD) and lime stabilization. Biosolids treated with the advanced methods had lower levels of tetracycline- and sulfonamide-resistant bacteria; however, while lime stabilized biosolids had consistently lower ARG levels, AD-treated biosolids did not have reliably lower ARG levels.

Biosolids undergo various types of treatment before being applied in agriculture, including with air-drying, aerobic and anaerobic digestion (AD), and lime stabilization. Looking specifically at AD, Burch et al. (2016) investigated the persistence of ARGs in waste solids at various anaerobic digester temperatures. ARG and *intI*1 levels decreased at all temperatures, with greater removal at higher temperatures. The kinetics fit a Collins-Selleck model, because the decrease in ARG was steep at the start, but then levelled off because some fraction of the ARGs were resistant to further decay. In many cases a two order of magnitude removal was observed, but since the levels of ARGs are so high in biosolids, the concentrations remaining in treated biosolids are still high.

2.2.5 Microbial regulations for biosolids in the United States

More than 2 million dry metric tons of biosolids are applied to land in the United States in 2019 (USEPA, 2020). Biosolids used for land application are currently regulated under 40 CFT Part 503 to ensure that levels of metals and pathogens pose minimal risk to human health. After initial treatment to reduce odorous compounds, biosolids are categorized into “Class A” and “Class B” with specified treatment requirements for pollutants, pathogens, and vector attraction reduction. Class A biosolids are commonly produced by AD, especially under thermophilic digestion (55°C), and can be used without restriction (Mclain et al., 2017). They must also meet the limit of either less than 3 *Salmonella* per 4g total solids or 100 MPN fecal coliforms per gram total solids. However, class B biosolids do not eliminate pathogens (the allowable level of fecal coliforms is $<$

2 million MPN fecal coliform per g total solids) and are restricted by certain regulations when applied to land (Zaleski et al., 2005). While current regulations were established on conservative risk assessment of soil- and food-transmitted enteric pathogens, the ARG distribution, diversity, and transport in soil microbiomes treated with biosolids still remain largely unknown. Therefore, the risk of disseminating ARGs into agricultural fields and products has yet to be elucidated (Mclain et al., 2017).

2.2.6 Elevated ARGs in soils after application of biosolids

Agricultural land application is one of the most common practices for recycling and reuse of nutrients in biosolids. More than 60% of biosolids from WWTPs were land applied in the United States (USEPA, 2000). Anaerobic digested biosolids were known to contain elevated levels of ARGs, yet the effect of biosolids applied soils on the resistome is less assertive. A number of studies have documented elevated levels of ARGs and/or antibiotic resistant bacteria (ARB) in soils after either manure application Tang et al. (2015) showed that in general, additions of manure to paddy soils resulted in increased ARG levels, and ARG concentration decreased with depth. Multiple studies were able to apply metagenomic techniques to characterize the impacts on the resistome in soils after land application of waste solids. The diversity and prevalence of ARGs were found to increase after addition of waste solids (Chen et al., 2017; Yang et al., 2018b). Increased availability of plasmids encoding for resistance genes was shown after biosolids application with a model recipient for horizontal gene transfer (HGT) (Leise Riber et al., 2014). Fahrenfeld et al. (2014) also showed evidence for HGT through a mass balance approach for ARGs in fields treated with waste solids; when observed ARG levels were higher than the level expected based on the mass balance, HGT was implicated. However, several studies also reported no significant effect of biosolids application on ARG sand ARB, as they were not persistent in the receiving soil systems (Brooks et al., 2007; Munir and Xagoraraki, 2011; Zerzghi et al., 2010). Soil microbiomes before land-applied with biosolids may already contain high levels of ARGs (Bondarczuk et al., 2016; Brooks et al., 2007). Moreover, occurrence, concentration, and spatial distribution of soil ARGs were also highly associated with land uses (Zhao et al., 2020).

2.2.7 Shifts to microbial community after land application of manure and/or biosolids

The diversity and prevalence of ARGs can increase after addition of waste solids (Chen et al., 2017). Recently Yang et al. (2018a) and Chen et al. (2017) were able to apply metagenomic techniques to characterize the impacts on the resistome in soils after land application of waste solids. The microbial community in the waste solids and soils were sufficiently distinct so both of the studies were able to identify biosolid-borne microbes. The prevalence of these microbes could then be detected in samples containing a mix of soil and biosolids. Riber et al. (2014) showed important shifts in the antibiotic resistance profile in soil amended with sludge and biosolids. The fraction of *Pseudomonas* resistant to gentamicin was elevated after application of sludge, and a greater fraction of cultured strains of *Pseudomonas* were multidrug-resistant in amended compared to unfertilized soil. Notably, HGT transfer of ARGs was higher after fertilization. Experiments using indigenous soil microbes as plasmid donors and a model *P. putida* strain as a recipient showed high availability of plasmids conferring resistance to gentamicin and tetracycline after application of sludge or manure.

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CHAPTER 3. PRELIMINARY ASSESSMENT OF LEAD CONCENTRATIONS IN TOPSOIL OF 100 PARKS IN LOS ANGELES, CALIFORNIA

3.1 Introduction

Lead (Pb) exposure has been a serious public health issue for at least a century, with soil being a primary pathway of exposure (Filippelli and Laidlaw, 2010; Mielke and Reagan, 1998). Pb contaminants in soil largely come from anthropogenic sources including Pb-based paint, leaded gasoline for automobiles, battery recycling, and emissions from factories (Johnston and Hricko, 2017). In recent years, the amount of Pb generated by anthropogenic sources such as those listed above has been reduced or phased out, which has reduced the frequency of soil-related Pb poisoning in humans (Needleman, 2004; Thomas et al., 1999). However, areas previously contaminated with Pb still pose an exposure risk due to high rates of persistence of Pb in soil (Semlali et al., 2004).

Pb contaminants tend to remain in surface soils due to the low mobility of Pb. (Li, 2006; Semlali et al., 2004). Growing evidence suggests that high levels of Pb-sorption in surface soil is largely mediated by organic matter, pH, and clay content (iron and manganese oxides) (Rieuwerts, 2007; Violante et al., 2010). In a study by Li (2006), selective sequential extraction, leachate extraction and desorption tests were used to demonstrate that Pb in top soil displayed little downward flux. High Pb concentrations in soils along highway corridors were mainly retained to the top soil (0.3 meter (m)), with concentrations reduced to the background levels at a depth of 0.6 m (Li, 2006). These data are in good agreement with a modeling study that suggested that less than 0.1% of the potentially-mobile Pb in topsoil (25 cm depth) migrated to lower soil layers each year (Semlali et al., 2004).

Soil serves as a reservoir of highly bio-accessible Pb, due to atmospheric deposition and low mobility in soil. Ingestion of Pb-contaminated soil following hand to mouth contact is a major Pb exposure pathway in children (Abadin et al., 2007; Mielke and Reagan, 1998; Ruby and Lowney, 2012). Exposure can also result from inhalation of atmospheric soil dust and atmospheric Pb (Zahran et al., 2013). Long-term Pb exposure to Pb can result in elevated blood lead levels (BLLs) and related neurobehavioral and cognitive deficiencies.

Significant associations between concentrations of Pb in soil and BLLs of children in New Orleans, Syracuse, and Detroit (Bickel, 2010; Johnson and Bretsch, 2002; Mielke et al., 1997). Laidlaw et al. (2017) analyzed the dose-response relationship in those three cities using the Integrated Exposure Uptake Biokinetic (IEUBK) model developed by the U.S. Environmental Protection Agency (USEPA). This analysis indicated that high BLLs correlated with relatively low soil Pb concentrations; as a result of this finding, the Centers for Disease Control and Prevention (CDC) has lowered the BLL threshold to 5 μg/dL. Nonetheless, no BLL concentration is considered to be safe for children (Centers for Disease Control and Prevention, 2012). Assumptions of safe minimal BLLs as well as other variables have led to a wide range of differing soil Pb regulatory guidelines.

Regulations governing acceptable levels of Pb in soil vary among different agencies within the U.S. government. Currently, per USEPA guidance, a Pb soil concentration of 400 ppm in both recreational and residential areas should trigger cleanup activities. However, this regulation does not consider the full extent of health effects resulting from Pb exposure (Laidlaw et al., 2017a). The Office of Environmental Health Hazard Assessment (OEHHA) of California revised their soil Pb threshold to 80 ppm Pb in residential areas, which was determined by modeling the degree of exposure resulting from an incremental increase in BLL of 1 μg/dL, using the LeadSpread model

(Office of Environmental Health Hazard Assessment, 2009). OEHHA guidelines also account for soil Pb concentrations that have no more than a 2.5% probability of decreasing IQ scores by more than 1 point in a 90% of children and fetuses (Office of Environmental Health Hazard Assessment, 2009). However, both soil thresholds from the USEPA and OEHHA are not enforceable since many factors may contribute to the need to cleanup soils. Currently, California Department of Toxic Substances Control (DTSC) recommends using the 95% upper confidence level (UCL) of the site's dataset to evaluate cleanup options (U.S. Environmental Protection Agency, 2002). The threshold concentrations are also intended to be used by community groups, land owners, and local agencies to estimate the degree of effort that is needed to remediate a Pb-polluted site.

Los Angeles has a long history of Pb contamination, being a car-dominant metropolitan area for decades. Los Angeles continues to be affected by the legacy of Pb emissions from vehicle traffic and battery industry. Between 1950 and 1982, over 100,000 metric tons of Pb were emitted by vehicle traffic traveling on freeways and major roads in Los Angeles (Mielke et al., 2010a). Furthermore, the smelters that recycled lead-acid batteries in southeast Los Angeles contaminated the air and soil of nearby communities for decades (Johnston and Hricko, 2017). Though Pb was banned from house paint in 1978, homes built before 1978 may contain high Pb levels in paints (Wiener et al., 2015). Currently, Pb is permitted in aviation fuel (avgas) for light aircrafts, which is often overlooked but represents the greatest source of air contamination. Avgas results in significantly higher air concentrations of Pb levels around many airports within Los Angeles (Miranda et al., 2016) and significantly higher Pb levels in soils near regional airports (McCumber and Strevett, 2017). Airborne emissions of leaded avgas may settle into soils, whereby Pb-contaminated soil can be ingested by children.

An investigation of Pb-contaminated soil in Los Angeles revealed that Pb concentrations near highways (average Pb concentration of 189 ppm; N = 299) and arterials (average Pb concentration of 224 ppm; N = 140) were higher than other areas consisting mostly of residential areas (average Pb concentration of 107 ppm; N = 111) (Wu et al., 2010). The study indicated that current Pb pollution in south Los Angeles results from both household paint and traffic patterns (Wu et al., 2010). However, Pb concentrations in park soils largely remain unknown, even though the most susceptible, children, are likely to come into contact with contaminated soils in parks. The purpose of this research was to evaluate Pb in soils of urban parks in Los Angeles, focusing on (1) parks close to hot spots found by Wu et al. (2010), (2) sites nearby potential Pb sources, and (3) parks chosen at random from a 1-km^2 grid.

3.2 Materials and Methods

3.2.1 Field sampling and site selection

All field sampling was conducted within a 549 square kilometer (km^2) area in Los Angeles, including western, south, and central of Los Angeles County. Field sampling was conducted in 100 parks from April 2016 to April 2017. To account for sampling variation, soil samples were collected from three locations within each park. The average park size was 41,000 m^2, with a range of 1,600 to 410,000 m^2. The minimum sampling density was seven samples per square kilometer. Detailed information, including park name, exact location, sampling density, and park size are shown in Table 2 and Figure 3.

Within each park, three plots in lawn patches near children's play areas were randomly selected for soil sampling (the plots had no tree canopy above them). The distance between each plot was at least 10 meters apart. A composite sample was obtained from each plot by performing five consecutive soil collections at a depth of 0 to 5 cm within an area of 0.5 m^2 to integrate the concentrations at that scale. Soil samples were stored in acid-washed glass bottles and transported to the laboratory in coolers with ice. All samples were refrigerated (4°C) until use.

The following approaches were used to select sampling locations throughout Los Angeles: (1) parks, recreational centers, and playgrounds in the vicinity of locations previously identified to contain high levels of Pb in soil (Wu et al., 2010) were considered as potential sites for field sampling. Locations that have high level of Pb in soil were assigned to the center of a circle two kilometers in diameter and any parks within the circles were sampled (N = 40); (2) parks near industrial sites, airports, and railways were considered possible sources of Pb (N = 45); and (3) two-step random sampling was performed to generate unbiased estimates of Pb in soils collected throughout study area (Wang et al., 2012). A continuous 1-km^2 grid was used as a primary unit and numbered units were randomly selected using a random number generator. All parks within each chosen unit in the grid were sampled (N = 15). Exact locations of parks that were chosen by each approach are shown in Figure 3.

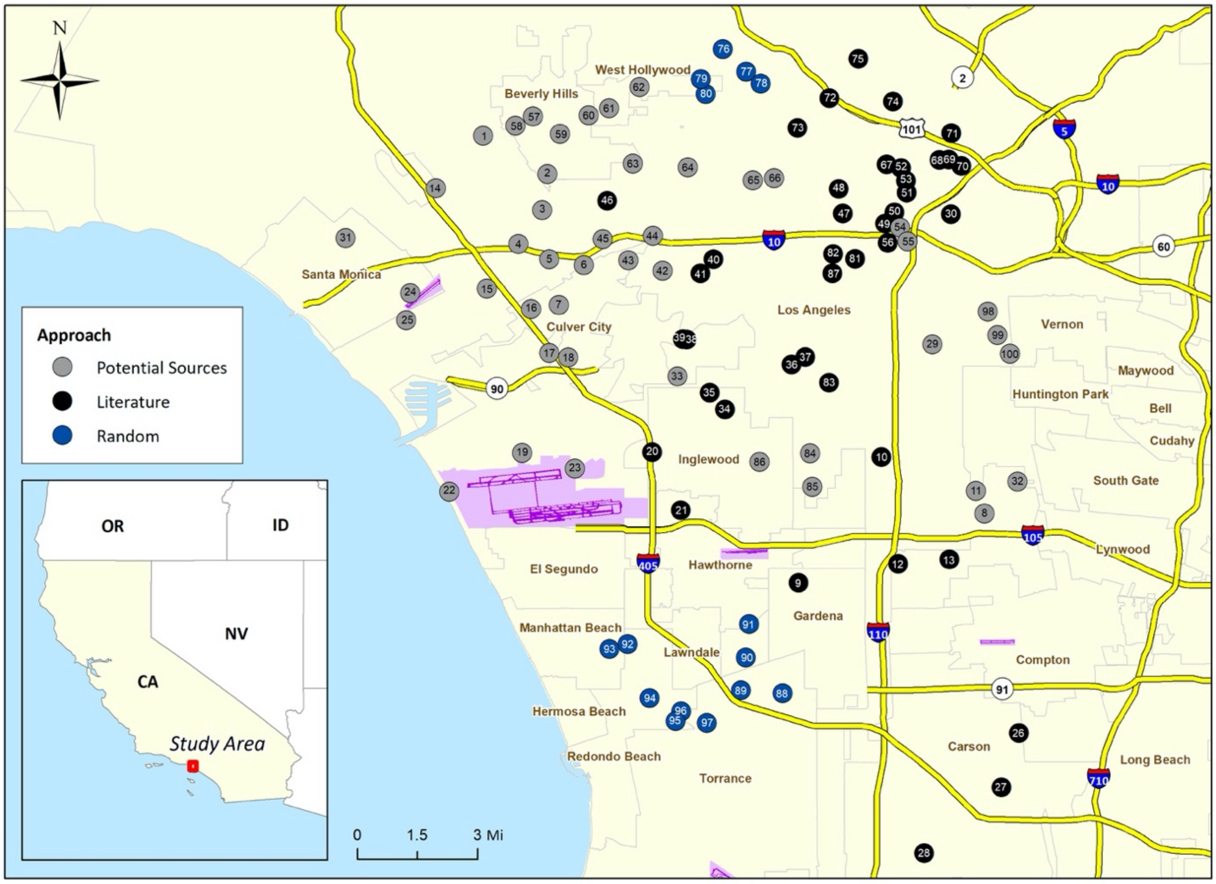

Figure 3. Map of the study area with park locations in Los Angeles with three sampling approaches: (1) literature from Wu et al. (2010), (2) potential Pb emission sources, and (3) random selection.

Table 2. Soil Pb concentrations in parks with their geographic characteristics sorted by average Pb concentrations.

ID	Park name	Pb concentrations in soil (ppm)			Average Pb concentration (ppm)	Standard deviation (ppm)	Park area (m^2)	Sampling density (N/km^2)	ZIP code
		Site 1	Site 2	Site 3					
Average values across all the parks					*65.5*	*63.9*	*40,951*	*288*	
6	Media Park	343	333	315	331	14.0	8,741	343	90232
60	Doheny Fountain	226	282	123	211	80.8	3,468	865	90210
86	Darby Park	61.1	245	278	195	117	53,823	56	90305
79	Plummer Park	291	120	77.5	163	113	26,305	114	90046
21	Lennox Park	194	64.2	215	158	81.5	19,304	155	90304
93	Polliwog Park	25.5	23.7	363	137	195	97,529	31	90266
54	Toberman Park	141	193	75	136	58.9	12,181	246	90015
72	Lemon Grove Recreation Center	244	84.7	73.0	134	95.7	18,373	163	90029
50	Terrace Park	108	157	127	130	25.0	5,585	537	90006
83	Havard Recreation Center	49.9	267	65.0	127	121	55,442	54	90047
76	Dorothy & Benjamin Smith Park	92.2	125	157	125	32.2	2,687	1116	90028
61	West Hollywood Park	22.4	56.9	293	124	147	36,098	83	90069
53	Mac Arthur Park	183	151	28.7	121	81.3	76,486	39	90057
100	Pueblo Del Rio Recreation Center	100	159	88.8	116	37.7	2,444	1227	90058
58	The Maltz Park	61.3	136	151	116	47.9	5,140	584	90210

ID	Park name	Pb concentrations in soil (ppm)			Average Pb concentration (ppm)	Standard deviation (ppm)	Park area (m^2)	Sampling density (N/km^2)	ZIP code
		Site 1	Site 2	Site 3					
55	Estrella Park	143	58.3	144	115	49.1	1,683	1782	90007
32	Jordan Downs Recreation Center	102	111	119	111	8.70	16,794	179	90002
63	La Cienega Park	157	67.6	105	110	45.1	40,266	75	90211
5	Woodbine Park	40.9	163	121	108	61.8	3,343	897	90034
69	Rockwood Community Park	90.3	109	122	107	15.8	2,804	1070	90026
91	Bodger Park	107	83.6	128	106	22.3	42,897	70	90250
81	Richardson Family Park	55.5	18.2	243	106	121	1,797	1670	90007
1	Holmby Park	122	39.2	146	102	55.8	40,307	74	90024
56	Hoover Recreation Center	60.1	101	135	98.4	37.3	12,990	231	90007
65	La High Memorial Park	53.4	78.7	156	95.9	53.2	11,817	254	90019
20	Ashwood Park	128	111	44.9	94.7	44.0	7,972	376	90301
99	Fred Roberts Recreation Center	93.0	96.4	89.2	92.9	3.63	12,909	232	90011
85	Jesse Owens Park	102	120	48.7	90.3	37.1	108,051	28	90047
59	Beverly Gardens Park	121	77.7	69.1	89.1	27.6	26,466	113	90210
66	Harold A Henry Park	83.6	86.9	93.5	88.0	5.01	8,579	350	90005
41	Rancho Cienega Recreation Center	45.1	105	101	83.7	33.5	126,262	24	90016
37	Chesterfield Square	57.1	118	75.9	83.6	31.2	9,389	320	90062

ID	Park name	Pb concentrations in soil (ppm)			Average Pb concentration (ppm)	Standard deviation (ppm)	Park area (m^2)	Sampling density (N/km^2)	ZIP code
		Site 1	Site 2	Site 3					
80	Poinsettia Recreation Center	39.3	63.5	144	82.2	54.7	27,114	111	90046
34	Edward Vincent Jr. Park	77.8	73.8	89.8	80.5	8.34	33,751	89	90302
57	Will Rogers Memorial Park	63.5	150	27.8	80.5	62.9	16,026	187	90210
73	Burns Park	23.3	56.7	159	79.6	70.6	7,891	380	90004
98	Ross Snyder Recreation Center	84.4	62.0	86.4	77.6	13.6	43,706	69	90011
16	Tellefson Park	67.4	68.7	79.5	71.8	6.63	7,810	384	90232
52	Levitt Pavilion	28.7	133	50.2	70.7	55.2	55,847	54	90057
49	Pico Union Vest Pocket Park	126	4.96	59.6	63.6	60.7	2,469	1215	90006
11	Ted Watkins Memorial Park	75.2	54.0	55.2	61.5	11.9	111,693	27	90002
62	Kings Road Park	58.4	73.4	49.6	60.4	12.0	2,675	1122	90069
36	Van Ness Recreation Center	34.2	103	35.6	57.7	39.5	35,046	86	90043
2	Roxbury Park	40.4	115	16.6	57.2	51.2	61,512	49	90212
75	Barnsdall Art Park	39.2	90.7	41.4	57.1	29.1	60,298	50	90027
89	McMaster Park	27.0	79.9	62.5	56.4	27.0	22,986	131	90504
64	Wilshire Green Park	56.5	47.1	63.7	55.8	8.32	6,435	466	90036
82	Loren Miller Recreation Center	99.2	25.2	30.9	51.8	41.2	11,695	257	90018
94	Perry Park	45.4	21.6	87.6	51.6	33.4	8,498	353	90278

ID	Park name	Pb concentrations in soil (ppm)			Average Pb concentration (ppm)	Standard deviation (ppm)	Park area (m^2)	Sampling density (N/km^2)	ZIP code
		Site 1	Site 2	Site 3					
44	Genesse Avenue Park	54.4	47.5	48.4	50.1	3.73	3,602	833	90016
45	Reynier Park	32.6	52.2	52.7	45.8	11.5	4,978	603	90034
87	Denker Recreation Center	23.4	84.2	29.7	45.8	33.5	13,719	219	90018
46	Robertson Recreation Center	93.9	24.5	17.2	45.2	42.3	6,232	481	90035
31	Douglas Park	45.1	43.3	47.1	45.2	1.90	18,616	161	90403
15	Mar Vista Recreation Center	52.6	25.1	55.8	44.5	16.9	80,533	37	90066
78	Hollywood Recreation Center	54.4	45.6	31.5	43.8	11.6	16,147	186	90038
33	Landra Park	56.6	42.2	32.5	43.7	12.1	62,322	48	90056
42	Baldwin Hills Recreation Center	31.8	51.5	46.4	43.2	10.2	44,111	68	90016
68	Unidad Park	23.8	65.3	29.3	39.5	22.5	1,562	1921	90026
35	North Park	18.5	15.3	81.9	38.6	37.5	10,967	274	90302
3	Cheviot Hill Park	38.0	40.5	36.5	38.4	1.99	165,112	18	90064
74	Bellevue Recreation Center	27.7	53.1	32.5	37.8	13.5	36,017	83	90026
8	109^{th} Street Recreation Center	48.1	34.1	27.9	36.7	10.3	14,285	210	90059
90	Alondra Park	51.3	29.9	26.3	35.8	13.5	100,767	30	90260
39	Norman Houston Park	32.5	35.7	36.0	34.7	1.92	39,174	77	90008
17	Culver Slauson Park	36.1	22.8	43.1	34.0	10.3	19,384	155	90230

ID	Park name	Pb concentrations in soil (ppm)			Average Pb concentration (ppm)	Standard deviation (ppm)	Park area (m^2)	Sampling density (N/km^2)	ZIP code
		Site 1	Site 2	Site 3					
40	Vineyard Recreation Center	51.6	32.2	15.9	33.2	17.88	5,544	541	90016
51	Hope and Peace Park	52.3	24.2	21.9	32.8	16.93	12,181	246	90057
67	Lafayette Recreation Center	25.2	47.8	25.0	32.7	13.09	42,492	71	90057
71	Echo Park	42.1	26.1	26.1	31.4	9.22	141,235	21	90026
29	South Park	24.6	38.6	27.4	30.2	7.38	77,295	39	90011
30	Grand Hope Park	20.6	21.3	46.2	29.4	14.55	12,788	235	90015
4	Palms Park	40.6	18.2	29.0	29.3	11.21	24,969	120	90064
10	Algin Sutton Recreation Center	12.4	33.0	41.6	29.0	14.98	66,773	45	90044
12	Athens Park	19.0	32.3	33.6	28.3	8.09	83,365	36	90061
7	Veteran Memorial Park	25.8	18.1	37.6	27.2	9.8	54,228	55	90230
97	Columbia Park	24.4	37.2	19.4	27.0	9.18	212,055	14	90504
77	De Longpre Park	17.9	35.5	22.9	25.4	9.08	6,515	460	90028
48	Seoul International Park	16.1	27.9	30.6	24.8	7.71	12,302	244	90006
38	Rueben Ingold Park	20.7	23.4	28.1	24.1	3.77	74,058	41	90043
28	Scott Park	26.8	23.4	19.5	23.2	3.63	32,658	92	90745
43	Syd Kronenthal Park	22.0	24.4	22.5	23.0	1.27	27,600	109	90232
9	Rowley Park	23.5	17.0	25.8	22.1	4.55	76,081	39	90249

ID	Park name	Pb concentrations in soil (ppm)			Average Pb concentration (ppm)	Standard deviation (ppm)	Park area (m^2)	Sampling density (N/km^2)	ZIP code
		Site 1	Site 2	Site 3					
47	Normandie Park Recreation Center	22.6	12.0	29.2	21.3	8.66	15,216	197	90006
88	Descanso Park	28.4	21.6	12.0	20.7	8.23	11,817	254	90278
14	Westwood Recreation Center	49.6	1.51	9.51	20.2	25.8	80,128	37	90025
96	El Nido Park	21.5	0.970	36.0	19.5	17.6	57,870	52	90278
22	Vista Del Mar Park	28.2	19.9	8.79	19.0	9.75	5,868	511	90293
92	Aviation Park	13.5	23.9	17.7	18.4	5.21	40,873	73	90278
27	Dolphin Park	12.7	23.1	18.8	18.2	5.21	50,990	59	90476
70	Vista Hermosa Natural Park	8.92	23.9	14.6	15.8	7.55	41,278	73	90026
24	Clover Park	7.83	18.6	17.6	14.7	5.98	76,081	39	90405
84	St Andrews Recreation Center	14.0	4.74	22.6	13.8	8.91	36,826	81	90047
23	LAX View Park	24.9	9.6	6.19	13.6	9.97	5,989	501	90045
25	Penmar Park	10.4	8.84	20.7	13.3	6.42	54,228	55	90291
18	El Marino Park	18.9	11.3	8.9	13.0	5.23	7,325	410	90230
13	Earvin Magic Johnson Park	13.6	10.6	10.6	11.6	1.75	408,733	7	90059
95	Franklin Park	19.9	6.05	7.69	11.2	7.56	16,309	184	90278
26	Anderson Park	13.6	8.23	8.49	10.1	3.05	35,734	84	90476
19	Westchester Recreation Center	6.11	5.49	5.88	5.83	0.310	136,784	22	90045

3.2.2 Soil characterization

Soil samples from 41 (randomly selected from the entire set of the 100 parks) were characterized to determine their physical and chemical properties. The pH of park soils was measured by pH meter following the transfer of 10 g of soil into 25 mL of deionized water, shaking the soil mixture for 20 minutes, and then allowing the mixture to settle for one hour. Water, organic matter, and fixed solids (the residue left after the sample was ignited at 550°C) content were determined by performing sequential loss on ignition (105°C for 24 hours and 550°C for 1 hour) (U.S. Environmental Protection Agency, 2001). The particle-size distribution of soil samples (three size ranges: <4 μm (clay), 4-63 μm (silt), and <63 μm (sand)) was determined by the hydrometer method (Ashworth et al., 2001). For particle sizing, dry soil samples were soaked in a 5% (w/w) sodium meta-phosphate solution overnight. After the suspension was transferred to a 1-L cylinder and inverted, hydrometer readings were recorded at standardized time increments.

3.2.3 Laboratory analysis

Top soil samples were oven-dried, ground with a mortar, and sieved to 2 mm. Dried samples were digested on hot plates using a combination of trace-metal grade nitric acid (HNO_3, 68-70% (v/v)), hydrogen chloride (HCl, 38% (v/v)), and hydrogen peroxide (H_2O_2, 30% (v/v)) according to USEPA Method 3050B (U.S. Environmental Protection Agency, 1996). Digested samples were diluted to 100 mL with deionized water containing 6.8-7% (v/v) HNO_3, 3.8% (v/v) HCl, and 0.9% (v/v) H_2O_2. Each digestate was analyzed in triplicate with a Graphite Furnace Atomic Absorption Spectrometer (GFAAS, Perkin-Elmer, AAnalyst 700). Calibration curves were created throughout each day of testing using at least five Pb standard solutions between the concentrations of 5–60 μg/L. A solution of digestion reagents alone was used as the diluent for the calibration curves.

Calibration curves had an average R^2 of 99.8% (range of 99.3%−99.9%). The primary Pb standard (Hach, Loveland, CO) in the laboratory was NIST-certified or traceable to NIST standard reference materials. The average recovery of matrix spike of the Pb standard solutions through the digestion process was 102.2% (range of 95.0%–112%). Because analytical recoveries were high, the matrix modifier was not used in this study, in agreement with USEPA Method 7010 (U.S. Environmental Protection Agency, 2007).

An additional 15 soil samples from Woodbine Park were characterized by X-ray fluorescence spectrometry (pXRF, Bruker S1 TITAN). Samples were heated in Pyrex dishes at 100°C overnight and screened to remove pebbles and debris prior to pXRF analysis.

3.2.4 Estimation of incremental increases in children's BLL

DTSC uses the LeadSpread model to estimate children's BLL resulting from inhalation, ingestion, and dermal exposure. The California Environmental Protection Agency (CalEPA) updated their toxicity criterion to include incremental changes in children's BLL of 1 μg/dL, which is predicted to reduce IQ score by up to 1 point (Office of Environmental Health Hazard Assessment, 2009). In this work, the incremental increases of 1 μg/dL in children's BLL resulting from Pb in soil was based on analyses conducted in LeadSpread 8.0. Default values of exposure factors were used by following the CalEPA's recommendations (Office of Environmental Health Hazard Assessment, 2009).

3.2.5 Statistical analysis and geographic information systems

Measured Pb concentrations were entered into ArcMap 10.4 to generate Pb distribution map. Statistical comparisons of soil Pb concentrations to soil properties were made using the Mann-Whitney U test. Spearman's correlation was also used to identify correlations between Pb concentrations and soil properties. Both are nonparametric methods that do not assume normal distribution. A p-value of < 0.05 was considered to be significant.

3.3 Results and Discussion

3.3.1 Descriptive statistics

A wide range of Pb concentrations were detected in Los Angeles park soils. A summary of results is shown in Table 3 and Figure 4. A comparison between soil Pb concentrations in dry weight and wet weight can be found in Table 4. It is important to note that Pb concentrations are reported in dry weight instead of wet weight to manage variable moisture content, as recommended by the USEPA (U.S. Environmental Protection Agency, 1993). Pb concentrations in soils were positively skewed, ranging from 0.969 to 363 mg/kg with an average of 65.5 mg/kg and a standard deviation of 63.9 mg/kg. The highest concentration (363 mg/kg) was found in Media Park soil while the lowest concentration (0.969 mg/kg) was found in Westchester Recreation Center soil. Based on the revised threshold screening concentration for Pb recommended by the CalEPA (80 ppm), soil samples from 35 parks were found to be higher than 80 ppm (based on the average Pb concentration measured at each park). Overall Pb concentrations were higher as compared to naturally occurring background levels in California (19.7 ppm) (Smith et al., 2013), with mean and median Pb concentrations approximately three times higher than naturally occurring background levels (Table 3).

Mean Pb concentrations measured in the parks may not be representative of actual risk level because of the high variation observed in triplicate measurements per park (89 parks contain > 10% relative standard deviation). For example, while 35 parks had a mean Pb concentration value greater than 80 ppm, 47 parks had at least one of three measurements exceed 80 ppm. The variation observed in these 47 parks suggests a 1–4 μg/dL increase of BLL, based on maximum observed values in each park (Figure 5).

Pb concentrations in 15 additional soil samples from Woodbine Park were determined by GFAAS and pXRF. Comparison of soil Pb concentrations between GFAAS and pXRF was consistent, with an R^2 value of 0.92, excluding samples in which no Pb was detected (below 27 ppm); slightly lower Pb concentrations were detected by pXRF (Figure 6). The detection limits of GFAAS and pXRF were 0.05 μg/L and 27 ppm, respectively.

Table 3. Summary of and Pb concentrations in soil samples collected in Los Angeles, CA. All units are ppm.

Analyte	Min	Max	Mean	Standard Deviation	Standard Error	Median	First Quartile	Third Quartile
Investigated values of Pb in soil (based on dry weight)								
Pb (N=300)	0.969	363	65.5	63.9	3.69	45.0	23.8	87.1
Natural background values of Pb in soil in California (Smith et al., 2013)								
Pb (N=258)	3.0	263	19.7	20.4	1.30	16.8	12.6	20.9

Table 4. Comparison of Pb concentrations in soils between dry weight and wet weight. All units are mg/kg or parts per million (ppm).

Analyte	Min	Max	Mean	Standard Deviation	Standard Error	Median	First Quartile	Third Quartile
Investigated values of Pb in soil (based on dry weight)								
Pb (N=300)	0.969	363	65.5	63.9	3.69	45.0	23.8	87.1
Investigated values of Pb in soil (based on fresh or wet weight)								
Pb (N=300)	0.458	344	54.3	53.4	3.08	35.5	20.2	71.1

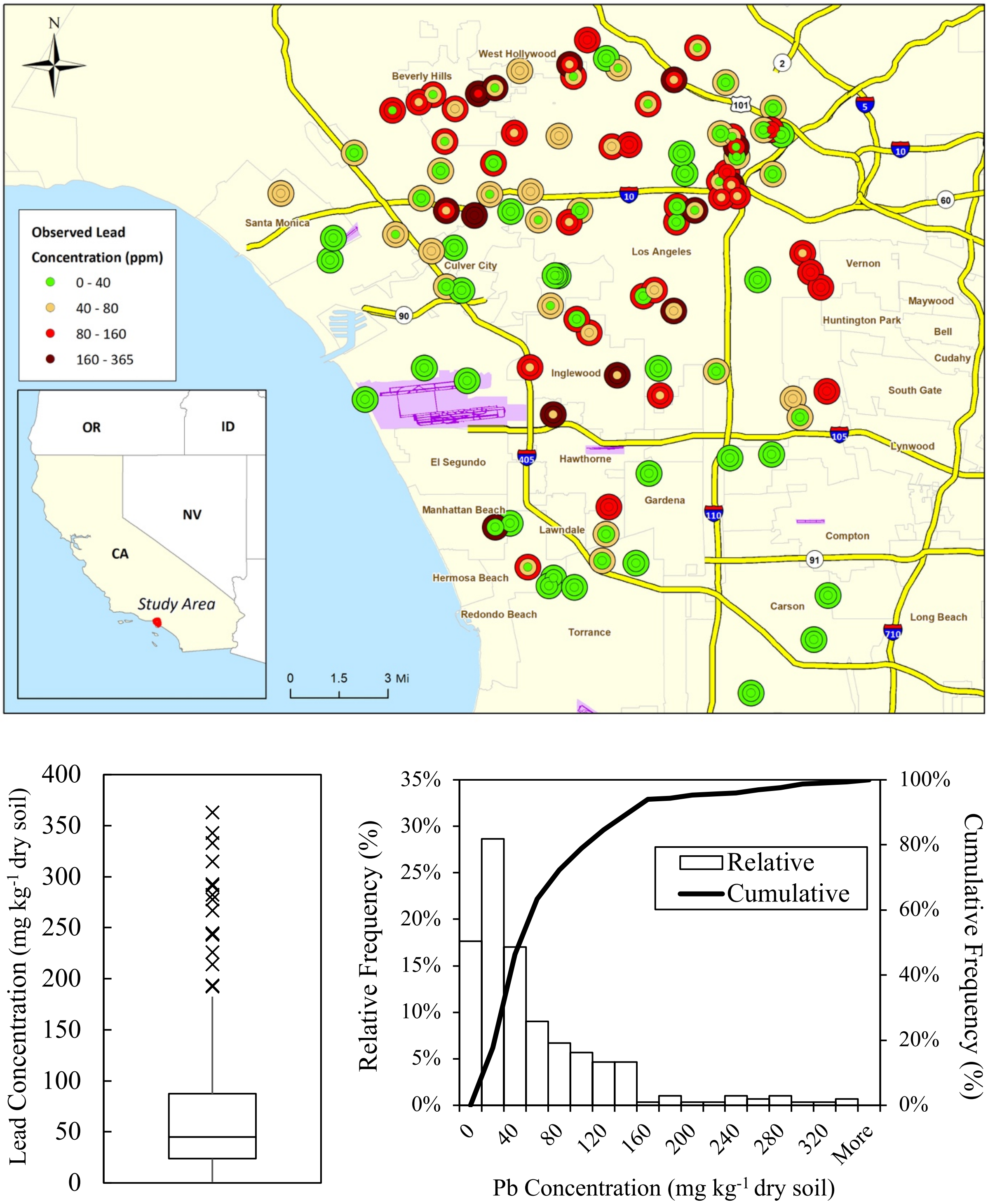

Figure 4. Map (top), box plot (bottom left), and histogram (bottom right) of Pb concentrations (ppm) in Los Angeles park soils. Concentric circles on the map represent three Pb concentration measurements from each park.

3.3.2 Estimation of incremental increases in children's BLL

The modeling results indicated that 47 parks listed in Figure 5 had a potential increase of BLL in children from 1 μg/dL to 4 μg/dL based on maximum observed values in each park. To assess Pb health risk, DTSC generally recommended a 95% UCL of the arithmetic mean Pb concentration for each site as an input variable in LeadSpread (U.S. Environmental Protection Agency, 2002). In this study, however, three samples in each park were insufficient to find a 95% UCL. It may be more appropriate to compare maximum detected concentrations with 150 ppm, an empirical value approximately equal to a 95% UCL of 80 ppm (Department of Toxic Substances Cotrol, 2011). Under the scenario of 95% UCL, 13 parks had samples results exceeding 150 ppm. Although parks have soil Pb concentrations above 150 ppm may assume to pose significant health effects on Children, further site evaluation is needed depending on site-specific conditions (Department of Toxic Substances Cotrol, 2011).

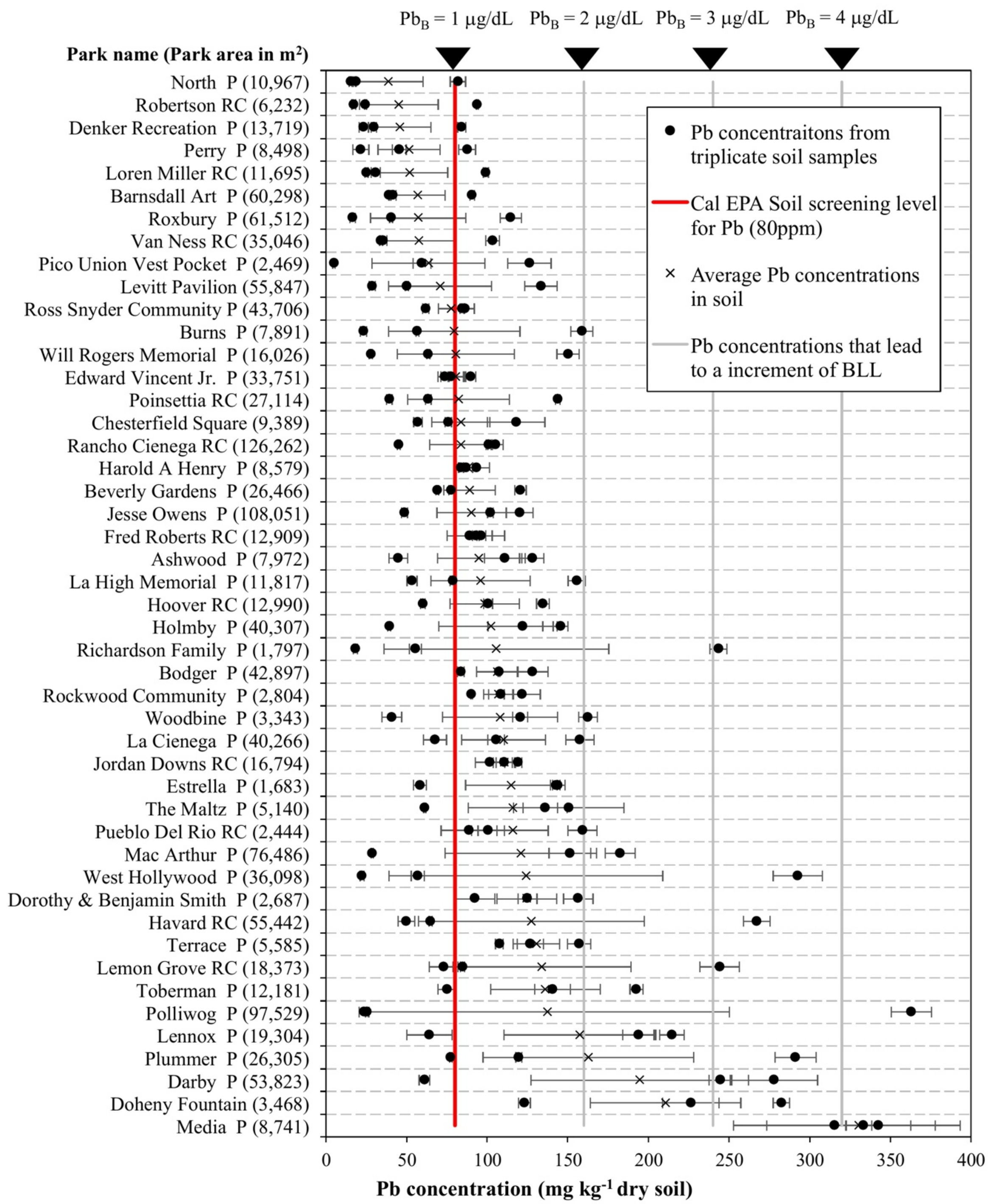

Figure 5. Pb concentrations in triplicate measurements where at least one of replicate was higher than the CalEPA threshold of 80 ppm. Vertical lines represent the 1 µg/dL increments of BLL based on the LeadSpread 8.0 model. Abbreviations: P, Park; RC, Recreation Center.

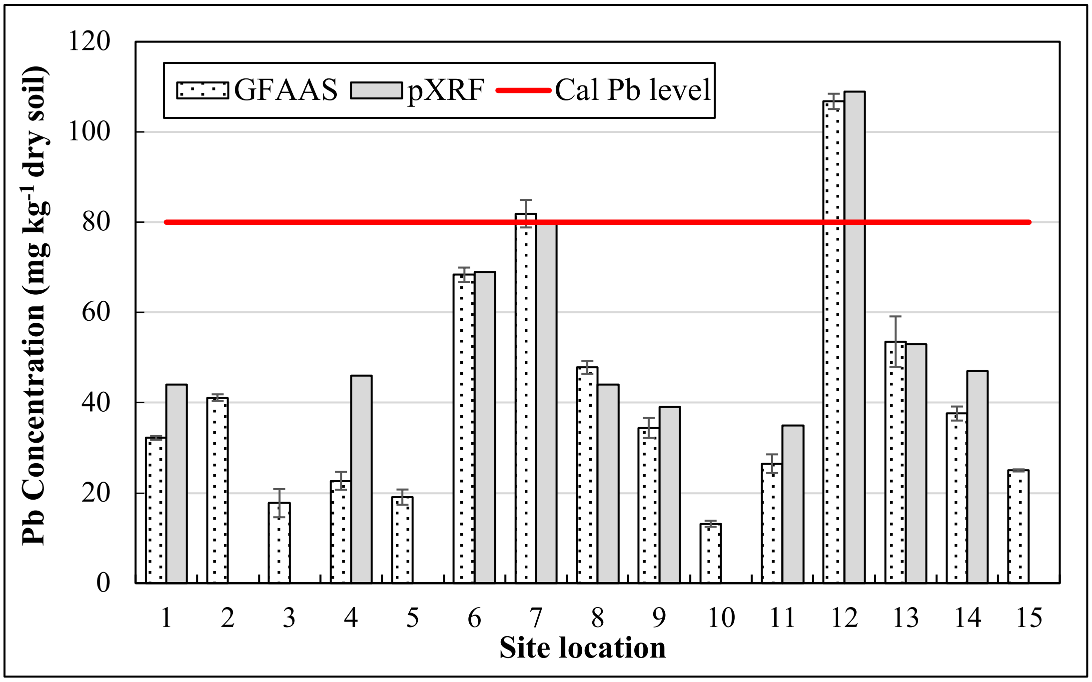

Figure 6. Comparison of soil Pb concentrations in Woodbine Park by GFAAS and pXRF.

3.3.3 Soil characterization

The descriptive statistics of soil pH, clay, sand, silt, water, organic matter, and fixed solids are summarized in

Table 5. Soil pH values ranged from 5.27 to 8.64. The highest pH value was detected in Rockwood Community Park soil while the lowest pH value was detected in Polliwog Park soil. According to the common classes of soil pH (U.S. Department of Agriculture, 1998), 14.8%, 32.5% and 53.7% of the soils measured in this study were acidic, neutral, and alkaline, respectively.

Based on the soil textural triangle from the United States Department of Agriculture (USDA), soil samples were predominately composed of sand. Of these soil samples, 52.8%, 25.2%, and 20.3% were identified as loamy sand, sand, and sandy loam, respectively (Figure 7). Only two samples were classified as sandy clay loam and clay loam, found in Dorothy & Benjamin Smith Park and Terrace Park, respectively.

Water content differed considerably across all soil samples, ranging from 1.4% to 52.8%. In the majority of samples, fixed solids were dominant in soil as compared to organic matter and water content.

Table 5. Soil texture of 123 soil samples collected from 41 parks in Los Angeles, CA.

Soil properties (N=123)	Min	Max	Mean	Median	Standard Deviation	Standard Error	Kurtosis	Skewness
pH (-)	5.27	8.64	7.38	7.44	67.1	6.05	0.49	-0.55
Clay (%)	0	29.6	6.66	6.18	4.73	0.429	4.19	1.55
Sand (%)	39.7	98.8	83.1	84.7	9.99	0.429	2.61	-1.29
Silt (%)	0	30.8	10.2	9.09	6.89	0.624	0.614	0.895
Water content (%)	1.37	52.8	14.9	13.5	9.27	0.859	1.62	0.999
Organic matter content (%)	0	39.2	13.3	10.8	9.40	0.851	-0.248	0.930
Fixed solids (%)	13.9	94.5	71.7	74.5	15.8	1.43	1.49	-1.04

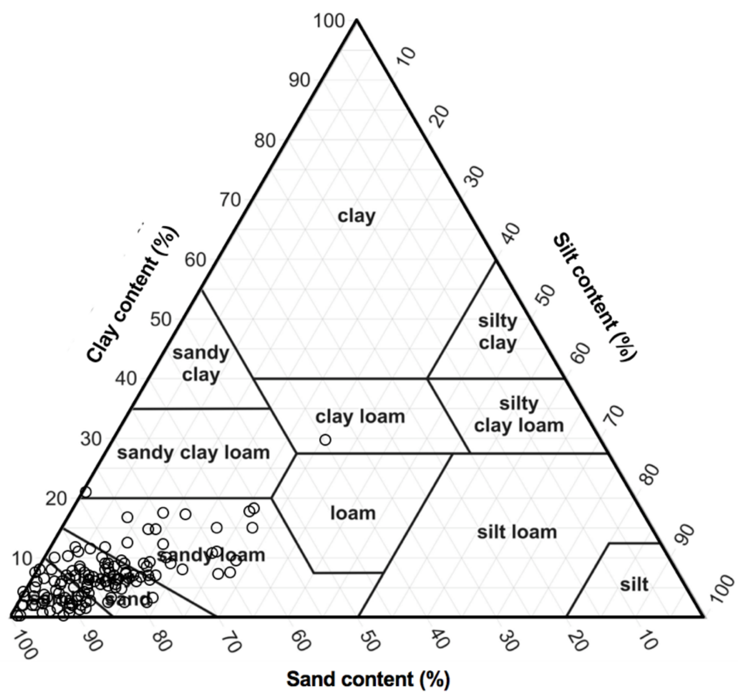

Figure 7. Soil texture of 123 soil samples collected from 41 parks in Los Angeles, CA.

Correlations between soil Pb concentrations and soil parameters were assessed in Figure 8 and Table 6. Spearman's ρ indicated that all correlations between soil Pb concentrations and soil parameters were not statistically significant, except for silt ($\rho = 0.226$, p value = 0.012) (Figure 9). Nonetheless, noticeable trends between soil Pb concentration and pH, clay, and moisture were observed, consistent with previous findings (Fifi et al., 2013; Krishnamurti and Naidu, 2003).

Soil physicochemical properties, which play important roles in the mobility of Pb, may influence the uptake of Pb by plants, which poses a hazard to children's health (Violante et al., 2010). Among soil properties, pH, clay, and organic matters have been proved to be key factors retaining Pb according to sequential extraction analysis (Fifi et al., 2013; Krishnamurti and Naidu, 2003). However, in this study, only silt content was found to have a significant positive correlation with soil Pb concentration ($\rho = 0.226$, p value= 0.012). Furthermore, sandy loam Pb concentrations ($N = 47$) were significantly higher (p value = 0.008) than those of loamy sand ($N = 48$) but were not significantly different than sand Pb concentrations (p value = 0.092). These observations are consistent with a recent study conducted in southern Finland that found that Pb was bound strongly within urban park soils containing low clay content (13%) and organic matter percentage (9.1%) (Setälä et al., 2017). Additionally, Acosta et al. (2014) reported that total Pb concentrations in soil correlated only with pH ($\rho = -0.45$, p value < 0.001) but not clay, silt, or organic matter percentages.

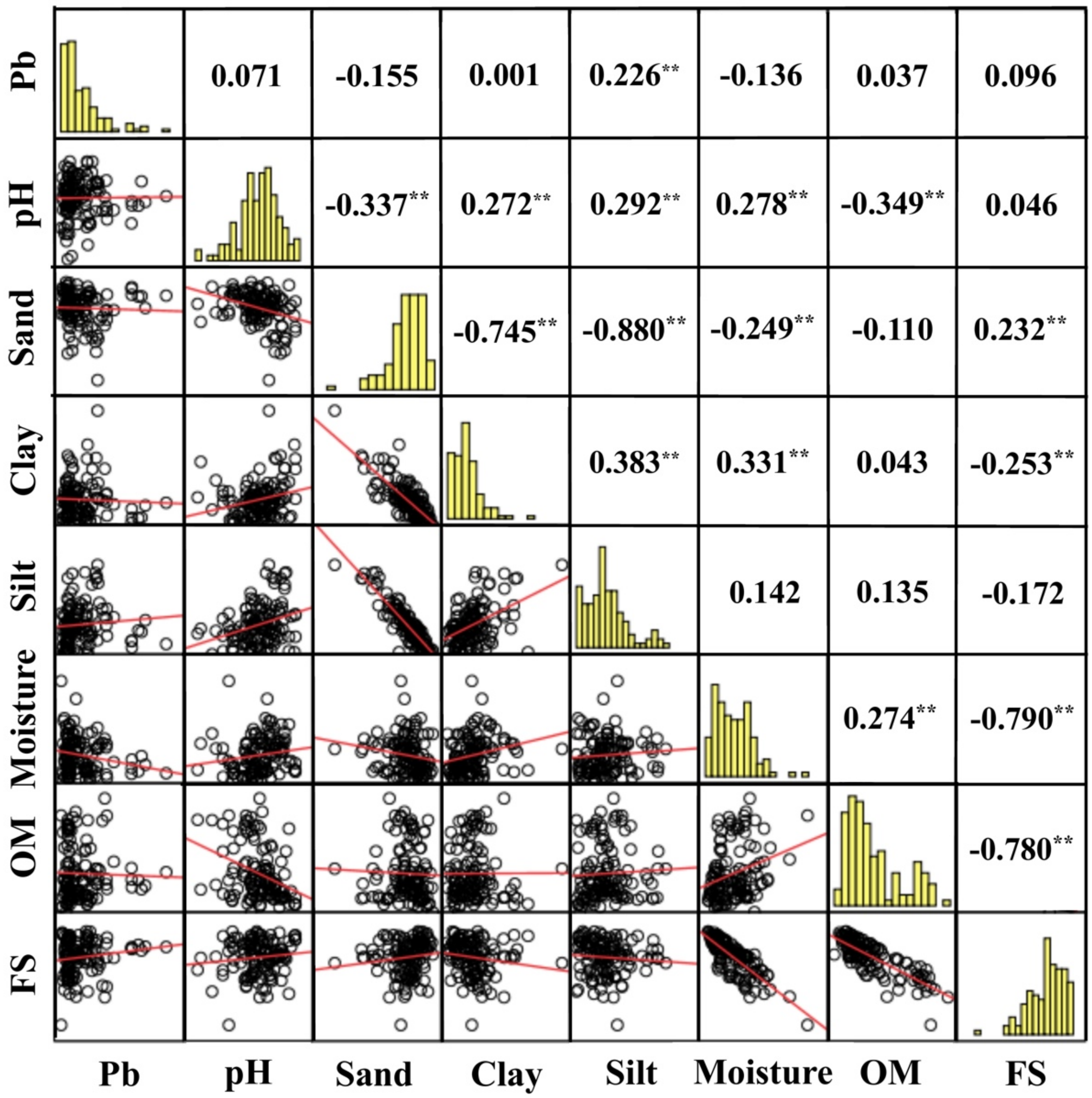

Figure 8. Scatter plot matrix of correlations between different soil Pb concentrations (ppm) and soil parameters including pH, sand content (%), silt content (%), water content (%), organic matter content (%) and fixed solids (%). Spearman's ρ, histograms, and p-values are also included. Pb, Moisture, OM, and FS refer to soil Pb concentration, water content, organic matter content, and fixed solids, respectively. Correlation was significant at the 0.05 level and the 0.01 level (two-tailed), denoted as * and **.

There could be several reasons that only silt was found to have a significant positive correlation with soil Pb concentration. In this work, approximately 85% of soil samples were neutral and alkaline (pH ≥ 7), indicating that pH is of limited importance concerning the availability and mobility of Pb in neutral-alkaline environments. The effect of clay and organic matter in soil on Pb adsorption capacity can also vary from different soil layers (Sipos et al., 2005). In this study, surface soil contained little clay (6.66% ± 4.73%) and organic matter contents (13.3% ± 9.4%), indicating a minor role of clay and organic matter content in the adsorption of Pb to soil. It is also possible that the high levels of Pb in urban soils might be dependent on grass roots, which reduced the leaching of Pb more deeply into the soil (Acosta et al., 2014).

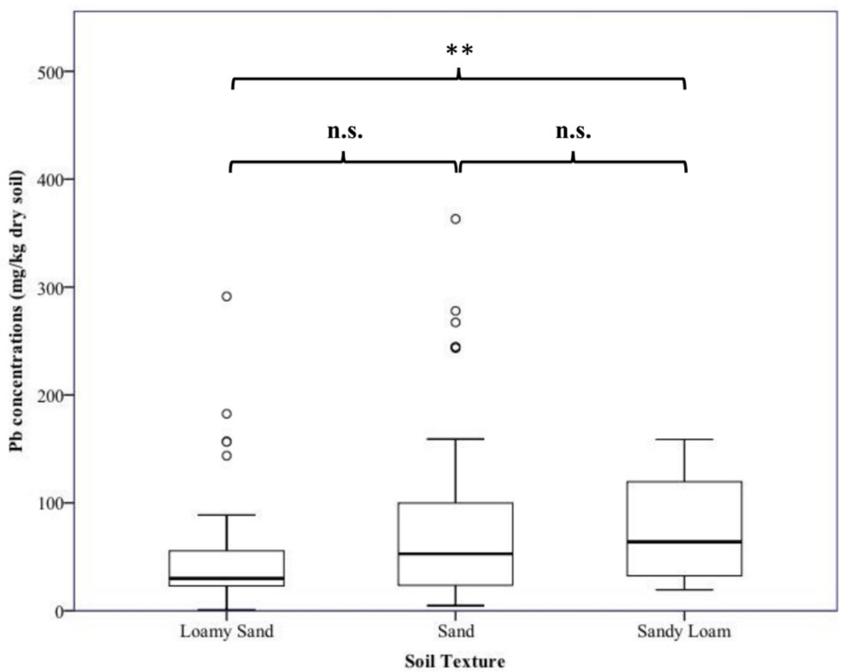

Figure 9. Box plots of soil Pb concentrations referencing USDA soil textural triangle. Correlation was not significant and significant at the 0.01 level (two-tailed), denoted as n.s. and **, respectively.

Table 6. Spearman's correlation coefficients among Pb concentrations and soil properties in the soil.

	Pb concentration (ppm)	pH	Sand (%)	Clay (%)	Silt (%)	Water content (%)	OM content (%)	FS content (%)
Pb concentration (ppm)	1							
pH (-)	0.071	1						
Sand (%)	-0.155	-0.337**	1					
Clay (%)	0.001	0.272**	-0.745**	1				
Silt (%)	0.226*	0.292**	-0.880**	0.383**	1			
Water content (%)	-0.136	0.278**	-0.249**	0.331**	0.142	1		
OM content (%)	0.037	-0.349**	-0.110	0.043	0.135	0.274**	1	
FS content (%)	0.096	0.046	0.232**	-0.253**	-0.172	-0.790**	-0.780**	1

* Correlation is significant at the 0.05 level (two-tailed)

** Correlation is significant at the 0.01 level (two-tailed)

3.3.4 Urban factors contributing to Pb contamination in soil

Children living in urban centers, (typically of a lower socioeconomic status and from marginalized populations) have a higher potential of being exposed to Pb (Rothenberg et al., 1996; Wu et al., 2010). The study indicated that high soil Pb levels are distributed across the greater Los Angeles area but also pose a serious threat to children in affluent community of Beverly Hills. One possible explanation for the high Pb levels in parks in the vicinity of Santa Monica Boulevard could be higher traffic volume and the historical railway transport lines. Soil from areas around major roads (Davis and Birch, 2011; Wu et al., 2010) and railway transport lines (Akoto et al., 2008; Liu et al., 2009; Ma et al., 2009; Wiłkomirski et al., 2011) have been shown to contain elevated Pb levels in many countries. The Balloon route of the Pacific Railroad passes by Santa Monica Boulevard near where samples were collected, which could likely contribute to current levels of Pb in soils (Figure 10). Further research in Beverly Hills is required to identify and confirm the possible contribution of railways to Pb pollution, in addition to leaded paints and vehicle traffic.

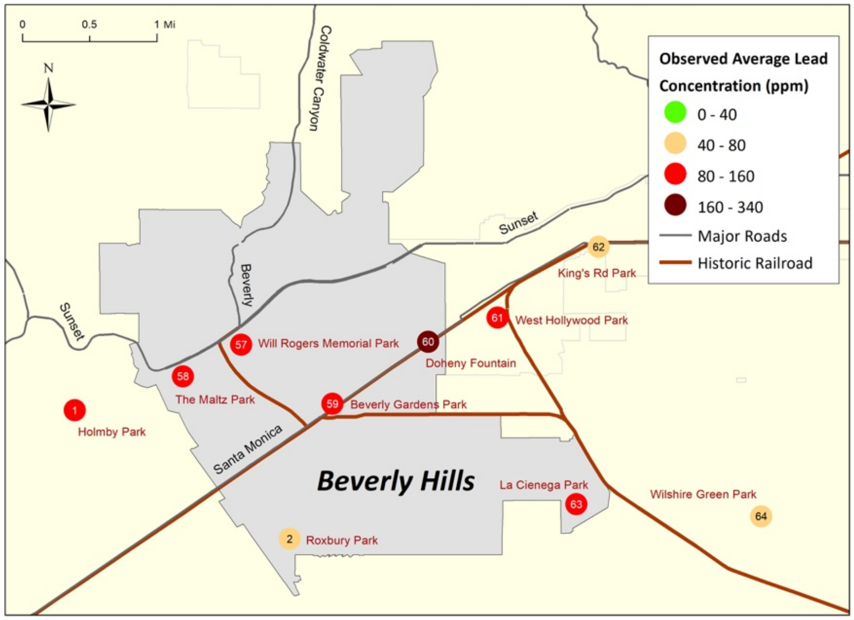

Figure 10. Potential Pb sources (major roads and historic railroads) and locations of high soil Pb levels in Beverly Hills.

The history of parks should also be considered as a factor that contributes to Pb pollution because older parks have been accumulating Pb for longer periods of time (Chen et al., 2005; Li et al., 2001). Since the late 19th century, the Department of Recreations and Parks in Los Angeles has created many recreational areas. Over that time, airborne Pb particles have deposited and accumulated on soil surfaces in urban parks, and residential and urban parks have become Pb reservoirs (Filippelli et al., 2005; Setälä et al., 2017). The age of 45 parks were obtained in this study. Pb concentration in the urban park soil significantly correlated ($\rho = -0.319$, $p = 0.033$) with park age, as shown in Figure 11a. These data are consistent with studies conducted in Beijing by Chen et al. (2005) and in Hong Kong by Lee et al. (2006) that both showed a significant relationship between the history of parks and Pb concentration. However, old parks may have been renovated or rebuilt more recently, and processes that would have introduced new soils or disturbed the original soil would have taken place. In renovated parks, the correlation between Pb concentration and park age following the last-known renovation was insignificant (ρ = -220, p = 0.121) (Figure 11b), as a newer or cleaner soil may have been brought into the parks.

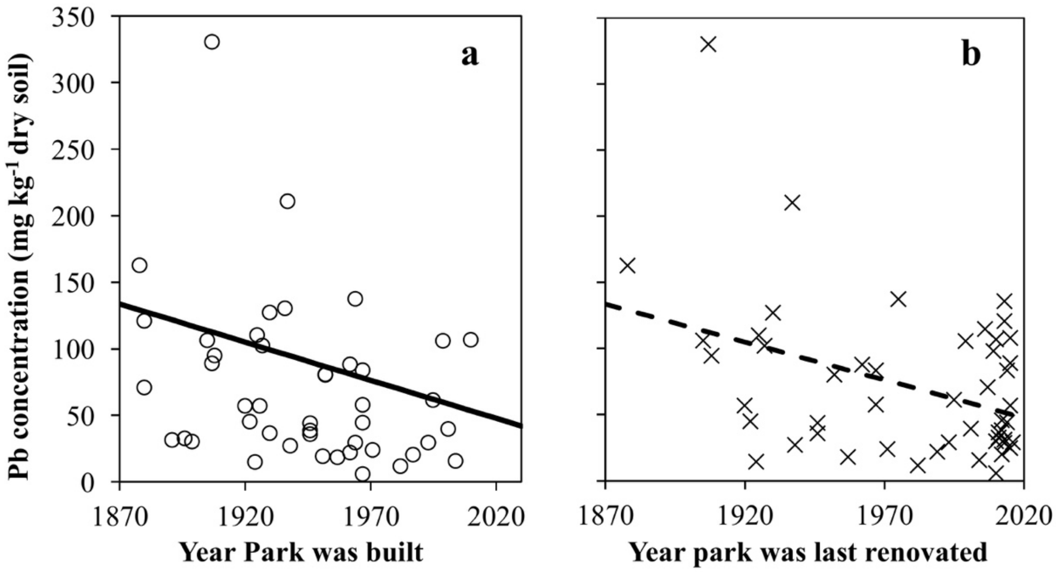

Figure 11. Relationship between Pb concentration in soils and (a) year of park construction and (b) year of last park renovation.

Vehicle traffic may have an impact on Pb pollution in park soils in the vicinity of major roads. One study reported that Pb contamination in soil up to 320 m away from the road (Viard et al., 2004). This work showed that the concentrations of Pb in soils decreased incrementally with roadside distance (Figure 12), although this was not statistically significantly. However, these data correlate with a previous study by Swaileh et al. (2003).

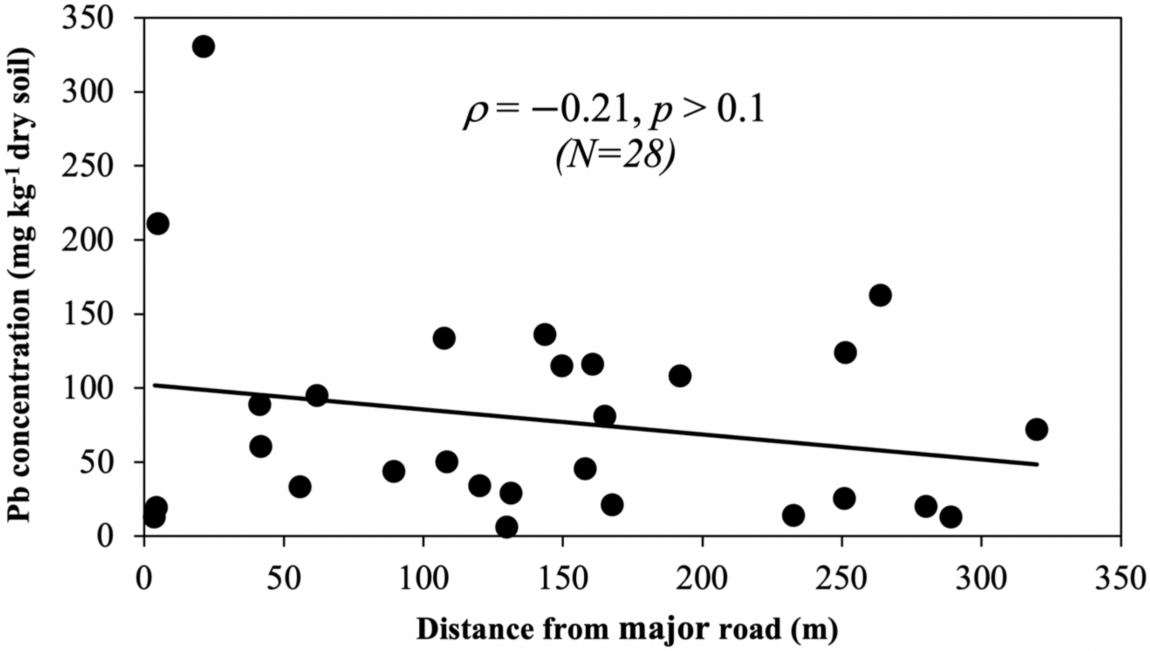

Figure 12. The relationship between the Pb concentrations in soils and the distance from major roads.

Industrial emissions have also contributed to soil Pb pollution in Los Angeles. In fact, Pb-acid battery recycling facilities have polluted the air and soil of neighborhoods in southeast Los Angeles County for years (Johnston and Hricko, 2017). Our investigation of three parks next to Vernon showed elevated Pb concentrations in soil (up to 156 ppm). These results agree with a report released by DTSC in 2016 in which 99% of properties within 1.7 miles from the facility have Pb levels above 80 ppm (Department of Toxic Substances Control, 2016).

3.3.5 Comparison of Pb concentrations in park soils with other cities around the world

While many studies have found higher levels of Pb in urban areas (Chen et al., 2005; Solt et al., 2015; Wu et al., 2010), our findings indicated higher levels of Pb were spread throughout a large area of Los Angeles, due to its large metropolitan area. Indeed, Pb levels in parks exhibited similar patterns to the study by Wu et al. (2010) conducted in Inglewood, Pico-Union and the Hollywood area.

Many previous studies focused on soils from different types of land use, such as residential, industrial, and roadside areas while only a few studies focused on Pb in park soils. Residential communities (Laidlaw et al., 2018; Pouyat et al., 2007) and roadside communities (Al Obaidy and Al Mashhadi, 2013; Sun et al., 2010) in other major cities have been found to contain areas of high Pb as compared with other types of land use. Similarly, both historical traffic and housing-unit related variables serve as crucial indicators of anthropogenic Pb inputs in Los Angeles (Mielke et al., 2010b). In fact, previous studies conducted in Los Angeles by Wu et al. (2010) showed higher total Pb concentrations in soils (average: 181 ppm; median: 81 ppm) than our results. This was likely because their soil samples were collected mostly from roadside areas that have higher average Pb concentrations.

Previous studies have emphasized large cities where Pb contamination would be expected to be high. However, there are only a few previous studies of urban parks for comparison purposes (Table 7). Parks within these cities appeared to have large variation, which is consistent with our results. Large variation can lead to difficulties making comparisons with regional studies. In this study, mean concentrations of triplicate samples may not provide a representative result for the associated parks. However, this study facilitates assessments of potentially high Pb levels in parks across Los Angeles and can be used to determine if further investigation or remediation is needed.

Table 7. Comparison of Pb concentration in park soils in major global cities.

City, Country	Mean	Median	Standard Deviation	min	Max	Reference
Khorramabad, Iran	7.38	-	7.94	0.05	35	(Solgi and Konani, 2016)
Los Angeles, United States	65.5	45.8	65.5	0.969	363	*This study*
Beijing, China	66.2	-	44.2	25.5	207.5	(Chen et al., 2005)
Shanghai, China	70.69	-	84.1	13.72	102.5	(Shi et al., 2008)
Galway, Ireland	78.4	58	72.0	25	543	(Zhang, 2006)
Shenyang, China	85.10	-	-	-	-	(Sun et al., 2010)
Hong Kong, China	93.4	-	37.3	5.27	404	(Li et al., 2001)
Athens, Greece	110.3	101.3	35.8	38.2	140.5	(Massas et al., 2010)
Guangzhou, China	195.95	224	116.07	38.4	348	(Lu et al., 2011)
Sicily, Italy	-	253	302	57	2516	(Manta et al., 2002)

3.4 Conclusions

Based on results from this study, park soils in Los Angeles contain high levels of Pb, with 35 parks exceeding the CalEPA guideline of 80 ppm. Children who frequent these parks are at an elevated risk of Pb poisoning that can result in increased BLL and related neurological problems. This work demonstrated that Pb levels are generally higher in Beverly Hills, Culver City, south Los Angeles, and central Los Angeles, but with large variations in 89 parks. High levels of Pb in park soils were most likely due to park age, vehicle traffic, and industrial emissions. However, further research is necessary to identify other potential sources of Pb contamination in park soils, such as railways. In addition, the impact of soil properties on Pb concentrations was not as significant as park age except for silt in urban top soils. Because most urban parks in this study were small (88% < 80,000 m^2), an additional investigation would be warranted to delineate soil Pb levels in large parks, including Polliwog Park, Jesse Owen Parks, and Rancho Cienega Recreation Center. In summary, this study provides information that can be used to guide remediation decisions for authorities and for the construction of Pb-safe environments.

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CHAPTER 4. PREVALENCE OF ANTIBIOTIC RESISTANCE GENES AND CO-SELECTION OF METAL(LOID)S IN STORMWATER BIOFILTERS IN SOUTHERN CALIFORNIA

4.1 Introduction

The widespread occurrence of antibiotic resistance (AR) has become one of the most critical public health issues, posing a global threat to human health. Newly developed antibiotics lose their effectiveness over time and threaten our future capability to treat bacterial infections. Increasing attention has been given to the role of environmental pathways for the emergence and proliferation of AR due to the potential for selective pressure in some settings (Singer et al., 2016). While AR originates in nature and exists at a baseline level in the environment (Czekalski et al., 2015), there are anthropogenic pollutants, such as heavy metals in the environment that can act as stressors, that also contribute to the development of AR through co-selection (Manaia et al., 2016).

Issues of particular concern for human health are the synergistic effects on the proliferation of AR that contaminants can have in the environment. For example, heavy metals, which are common co-contaminants with antibiotic resistance genes (ARGs), may co-select by several mechanisms. First, co-resistance occurs when genetic elements conferring resistance to metals and antibiotics are spatially linked on the same plasmids. Second, cross-resistance occurs when the same mechanism used by the cell for heavy metal resistance also is effective against antibiotics. For example, efflux pumps will remove both heavy metals and antibiotics from the cell. Third, co-regulation for co-selection whereby a shared regulatory response for both types of exposure can occur in response to one type of exposure, thereby conferring resistance to the other. All three co-selection mechanisms are similar in that the presence of a stressor may induce indirect selection for bacteria with resistance to multiple chemically-unrelated substances (Baker-Austin et al., 2006). Co-selection is expected to depend on the concentrations and speciation of the antibiotics and metals in addition to the composition of the microbial community and level of horizontal gene transfer (HGT). HGT is facilitated by mobile genetic elements (MGEs), such as plasmids and integrons, which can transfer genetic materials to a variety of microorganisms (Schlüter et al., 2007). This process, alongside direct and indirect selection for bacteria in metal-polluted streams, may represent a critical pathway of dissemination of antibiotic resistance.

Correlations between heavy metals and antibiotic resistance proliferation have been widely reported in many highly contaminated environments due to continuous and/or accidental pollution. Studies investigating co-selection in soils frequently indicate the correlation of increased metal concentrations with increased ARG levels. These environments include effluents and biosolids from waste treatment plants (Di Cesare et al., 2016; Gao et al., 2012; Jang et al., 2018; Mao et al., 2015), agricultural soils affected by land application of biosolids or manure waste (Chee-Sanford et al., 2009; Ji et al., 2012), feedlots (He et al., 2016), water bodies associated with waste discharges (Graham et al., 2011), and metal-spiked experiments in microcosms (Knapp et al., 2011; Stepanauskas et al., 2006; Wang et al., 2020) and in agricultural fields (Hu et al., 2017). In these cases, the levels of AR increased due to heavy metal co-selection; however, slightly-contaminated soils, particularly stormwater biofilters, are poorly understood even though urban stormwater and associated surface runoff have been identified as reservoirs of AR (Dorsey et al., 2013; Garner et al., 2017).

Stormwater biofilters (also referred to bioretention systems or bioswales; hereafter, we use the term “biofilter”) are an example of a green infrastructure or low impact development that is designed to capture and treat stormwater pollutants in urban areas. Benefits of biofilters include protecting wildlife habitat, improving stormwater runoff water quality, and reducing flood risks. In densely populated Southern California, runoff from both wet and dry weather is a major source of pollution that significantly impacts the region's water quality and poses risks to local health and safety (Ambrose and Winfrey, 2015). Although biofilters are known to remove contaminants, such as heavy metals and nitrogen (Blecken et al., 2009), few studies have been conducted on microbial pollutants that are potentially deleterious to human health. It is still unclear whether biofilters are sufficiently capable of removing emerging contaminants including antibiotic resistant bacteria (ARB) and ARGs.

Urban stormwater runoff has been commonly reported to contain noticeable levels of pathogens (Dorsey et al., 2013; Garner et al., 2017; Sidhu et al., 2012). Fecal contaminants found in stormwater are frequently observed to carry elevated levels of ARB and antibiotics (Karkman et al., 2019). While degradation of antibiotics are dependent on physicochemical properties and molecular structure (Cycoń et al., 2019), heavy metals are distinguished by their persistence in the environment (Hung et al., 2018). After infiltrating an environment, ARB or ARG levels may increase after initial infiltration into biofilters due to HGT and co-selective pressure caused by heavy metals, depending on the metal’s bioavailability and speciation (Isaac Najera et al., 2005). A detailed study in this area is needed considering the significant consequences of impaired urban stormwater quality.

The objectives of this study consisted of several tasks as following: (1) determine the prevalence of ARGs, MGE, bioavailable heavy metals, and total heavy metals within biofilters; (2) examine other factors that potentially contribute to AR, including geochemical conditions of biofilters, temporal effects, and AR management strategies; (3) perform correlation analysis to investigate the heavy metal co-selective effects on ARGs within biofilters; and (4) establish multiple linear regression models between gene abundance and a combination of metal(loid) levels and soil properties on biofilters. This model will further delineate the levels of ARGs for risk assessment and provide useful references for better developments in urban biofilters.

4.2 Materials and Methods

4.2.1 Study area and sample collection

Urban biofilters were sampled for the beginning, middle, and end of the wet season (Fall: October/November, 2018; Winter: February/March, 2019; Spring: April, 2019) at three University of California campuses located in southern California (University of California, Santa Barbara [UCSB], University of California, Irvine [UCI], and University of California, San Diego [UCSD]). Irvine, Santa Barbara, and San Diego have semi-arid and Mediterranean climates. Within each of the three campuses, two biofilter sites were sampled (Figure 13). Each sampling site was represented by four subsamples of soil covering the length of the bioswales for Manzanita (MZ) (UCSB), Sierra Madre (SM) (UCSB), Culver (CUL) (UCI), Verano (VER) (UCI), Sanford (SAN) (UCSD), and each individual sub-basin for the infiltration basin at Altman Clinical and Translational Research Institute (ACT) (UCSD). A total of seventy-two soil samples across the three time points were collected.

Each biofilter was represented by four subsamples corresponding to four different locations. After aboveground vegetation, rocks, and mulch were removed, each of three soil cores at each location was collected and combined into a composite soil sample of surface soils (0−10 cm), using cylindrical stainless-steel coring cups (5.08 cm diameter x 10 cm length) attached to a slide hammer. Soil cores were stored in sterile 15 mL Falcon tubes and transported in coolers (4°C) before being stored at -20°C in the laboratory. For ARG quantification analysis, three sieved soil subsamples of 0.25 ± .01 g from each tube were measured into sterile 2 mL screwcap tubes preloaded with garnet beads and bead solutions (Qiagen, Valencia, CA, USA). Screwcap Tubes were stored at -20°C until DNA extraction. In parallel, a portion of soil samples were taken to the laboratory for soil characterization.

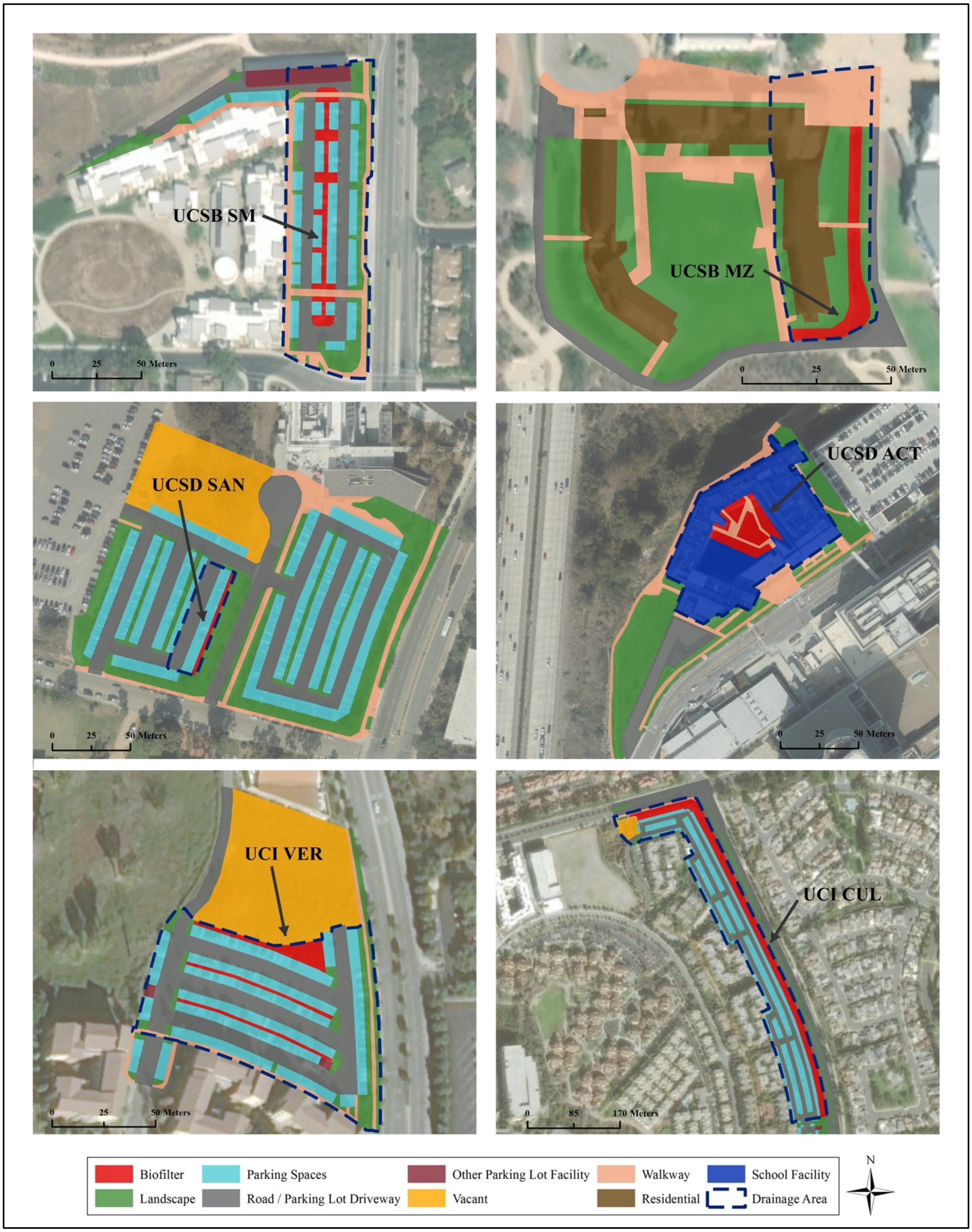

Figure 13. Land-use types near six biofilter sites located at UCI, UCSD, and UCSB. Exact drainage areas of biofilters were not shown on this map. Abbreviation: UCSB, University of

California, Santa Barbara; UCI, University of California, Irvine; UCSD, University of California, San Diego; MZ, Manzanita; SM, Sierra Madre; CUL, Culver; VER, Verano; ACT, Altman Clinical and Translational Research Institute; SAN, Sanford.

4.2.2 Soil characterization

Biofilter samples from the three UC campuses were characterized to determine their physical and chemical properties. Soil samples were sieved (2 mm) and shipped at 4°C to the Analytical Laboratory at University of California, Davis for determination of soil texture, cation exchange capacity (CEC), and total nitrogen and total carbon (total N and total C, respectively) according to previously published methods (Association of Official Analytical Chemists (AOAC), 1997; Rible and Quick, 1960; Sheldrick and Wang, 1993). Gravimetric soil moisture and organic matter were determined in triplicate by sequential loss on ignition (LOI) at 105°C for 24 h and at 550°C for 4 h, respectively (Gardner, 1986; Nelson and Sommers, 1996). The pH of biofilter samples was measured with a pH meter (Oakton Ion 700 benchtop meter, Cole Parmer, Vernon Hills, IL) following the transfer of 10 g of soil into 10 g of deionized water and allowing the mixture to settle for 10 minutes. The inorganic nutrients in the soil samples were extracted with 30 mL of 2 M KCl solution following the standard method (Mulvaney, 1996). Soil extracts were filtered through Whatman filtration papers (ashless, grade 42, 42.5 µm diameter, Sigma-Aldrich, St. Louis, MO) and analyzed for dissolved nitrate and phosphate at the UCSB Marine Science Institute (MSI) Analytical Lab using QuikChem8500 Series 2 Flow Injection Analysis system (Lachat Instruments, Milwaukee, WI).

4.2.3 Determination of exchangeable and total heavy metal content

To determine the bioavailable (soluble and exchangeable) fraction and total heavy metals in the biofilters, a sequential extraction procedure developed by (Tessier et al., 1979) and acid digestion according to United States Environmental Protection Agency (USEPA) Method 3050B (U.S. Environmental Protection Agency, 1996) were used. In brief, air-dried soil samples were extracted with 1 M $MgCl_2$ at an initial pH 7.0 (25 mL) and was shaken at 250 rpm for 2 h at ambient temperature. The residue from previous step was digested on hot plates using a combination of concentrated nitric acid ([HNO_3], 68%–70% (v/v)) and hydrogen peroxide ([H_2O_2], 30% (v/v)). Digested samples were diluted to 50 mL with a solution containing 6.8%–7% (v/v) HNO_3, and 0.9% (v/v) H_2O_2. All chemicals were analytical grade or higher, and glassware was acid-washed and rinsed before use. The concentrations of nine metal(loid)s, including arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), selenium (Se), vanadium (V), and zinc (Zn), in each extract were analyzed in triplicate with inductively coupled plasma-mass spectrometry ([ICP-MS], 7700 Series, Agilent Technologies, Santa Clara, CA, USA) at the University of California-Riverside Water Technology Center laboratory. Total heavy metals were calculated by adding bioavailable and acid-digested fractions described above. A reagent blank per batch was processed in the same manner as the samples and analyzed to correct the instrument readings as part of the quality control protocol.

4.2.4 DNA extraction and real-time quantitative Polymerase Chain Reaction of ARGs

DNA was extracted from all biofilter samples using DNeasy PowerSoil Kits (Qiagen, Valencia, CA, USA) following the manufacture's guideline. The final DNA extracts were store at -20°C for subsequent real-time qPCR analysis. Meanwhile, the purity and quantity of total DNA extracts were determined using UV absorption by a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA). DNA extracts were considered as relatively free of contamination as the A260/280 ratio was above 1.8 per the instrument manual.

All samples were analyzed for ARG (*sul*1, *sul*2, *tet*A, *tet*W, and *erm*F), class 1 integron-integrase gene (*intI*1), and *16S rRNA* (a proxy for total cells) gene abundances. Real-time polymerase chain reaction (qPCR) was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) in the StepOne Plus qPCR system (Applied Biosystems, Foster City, CA, USA). Primer concentrations (Table 8) and thermocycling conditions (Table 9) were optimized as described in (Echeverria-Palencia et al., 2017). DNA standards were designed using sequences from the National Center for Biotechnology Information (NCBI) database and obtained through Integrated DNA Technologies (IDT) (Coralville, IA, USA). Standard concentrations of the designed DNA fragments were analyzed next to biofilter samples in triplicate. To minimize effects of qPCR inhibition, soil DNA samples were diluted as shown in (Echeverria-Palencia et al., 2017).

4.2.5 Statistical analysis and geographic information systems

Site locations and surrounding land uses were entered and digitalized into ArcMap Version 10.7 (ESRI, Redlands, CA, USA) to generate biofilter map across three UC campuses. All statistical analysis and graphical outputs were performed by RStudio Version 1.3 (RStudio, Inc., Boston, MA, USA) and SPSS Version 23 (IBM Co., Armonk, NY, USA), respectively. Statistical comparisons of ARG levels among soil types were performed using the Mann−Whitney U test followed by Bonferroni correction to adjust the probability. Spearman's correlation was undertaken to identify correlations among ARGs, metal(loid) concentrations, and soil properties.

The p-values were adjusted according to Benjamini–Hochberg method in consideration of false discovery rate (Benjamini and Hochberg, 1995). Both are nonparametric methods that do not assume normal distribution since our data were mostly not normally distributed as tested by the Kolmogorov-Smirnov method ($p < 0.05$). A p-value of < 0.05 was set to be significant. Hierarchical cluster analysis (HCA) was performed to identify similar groups among metal(loid)s and biofilter sites based on the Euclidean distance and the between linkage method. Lastly, stepwise multiple linear regression (MLS) was built to acquire relationships between relative ARG abundances and environmental factors. Metal(loid) concentrations below the detection limit (BDL) were manually designated a value of half the detection limits (Helsel and Gilloom, 1986) for further analysis. Suggestions including replacing the values by zeros and the detection limits were not considered to better perform MLS and to differentiate values close to BDL, respectively.

Table 8. Primer sequences and concentrations of qPCR reactions. Abbreviations: F, Forward; R, Reverse.

Gene	Primer	Concentration (nM)	Sequence (5'-3')	Amplicon size (bp)	References
*sul*1	*sul*1-F	200	CGCACCGGAAACATCGCTGCAC	258	Pei et al., 2006
	*sul*1-R		TGAAGTTCCGCCGCAAGGCTCG		
*sul*2	*sul*2-F	200	CTCCGATGGAGGCCGGTAT	190	Luo et al., 2010
	*sul*2-R		GGGAATGCCATCTGCCTTGA		
*tet*A	*tet*A-F	200	GCTACATCCTGCTTGCCTTC	250	Ng et al., 2001
	*tet*A-R		CATAGATCGCCGTGAAGAGG		
*tet*W	*tet*W-F	200	GAGAGCCTGCTATATGCCAGC	210	Aminov et al., 2001
	*tet*W-R		GGGCGTATCCACAATGTTAAC		
*erm*F	*erm*F-F	500	TCGTTTTACGGGTCAGCACTT	246	Knapp et al., 2010
	*erm*F-R		CAACCAAAGCTGTGTCGTTT		
*intI*1	*intI*1-F	200	GGCTTCGTGATGCCTGCTT	424	Luo et al., 2010
	*intI*1-R		CATTCCTGGCCGTGGTTCT		
16S rRNA	16S rRNA-F	500	ATGGCTGTCGTCAGCT	351	Pan et al., 2018
	16S rRNA-R		ACGGGCGGTGTGTAC		

Table 9. Thermocycling conditions of real-time quantitative PCR.

Gene	Holding		Denaturation		Annealing		Extension		R^2	Efficiency (%)
	Temp (°C)	Time (min)	Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	Temp (°C)	Time (sec)		
*sul*1	95	10	95	15	65	30	72	30	0.996 – 1	90.0 – 97.5
*sul*2	95	10	95	30	60	30	72	30	0.999 – 1	86.0 – 90.3
*tet*A	95	4	95	5	55	30	72	30	0.995 – 0.997	87.5 – 91.0
*tet*W	95	4	95	30	60	30	72	30	0.999 – 1	97.3 – 100
*erm*F	95	10	94	20	60	30	-	-	0.997 – 1	94.8 – 100
*intI*1	95	10	95	15	55	30	72	30	0.999 – 1	90.7 – 94.3
16S rRNA	94	4	94	40	60	45	72	60	0.991 – 1	88.4 – 94.9

4.3 Results and Discussion

4.3.1 Prevalence of ARGs in biofilters in Southern California

Soil DNA extracts were analyzed for selected ARGs, MGE (*intI*1), and 16S rRNA (surrogate of total bacteria) in Table 10. Relative gene abundances (normalized to 16S rRNA genes) were often used to account for efficiencies during DNA extraction and size of the microbial community (Ji et al., 2012). In this study, absolute gene abundances based on total mass input were also listed. All genes were detected except for *erm*F genes, which were not detected across any biofilter sample. The relative gene abundances of selected ARGs and *intI1* ranged from 10^{-7} to 10^{-4} genes per 16S rRNA gene copies (hereafter we abbreviate as genes/16S), meaning *intI*1 and ARGs representing roughly 0.00001% to 0.01% of the total bacteria.

Relative abundances of the *intI1* gene have been identified as good indicators of pollution and are commonly linked to genes conferring resistance to antibiotics and heavy metals (Gillings et al., 2015). For example, relative gene abundances of *intI*1 had significant correlation with relative gene abundances of *sul*1 ($\rho = 0.78$, $p < 0.01$) and *tet*W ($\rho = 0.33$, $p < 0.01$) in the present study, suggesting the potential of HGT driven by heavy metals.

Ranges of relative *sul*1 and *intI*1 gene abundances from UC biofilter soil samples were compared in soils to those found in biosolids from waste water treatment plant (Hung, 2020), San Diego park soils (Cristina M Echeverria-Palencia et al., 2017), commercially available manure fertilizers (Cira et al., n.d.), and pristine soils from nearby hiking trails (Cira et al., n.d.) as shown in Figure 14. From this analysis, both relative *sul*1 and *intI*1 levels in biofilters in this study may be considered similar to slightly impacted sites as they were statistically greater than those detected in pristine soils collected from Santa Monica Mountains near Los Angeles. Relative gene abundances of *sul*1 were statistically smaller than relative *sul*1 gene measured in biosolids, manure fertilizer, and San Diego park soils. Similar trends were found in the relative gene abundances of *intI*1 except for San Diego park soils as that information was not available. These gene disparities between soil environments should receive more attention. Particularly in soils containing relatively low concentrations of metal(loid)s, biofilters are likely to be new hot spots that present risks to the environment in the long-term.

Management of biofilters could have also played a critical role in the ARG levels. The use of garden amendments and reclaimed water could contain ARGs and thus be capable of increasing soil ARGs (Echeverria-Palencia, 2018; Fahrenfeld et al., 2013; Wang et al., 2014). In the present study, MZ, SM, CUL, and SAN were irrigated with reclaimed water irrigation while VER and ACT were irrigated with potable water (Table 11). Among all biofilters, MZ was the only biofilter reported not to receive garden amendments and had relatively lower relative ARG abundances compared to the other biofilters. Except for fertilizers, operations and practices of biofilters do not appear to affect levels of ARGs in biofilters

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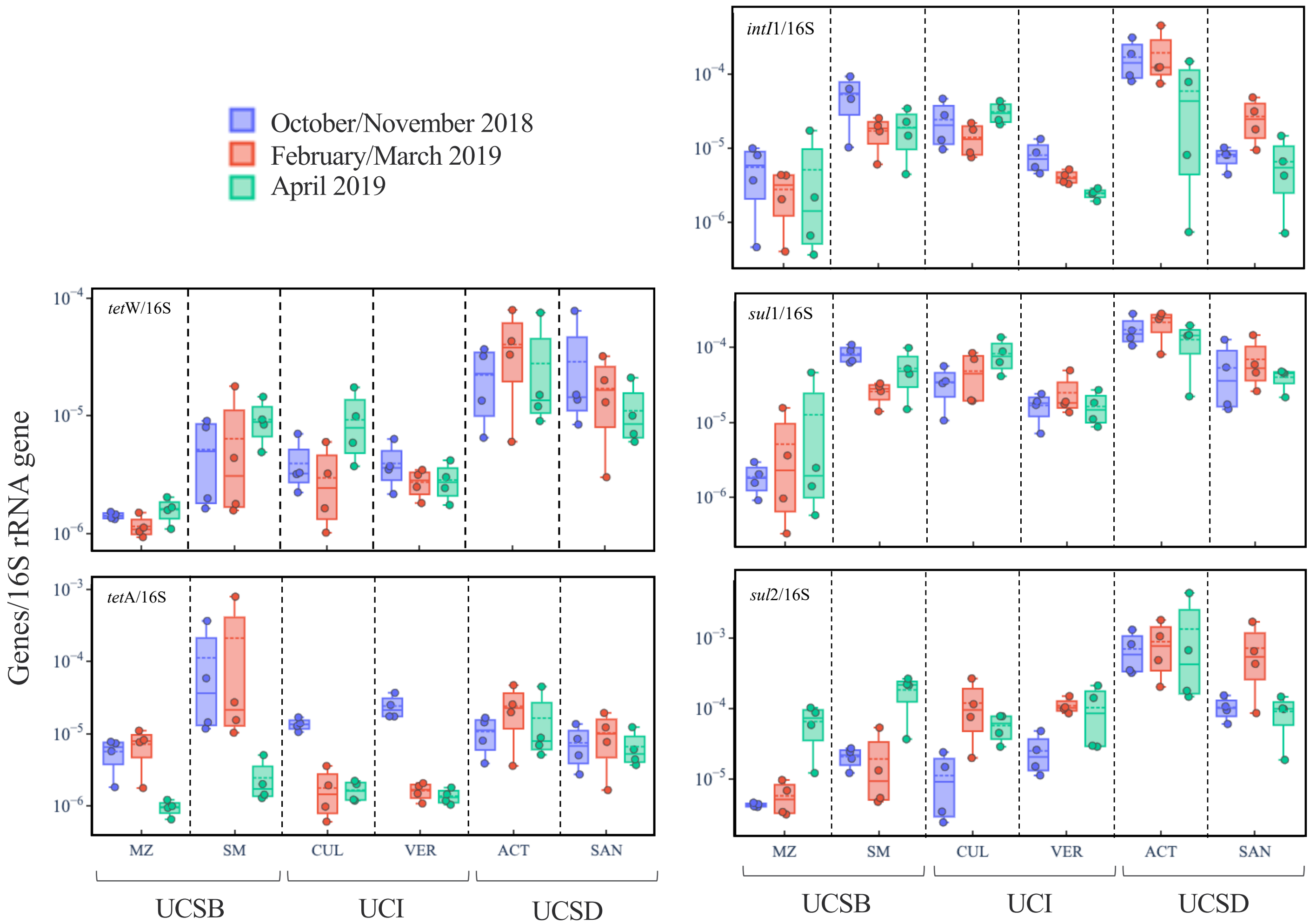

Figure 15). Levels of relative gene abundances of *sul*1, *sul*2, *tet*W, and *intI*1 were the highest at ACT compared to those in other biofilters, possibly due to condensation drainage from air conditioners. Other factors including year built, areas, and impervious drainage areas that may potentially affect the ARG levels within the biofilters were not obvious. A larger sample size may be required to study the effects of those factors.

Table 10. Summary of gene contents in biofilter samples (N = 72) collected from UCSB, UCI, and UCSD. Genes below the detection limit (BDL) were manually designated a value of half the detection limit for further analysis. Abbreviations: BDL, Below Detection Limit; N.A., Not Available.

	16S rRNA	*intI*1	*sul*1	*sul*2	*tet*A	*tet*W	*erm*F
Absolute abundance (genes/g soil)							
Mean	9.68×10^{8}	1.46×10^{4}	2.78×10^{4}	7.27×10^{4}	2.54×10^{-4}	3.63×10^{3}	BDL
Median	7.91×10^{8}	7.32×10^{3}	1.72×10^{4}	3.33×10^{4}	7.75×10^{-5}	BDL	BDL
SD	7.58×10^{8}	2.06×10^{4}	3.09×10^{4}	1.06×10^{5}	6.10×10^{-4}	2.99×10^{3}	N.A.
Kurtosis	-0.226	26.6	6.31	15.4	30.4	19.1	N.A.
Skewness	0.741	4.37	2.32	3.50	5.03	4.00	N.A.
min	2.96×10^{7}	382	741	BDL	BDL	BDL	BDL
Max	2.96×10^{9}	1.51×10^{5}	1.66×10^{5}	6.57×10^{4}	4.38×10^{-3}	2.12×10^{4}	BDL
Relative abundance (genes/16S)							
Mean	N.A.	3.59×10^{-5}	5.97×10^{-5}	2.30×10^{5}	2.56×10^{-5}	1.10×10^{-5}	BDL
Median	N.A.	1.00×10^{-5}	3.43×10^{-5}	1500	6.53×10^{-6}	BDL	BDL
SD	N.A.	7.05×10^{-5}	6.75×10^{-5}	1.31×10^{5}	1.01×10^{-4}	1.66×10^{-5}	N.A.
Kurtosis	N.A.	19.4	2.91	70.0	48.9	9.02	N.A.
Skewness	N.A.	4.05	1.79	8.32	6.80	2.94	N.A.
min	N.A.	3.63×10^{-7}	3.32×10^{-7}	BDL	BDL	BDL	BDL
Max	N.A.	4.51×10^{-4}	2.81×10^{-4}	1.11×10^{6}	7.92×10^{-4}	7.87×10^{-5}	BDL

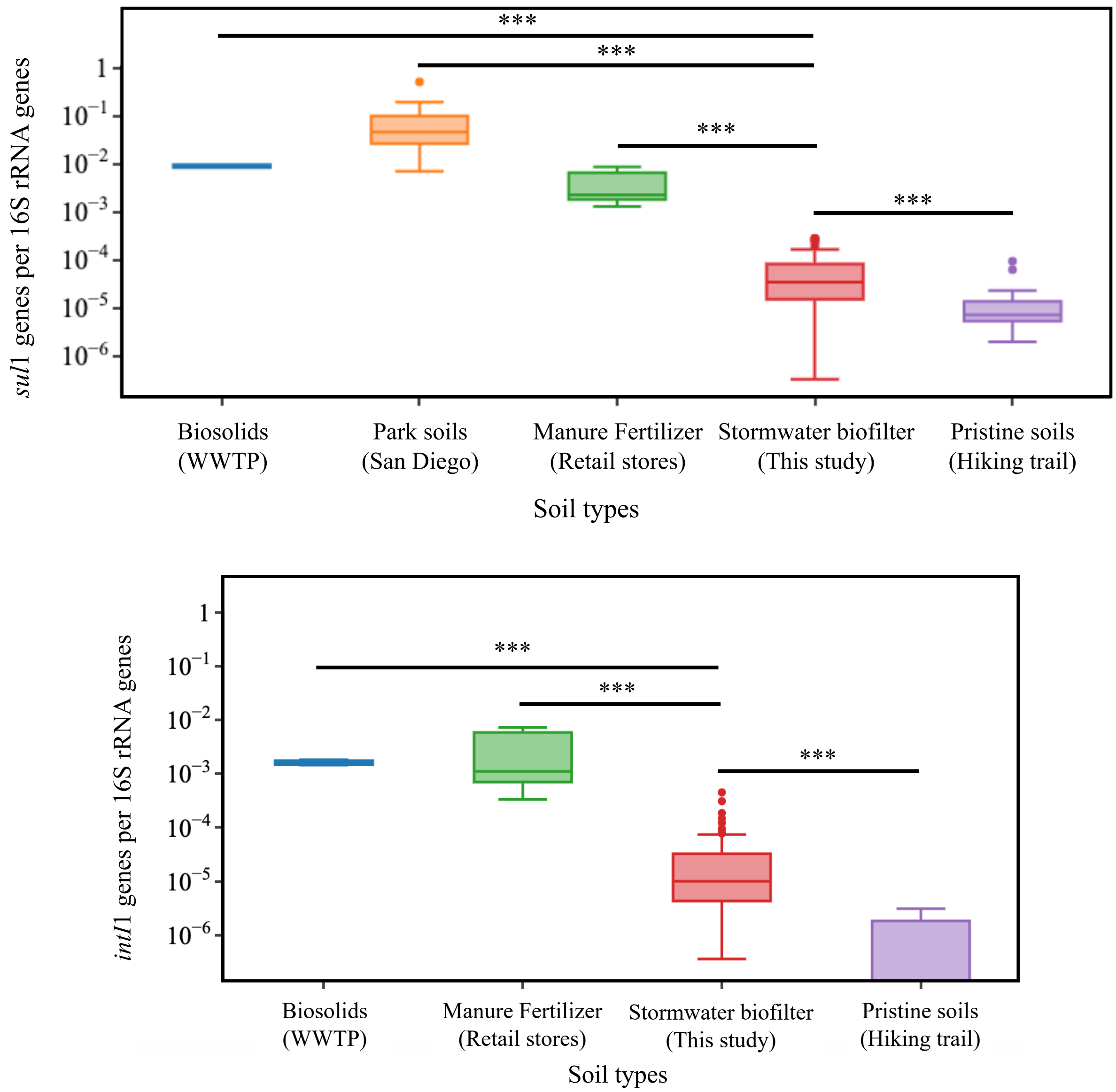

Figure 14. Relative *sul*1 (top) and *intI*1 (bottom) gene abundances among different soil types. Statistical differences between soil types significant at $p < 0.01$, $p < 0.05$, and $p < 0.1$ level are marked with ***, **, and *, respectively.

Table 11. Site characteristics of biofilters distributed in three UC campuses.

Site	Campus	NTS area (m^2)	NTS type	Runoff contributing area	Impervious drainage area (m^2)	Year built	Type of irrigation water	Potential source of AR
MZ	UCSB	363	Bioswale	Buildings, lawns, and paved walkways	1,997	2001	RW	RW, runoff
SM	UCSB	126	Bioswale	Parking lot	1,571	2015	RW	RW, runoff, fertilizer
CUL	UCI	1,330	Bioswale	Parking lot and adjacent landscaped area	21,400	2007	RW	RW, fertilizer, runoff
VER	UCI	146	Bioswale	Parking lot and unmanaged greenspace	1,878	2012	PW	runoff, fertilizer
ACT	UCSD	1,725	Infiltration basin	Buildings and AC condensate	4,080	2016	PW	runoff, fertilizer
SAN	UCSD	81	Bioswale	Parking lot	432	2011	RW	RW, runoff, fertilizer

Abbreviations: MZ, Manzanita; SM, Sierra Madre; CUL, Culver; VER, Verano; ACT, Altman Clinical and Translational Research Institute; SAN, Sanford; NTS, Natural Treatment System; RW, Reclaimed Water; PW, Potable Water.

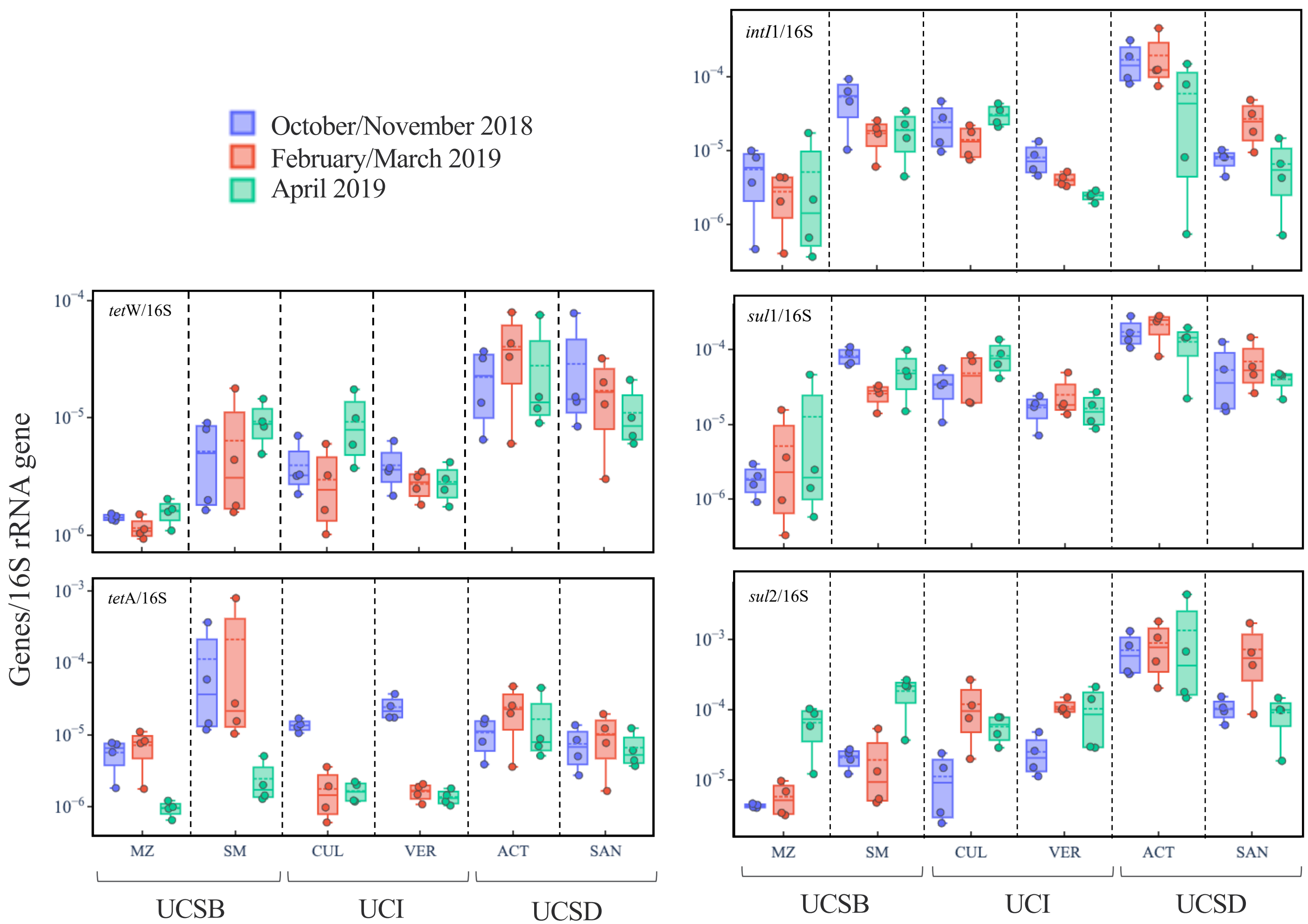

Figure 15. Temporal and spatial variation of the relative gene abundances in six biofilter sites on October/November 2018 (blue), February/March 2019 (red), and April 2019 (green). Abbreviations: MZ, Manzanita; SM, Sierra Madre; CUL, Culver; VER, Verano, ACT, Altman Clinical and Translational Research Institute; SAN, Sanford.

4.3.2 Soil characteristics of campus biofilters in Southern California

The descriptive statistics of soil properties in biofilters are summarized in Table 12. Based on the soil textural triangle from the United States Department of Agriculture (USDA), biofilter samples were predominately sand (Figure 16). Of these biofilter samples, most were characterized as sandy loam with a few identified as loamy sand and sandy clay loam. Soil pH values changed respective of time points but irrespective of locations and ranged from 6.59 to 9.05. According to the common classes of soil pH defined by United States Department of Agriculture (USGS), soil pH values ranged from slightly acidic to strongly alkaline. Both soil moisture and soil organic matter (SOM) also differed considerably across all soil samples, ranging from 5.77% to 39.5% and from 1.22% to 15.5%, respectively.

Spearman's correlations between gene abundances and soil properties were examined (Table 13). The percentage of sand and silt, SOM, bulk density, total C, and total N within biofilters were significantly correlated with relative gene abundances of *sul*1 (sand: $\rho = 0.398$, $p < 0.01$; silt: $\rho = -0.550$, $p < 0.01$; SOM: $\rho = -0.363$, $p < 0.01$; bulk density: $\rho = 0.423$, $p < 0.01$; total C: $\rho = -0.349$, $p < 0.01$; total N: $\rho = -0.425$, $p < 0.01$), *sul*2 (sand: $\rho = 0.614$, $p < 0.01$; silt: $\rho = -0.519$, $p < 0.01$; SOM: $\rho = -0.547$, $p < 0.01$; bulk density: $\rho = 0.519$, $p < 0.01$; total C: $\rho = -0.556$, $p < 0.01$; total N: $\rho = -0.630$, $p < 0.01$), and *tet*W (sand: $\rho = 0.490$, $p < 0.01$; silt: $\rho = 0.456$, $p < 0.01$; SOM: $\rho = -0.572$, $p < 0.01$; bulk density: $\rho = 0.393$, $p < 0.01$; total C: $\rho = -0.553$, $p < 0.01$; total N: $\rho = -0.649$, $p < 0.01$). Relative abundance of *intI*1 also showed significant correlations with the percentage of silt ($\rho = -0.331$, $p < 0.01$), moisture ($\rho = 0.262$, $p < 0.01$), pH ($\rho = 0.366$, $p < 0.01$), and the phosphorous concentration ($\rho = 0.377$, $p < 0.01$). Both the percentage of clay and CEC also had significant negative correlations with relative gene abundances of *sul*2 (clay: $\rho = -0.452$,

$p < 0.01$; CEC: $\rho = -0.510$, $p < 0.01$) and *tet*W (clay: $\rho = -0.292$, $p < 0.05$; CEC: $\rho = 0.487$, $p < 0.01$), respectively.

These results were partially in agreement with previous investigation in Antarctic soils where soil texture influences the relative abundance of ARGs (Wang et al., 2016). Moreover, surface sediment near mariculture and in Dongjiang River basin from two Chinese studies indicated that nutrients explained certain variation of ARGs and some ARGs showed certain correlations with total organic carbon and total N (Su et al., 2014; Zhou et al., 2018). Mexican soils frequently irrigated with wastewater also showed significant correlations between ARG levels and sulfur and phosphorus concentrations (Jechalke et al., 2015). While most ARGs in Mexican soils were positively correlated with total organic carbon, no significant correlations were found between soil pH and ARGs. Although there are discrepancies of correlation results between previous reports and current study, soil properties must also be considered when comprehensively examining co-selective effect of heavy metal on ARG prevalence.

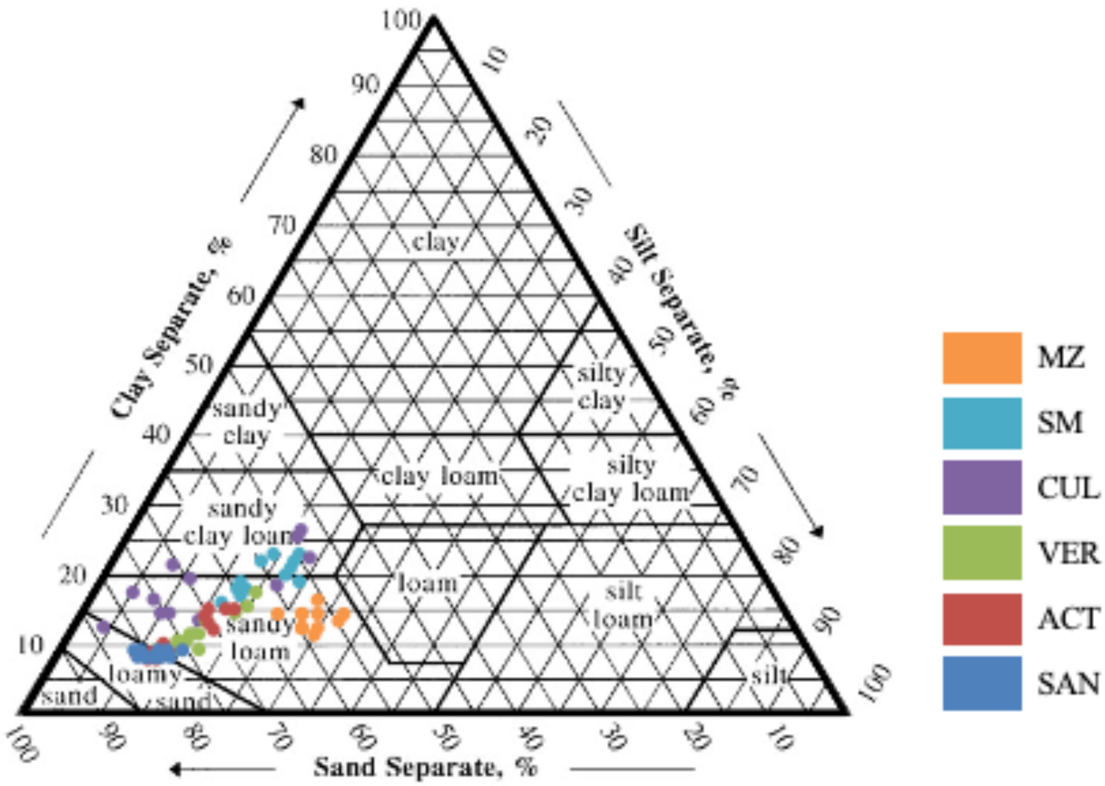

Figure 16. Soil texture of 72 biofilter samples collected from 6 biofilter sites in three UC campuses (MZ = orange, SM = blue green, CUL = purple, VER = green, ACT = red, SAN = blue). Abbreviation, MZ, Manzanita; SM, Sierra Madre; CUL, Culver; VER, Verano; ACT, Altman Clinical and Translational Research Institute; SAN, Sanford.

Table 12. Soil properties in biofilter samples collected from UCSB, UCI, and UCSD (N = 72). N.A. indicates insufficient amount of soil for triplicate subsamples to be processed. Abbreviations: SOM, Soil Organic Matter; CEC, Cation Exchange Capacity; C, Carbon; N, Nitrogen.

	Sand (%)	Silt (%)	Clay (%)	Moisture (%)	SOM (%)	Bulk Density (g/cm^3)	pH (-)	CEC (meq/100g)	Total C (%)	Total N (%)	PO_4 (ppm)	NO_3 (ppm)	NH_4 (ppm)
Mean	69	17	14	17.2	4.40	0.708	7.94	11.2	1.1	0.081	9.57	12.9	1.88
Median	71	16	13	15.8	3.80	1.07	7.93	11.9	0.73	0.039	4.99	5.71	1.47
SD	9.4	6.7	4.8	7.52	2.81	0.651	0.545	9.60	1.3	0.10	14.6	23.8	1.42
Kurtosis	-1.4	-0.45	-0.39	0.144	3.60	-1.87	-0.392	-0.406	2.8	2.8	10.1	20.6	6.01
Skewness	-0.18	0.50	0.69	0.869	1.70	-0.0985	-0.0756	0.393	1.5	1.6	3.14	4.32	2.12
min	53	4.0	8.0	5.77	1.22	1.00	6.59	7.70	0.13	0.020	1.18	1.23	0.289
Max	84	32	26	39.5	15.5	1.61	9.05	36.8	6.2	0.43	77.4	150	7.65

Table 13. Spearman's correlation between gene abundances and soil properties within biofilters. Significance levels at $p < 0.01$ and $p < 0.05$ are marked as ** and *, respectively. Abbreviations: SOM, Soil Organic Matter; CEC, Cation Exchange Capacity; C, Carbon; N, Nitrogen.

	Sand (%)	Silt (%)	Clay (%)	Moisture (%)	SOM (%)	Bulk Density (g/cm^3)	pH (-)
*intI*1/16S	0.152	-0.331**	0.209	0.262*	-0.160	0.291	0.366**
*sul*1/16S	0.398**	-0.550**	0.010	-0.035	-0.363**	0.423**	0.325**
*sul*2/16S	0.614**	-0.519**	-0.452**	-0.211	-0.547**	0.519**	-0.051
*tet*A/16S	-0.020	-0.048	0.051	0.258*	-0.125	0.103	0.074
*tet*W/16S	0.490**	-0.456**	-0.292*	-0.033	-0.572**	0.393*	0.062

	CEC (meq/100g)	Total C (%)	Total N (%)	PO_4 (ppm)	NO_3 (ppm)	NH_4 (ppm)
*intI*1/16S	0.194	-0.036	-0.108	0.377**	-0.177	-0.214
*sul*1/16S	-0.185	-0.349*	-0.425**	0.174	-0.33**	-0.111
*sul*2/16S	-0.510**	-0.556**	-0.630**	-0.141	-0.332**	-0.074
*tet*A/16S	-0.079	-0.273	-0.252	0.156	-0.128	-0.377**
*tet*W/16S	-0.487**	-0.553**	-0.649**	-0.124	-0.376**	-0.240*

4.3.3 Bioavailable and total metal(loid)s of campus biofilters in Southern California

Biofilter samples were also determined for bioavailable and total metal(loid) concentrations (As, Cr, Cd, Cu, Ni, Pb, Se, V, and Zn) as shown in Table 14. While a wide range of total metal(loid) concentrations were detected in campus soils, most values were lower as compared to naturally occurring background levels from surface soils (0–5 cm) in the Unites States (Smith et al., 2013) with several exceptions. High total As (40.9 mg/kg), total Cr (96.8 mg/kg), and total Ni levels (83.5 mg/kg) were found at several sites, but they were within the screening levels for soil metal(loid)s in residential areas recommended by California Department of Toxic Substance Control (Cal DTSC, 2020). However, based on the screening concentrations for arsenic recommended by the DTSC (0.41 ppm or mg/kg), all soil samples from six biofilter sites were found to be higher than 0.41 ppm.

Assessment of temporal variations of metal(loid)s in biofilters is also of great concern. Soils represent a major sink for metal(loid)s, which tend to accumulate over time (Chen et al., 2005). Temporal changes of heavy metal pollution in urban stormwater runoff have been identified extensively (Li et al., 2012). Meanwhile, stormwater runoff has been demonstrated to contain elevated levels of heavy metals as a result of runoff from impervious urban surfaces and building roofs (Li et al., 2012). It is likely that stormwater runoff may contribute to the elevated levels of heavy metals in biofilters. Yet, little research has been devoted to investigating seasonal variations in heavy metals and their co-selective effects on AR in urban biofilters. These variations are especially relevant to rain events in which substances, such as heavy metals, are transported to biofilters through stormwater runoff (Figure 17). In this study, the levels of heavy metals in biofilters varied over the course of time (Figure 18). Total heavy metals in biofilters were observed to be the highest in winter (February/March) among all sampling periods for As, Cr, Cu, Ni, Se, V, and Zn, which correlated with the winter rainy season. Moreover, levels of Pb in winter appeared to be the lowest among those in other time points. There was no temporal difference for Cd.

In addition to temporal patterns of metal(loid)s in biofilters, variation of metal(loid) concentrations in biofilters among campuses were also observed (Figure 18). For example, while UCSD exhibited the highest As, Pb, Se, and V values in biofilters, UCI had the highest Cd, Cu, Cr, Ni, and Zn concentrations. The levels of metal(loid)s in biofilters changed both spatially and temporally, which impacted the heavy metal driven co-selection.

Table 14. Bioavailable and total metal(loid) concentrations (mg/kg dry soil) in biofilter samples collected from UCSB, UCI, and UCSD (N = 72). Metal(loid)s below the detection limit (BDL) were designated a value of half the BDL for further analysis. Abbreviations: BDL, Below Detection Limit; N.A., Not Available.

	Bioavailable metal(loid) concentration (mg/kg dry soil)									Total metal(loid) concentration (mg/kg dry soil)								
	Pb	Cd	Se	As	Zn	Cu	Ni	Cr	V	Pb	Cd	Se	As	Zn	Cu	Ni	Cr	V
Mean	0.418	0.0246	0.0326	0.332	0.154	0.127	1.11	0.47	4.63	6.06	0.328	0.335	5.72	52.5	17.5	15.0	17.3	31.1
Median	0.0156	BDL	BDL	0.23	BDL	0.08	BDL	0.06	2.26	5.71	0.173	0.283	4.68	48.6	14.1	12.6	12.7	20.5
SD	0.892	0.0670	0.0635	0.430	0.369	0.217	1.81	1.10	11.1	7.10	0.655	0.358	5.45	42.4	14.9	11.8	16.9	29.3
Kurtosis	3.05	23.71	4.75	23.6	14.2	20.1	0.134	4.53	31.6	7.06	40.1	0.0205	4.46	1.35	3.09	15.6	8.82	3.56
Skewness	2.15	4.60	2.32	4.27	3.54	3.87	1.29	2.46	5.32	2.04	5.72	0.896	1.89	0.879	1.39	3.14	2.70	1.87
min	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL	0.37	BDL	0.238	2.01	0.45	2.64
Max	2.82	0.438	0.281	3.05	2.08	1.46	5.54	3.85	79.5	40.9	5.09	1.30	28.8	207	78.8	83.5	96.8	150
Screening levels for soil heavy metals in residential areas (DTSC HERO, 2020)										80.0	71.0	N.A.	0.41	N.A.	N.A.	820	230	N.A.
Background values of heavy metals in soil in United States (Smith et al., 2013)										25.8	0.3	0.3	6.4	66	17.9	17.7	36	60

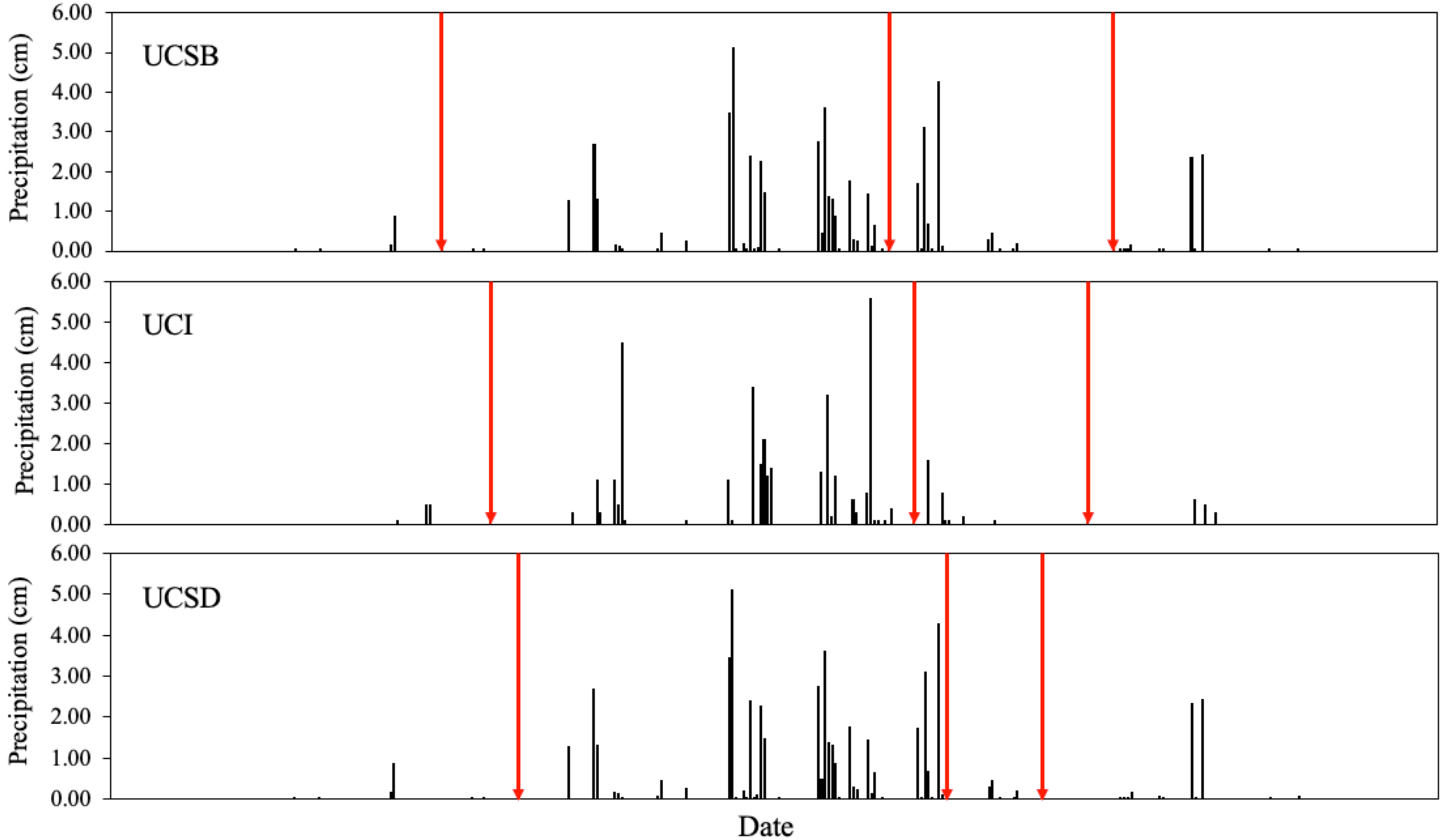

Figure 17. Daily rainfall data reported in areas near each campus. Red lines indicated the dates of sampling.

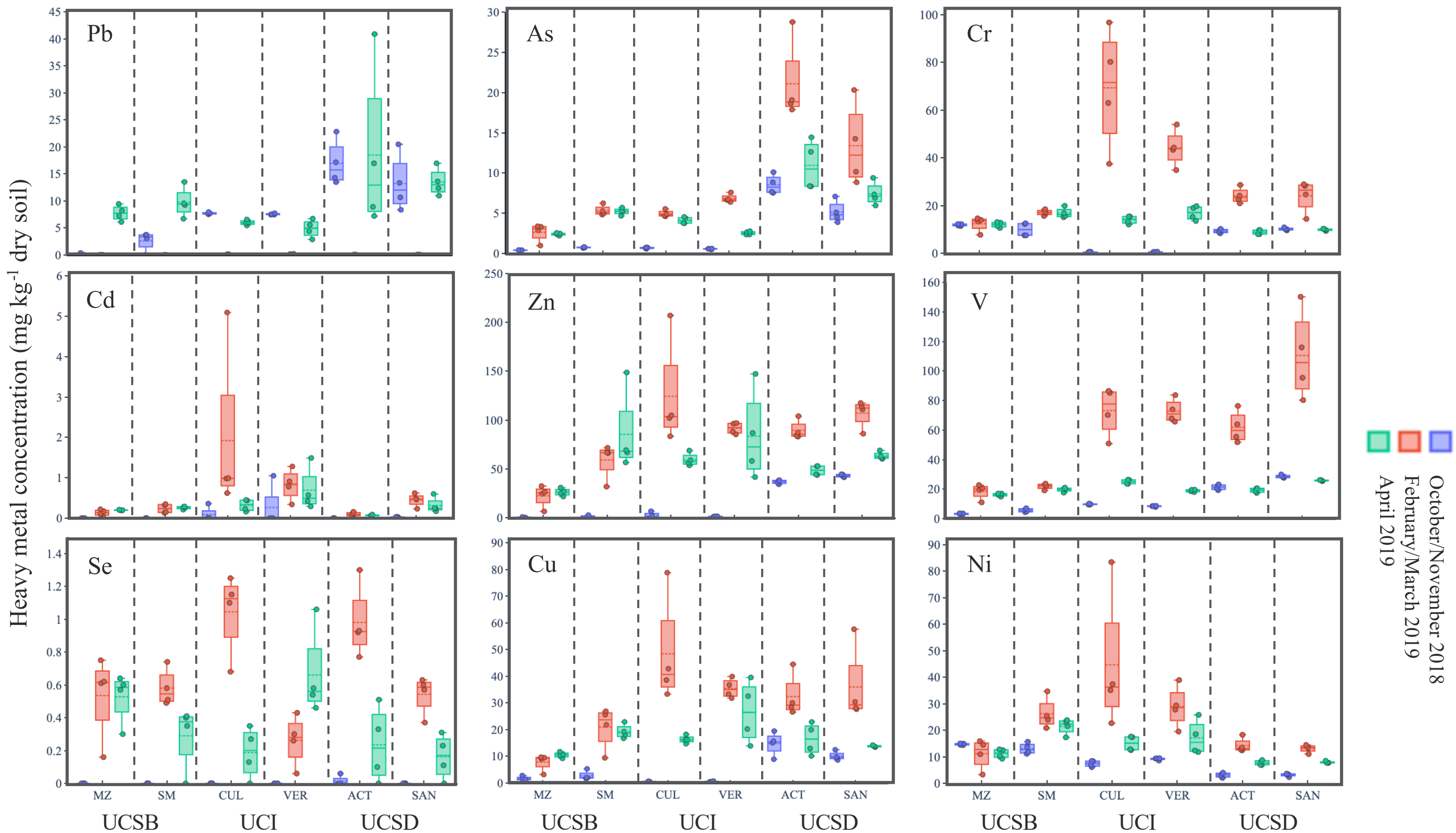

Figure 18. Temporal variation of nine metal(loid) concentrations, including arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), selenium (Se), vanadium (V), and zinc (Zn) in six biofilter sites. Time points included October/November 2018 (blue), February/March 2019 (red), and April 2019 (green). Abbreviations: MZ, Manzanita; SM, Sierra Madre; CUL, Culver; VER, Verano; ACT, Altman Clinical and Translational Research Institute; SAN, Sanford.

4.3.3 Correlations between bioavailable metal(loid)s, total metal(loid)s, and ARGs

To find out whether bioavailable and total heavy metals impacted the levels of ARGs, Spearman's correlations between total and bioavailable heavy metals and ARGs in biofilters are shown in Figure 19. Many significant correlations were found between ARGs and total heavy metal levels in biofilters. Total metal(loid)s of As, Pb, V, and Zn exhibited significant correlations individually with selected ARGs: *sul*1/16S, *sul*2/16S, and *tet*W/16S. Additional correlations were also detected between *intI*1/16S and total As concentrations ($\rho = 0.32$, adjusted $p < 0.05$) and total Pb concentrations ($\rho = 0.31$, adjusted $p < 0.05$). Yet, *tet*A/16S showed significantly negative correlations with total concentrations of Cd, Se, Zn, Cu, Ni, and Cr. While most heavy metals showed positive correlations with gene abundances normalized to cell population, negative correlations of heavy metals with gene abundances were still found.

Many previous studies have found significant correlations between ARGs and total heavy metals in soils. Mn, Cu, Zn, Cd, Ni, and Pb have previously been found to be correlated with *sul*1/16S and *tet*W/16S (Knapp et al., 2017, 2011; Zhang et al., 2018a). Moreover, the mobile genetic element *intI*1 was shown to be positively related with Cu and Zn content (Zhang et al., 2018). Overall, previous works were mainly consistent with the results reported in this study. However, Ji et al. (2012) demonstrated no significant correlations between heavy metals and *sul*1/16S and *tet*W/16S in manure and agricultural soils. This inconsistency between correlation results may originate from the fact that most studies investigated environments with elevated levels of pollution.

It is also suggested that metals were more likely to influence ARGs as groups in complex soil environments since many heavy metals were inter-correlated. Many strong and positive correlations were found among all total heavy metals except for Pb–Se, Pb–Ni, and Pb–Cr, all of which showed negative relationships. One possibility is that Pb is one of the most toxic elements to cells and serves as a limited role in cellular function (Seiler and Berendonk, 2012). Total heavy metals have been clustered into four major groups as shown in HCA in Figure 20a: (1) Zn, Cu, and V; (2) Ni, Cr, and Cd; (3) Se; (4) As; (5) Pb. Metals including Zn, Cu, Cr, and Ni serve as micronutrients in various physiological functions of cells. However, metal(loid)s are not equally toxic to bacteria. The three outliers, As, Se, and Pb are fused in rather far away at much higher distance as they have significantly reduced relevance as microelements. To sum up, soil environments with lower metal(loid)s, like biofilters, exhibit inter-correlated patterns of total heavy metal that can drive co-selection of AR.

Similarly, many bioavailable metal(loid)s had significant relationships with ARGs. In particular, bioavailable Zn was significantly correlated with relative abundance of *intI*1 genes ($\rho = 0.39$, adjusted $p < 0.05$), *sul*1 genes ($\rho = 0.33$, adjusted $p < 0.01$), and *tet*A genes ($\rho = 0.39$, adjusted $p < 0.05$). Levels of bioavailable As were also found to be significantly associated with *sul*1/16S ($\rho = 0.30$, adjusted $p < 0.01$) and *sul*2/16S ($\rho = 0.54$, adjusted $p < 0.001$) Bioavailable Ni concentrations, however, were negatively correlated with *sul*2/16S ($\rho = -0.43$, adjusted $p < 0.01$). Overall, most correlation coefficients between bioavailable metal(loid)s and ARGs appeared to be weak. HCA also reveals that most bioavailable heavy metals did not fall into any groups (Figure 20b). Unlike total heavy metals, limited studies have reported the correlations between bioavailable heavy metals. Bioavailable heavy metals, rather than total heavy metals, may play a more important role for the microbial communities since they may be able to penetrate cytoplasmic membrane and trigger metal resistance (Roosa et al., 2014). Yet, bioavailability of metal(loid)s depend on multiple factors, such as the origin and nature of heavy metals, soil physicochemical processes, and soil microbial species (Olaniran et al., 2013).

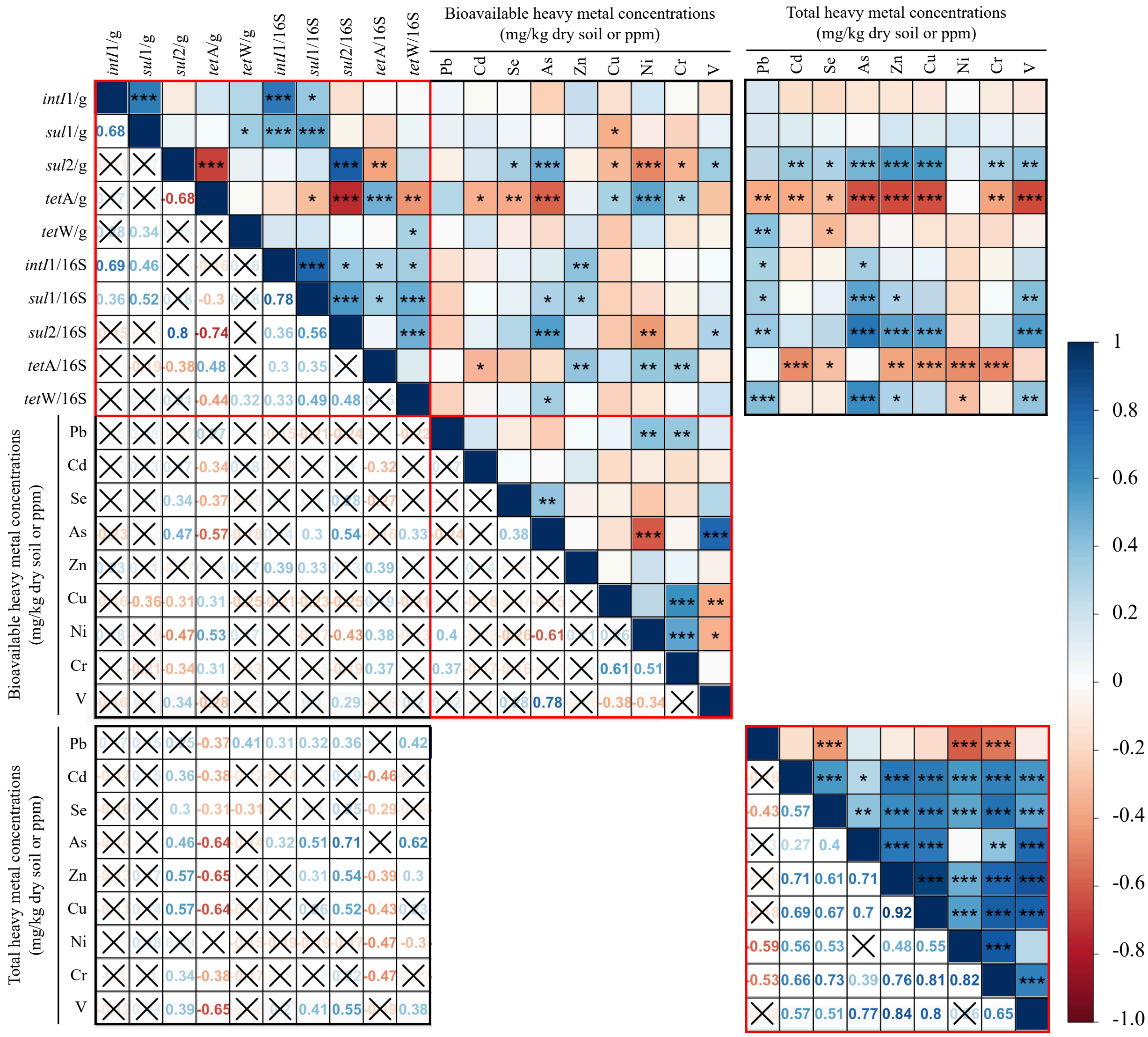

Figure 19. Spearman's correlation coefficients for combination of antibiotic resistance genes (ARGs), mobile genetic elements (MGEs), bioavailable metal(loid)s, and total metal(loid)s (Positive = blue, negative = red, X = not significant). Correlations significant at $p < 0.001$, $p < 0.01$, and $p < 0.05$ are marked with ***, **, and *, respectively.

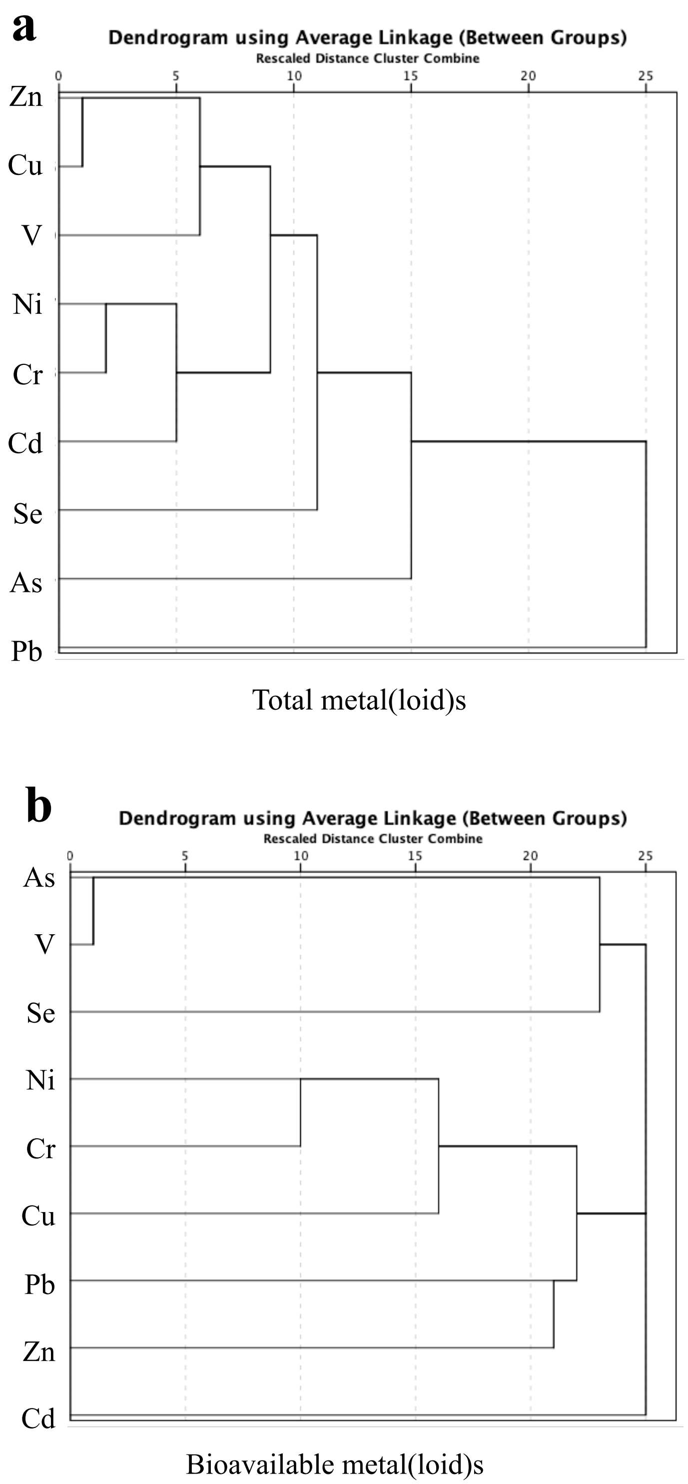

Figure 20. Dendrogram of HCA for (a) total metal(loid)s and (b) bioavailable metal(loid)s.

4.3.4 Linking ARGs to levels of heavy metals

Issues of particular interest and complexity are the persistence of ARGs and their synergistic effect that heavy metals can pose on the proliferation of AR. Heavy-metal driven co-selection on AR that occurs in soil environments is still not completely understood. Sulfonamides and Tetracyclines are both commonly used in productive animals. Therefore, sulfonamide and tetracycline resistance genes are often found in agricultural settings (Zhou et al., 2017). Recent studies that showed significant correlations between ARGs and heavy metals in soils are summarized in Table 15. These soil samples originated from many places, including those in sludge, feedlots, fertilizers, urban parks, and natural environments. Although correlations do not necessarily represent causation, these findings suggest that heavy metals are likely to exert selective pressure on the emergence of ARGs, with no exception in those containing relatively low levels of heavy metals. For instance, a Scottish study showed that six out of eleven ARGs have significant correlations with soil heavy metal levels in early antibiotic era from the 1940s to the 1970s (Knapp et al., 2011).

As mentioned earlier in this paper, levels of heavy metals varied greatly in soil environments. Thus, the concept of minimum co-selective concentrations (MCC) was first adopted and evaluated in many environmental compartments by Seiler and Berendonk (2012). Metals exceeding their MCCs in the environment were likely co-selective for ARGs and provided valuable data for risk assessment purposes. Although MCCs were not available for biofilter settings, most Zn and Cu measurements in the present study were found to reach levels higher than their MCCs in the different soil environments. Furthermore, it is also suggested that the MCCs (As, Cu, Ni, Pb, and Zn) within biofilters are lower than MCCs reported in other soil environments. Attention should be drawn to those soil environments occasionally impacted by surface runoff, such as biofilters, as stormwater might additionally carry metals to soils. Heavy metals in biofilters are more likely to exceed their respective MCC levels, which might facilitate co-selection of AR (Seiler and Berendonk, 2012).

Table 15. Correlations between ARGs and heavy metals in soils reported by previous studies (Cui et al., 2016; He et al., 2014; Ji et al., 2012; Knapp et al., 2017, 2011; Zhang et al., 2018b) Bordered genes indicate correlations are overlapped with present study. Abbreviation: Ct, Count.

Element	List of ARGs significantly correlated with the element			
	Absolute gene abundances (per gram)		Relative gene abundances (per 16S)	
	Ct.	Type	Ct.	Type
Total heavy metals				
aluminum (Al)	6	*bla*$_{TEM}$, *bla*$_{OXA}$, *tet*M, *tet*W, *sul2*, *sul*3	1	*bla*$_{TEM}$
arsenic (As)	1	*bla*$_{SHV}$	11	*bla*$_{SHV}$, *tet*BP, *fex*A, *fex*B, *cfr*, $\boxed{sul1}$, $\boxed{intI1}$, *tet*H, *tet*O, *tetQ*, $\boxed{tetW}$
Cadmium (Cd)	1	*bla*$_{OXA}$	0	
chromium (Cr)	1	*tet*T	5	*bla*$_{CTX}$, *bla*$_{OXA}$, *tet*M, *tet*O, *tet*S
cobalt (Co)	0		1	*tet*M
copper (Cu)	5	*bla*$_{TEM}$, *bla*$_{OXA}$, *tet*M, *tet*T, *dfr*A12(-)	20	*tet*M, *tet*W, *bla*$_{OXA}$, *erm*B, *erm*F, *sul*A, *sul*3, *tet*A, *tet*B, *tet*Q, *tet*X, $\boxed{sul1}$, $\boxed{sul2}$, *cfr*, *fex*A, *fex*B, *cfr*, *int*I1, *tet*O, *tet*S
mercury (Hg)	1	*tet*2	2	*tet*2 (-), *sul*A
manganese (Mn)	8	*bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{OXA}$, *tet*4, *tet*M, *tet*W, *sul*1, *sul2*	9	*bla*$_{TEM}$, *tet*2 (-), *fex*A, *fex*B, *cfr*, *sul*1, *tet*O, *tet*S, *tet*W
nickel (Ni)	3	*bla*$_{TEM}$, *bla*$_{OXA}$, *tet*T	3	*bla*$_{SHV}$, *tet*2 (-), $\boxed{tetW}$
lead (Pb)	4	*bla*$_{OXA}$, *tet*2 (-), *dfr*A12 (-), *erm*ATR	3	*bla*$_{TEM}$, *bla*$_{OXA}$, *tet*2 (-)
selenium (Se)	0		1	*tet*3
strontium (Sr)	0		10	*fex*A, *fex*B, *cfr*, *sul*1, *int*I1, *tet*O, *tetQ*, *tetS*, *tet*W, *tet*T
uranium (U)	2	*bla*$_{TEM}$, *bla*$_{CTX}$	3	*bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{SHV}$
vanadium (V)	8	*bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{OXA}$, *tet*2 (-), *tet*4, *tet*W, *sul*1, *sul*2	1	*tet*2 (-)
zinc (Zn)	4	*bla*$_{TEM}$, *bla*$_{OXA}$, *tet*T, $\boxed{tetW}$	11	*sul*A, *sul*3, *tet*L, $\boxed{tetW}$, *tet*Q, $\boxed{sul1}$, $\boxed{sul2}$, *fex*A, *fex*B, *cfr*, $\boxed{intI1}$
Bioavailable heavy metals				
arsenic (As)	0		8	*tet*A, *tet*L, *tet*M, *tet*W, *tet*Q, $\boxed{sul1}$, *cfr*, *fex*A
copper (Cu)	0		2	*tet*A, *flo*R
iron (Fe)	0		1	*tet*M
zinc (Zn)	0		10	$\boxed{intI1}$, *tet*B, *tet*M, *tet*Q, *tet*X, $\boxed{sul1}$, *sul*2, *cfr*, *cml*A, *flo*R

4.3.5 The influence of soil properties and metal contents on antibiotic resistance

Although significant correlations between metal(loid)s and ARGs existed, Spearman's correlation coefficients (ρ) were weak in general. It may be necessary to consider all potential factors contributing to AR due to the complexity of soil environments (Knapp et al., 2011). In this study, multiple linear regression was performed to identify the multiple effects of soil properties, bioavailable metal(loid)s, and total metal(loid)s on ARGs and MGE in biofilters (Table 16). Only variables had significant spearman correlations with gene abundances previously were considered in this analysis. We used stepwise regression analysis to estimate relative gene abundances, which had strong significant relationships (Figure 21): (1) *intI*1/16S ($R^2_{adj} = 0.329$, $p < 0.01$), (2) *sul*1/16S ($R^2_{adj} = 0.670$, $p < 0.01$), (3) *sul*2/16S ($R^2_{adj} = 0.584$, $p < 0.01$), (4) *tet*A/16S ($R^2_{adj} = 0.228$, $p < 0.01$), and (5) *tet*W/16S ($R^2_{adj} = 0.554$, $p < 0.01$). Relative *intI*1 genes had no obvious improvement of regression coefficients for both bioavailable and total heavy metal measurements, suggesting that multi-collinearity of metal(loid)s and therefore relative *intI*1 genes in biofilters were mainly governed by physiological soil traits. Compared to previous correlation results, inclusion of both physiological soil and heavy metal factors in multiple linear regression models caused a great improvement in the relationship with ARGs and MGEs. Variations of gene levels in biofilters could still be explained up to 67% by the model despite the low metal(loid) concentrations in this work. More importantly, other factors in addition to heavy metals including soil physio-chemical properties were shown to drive the selection of AR.

Table 16. Summary of multiple regression with a linear combination of bioavailable and total heavy metal concentrations and soil properties. A stepwise method was adopted. Several soil properties were not available and hence were excluded in the multiple linear regression analysis. All variables except for pH were log-transformed to ensure better normal distribution prior to multiple linear regression analysis.

Element	Equation	R^2	R^2_{adj}	p-value
*int*I1/16S	$Log_{10}(intI1) = –7.56 + 0.432(pH) – 1.858\ Log_{10}(Silt) + 1.186\ Log_{10}(Moisture)$	0.358	0.329	0.000001
*sul*1/16S	$Log_{10}(sul1) = –1.84 – 1.87\ Log_{10}(Silt) + 2.20\ Log_{10}(Tot\ As) + 0.198\ Log_{10}(Bio\ Pb) – 1.22\ Log_{10}(Tot\ V) + 0.896\ Log_{10}(SOM) – 0.155\ Log_{10}(Tot\ Zn)$	0.698	0.670	3.54×10^{-15}
*sul*2/16S	$Log_{10}(sul2) = –12.5+ 4.05\ Log_{10}(Sand) + 1.05\ Log_{10}(Tot\ As) – 0.225\ Log_{10}(Tot\ Se)$	0.601	0.584	3.37×10^{-13}
*tet*A/16S	$Log_{10}(tetA) = 6.01 – 0.710\ Log_{10}(NH_4) + 1.15\ Log_{10}(Moisture) – 0.435\ Log_{10}(Tot\ Ni)$	0.260	0.228	0.000125
*tet*W/16S	$Log_{10}(tetW) = –5.42 + 1.13\ Log_{10}(Total\ As) – 0.443\ Log_{10}(Total\ Cu) – 0.292\ Log_{10}(Total\ N) + 0.122\ Log_{10}(Bio\ Cu)$	0.579	0.554	5.43×10^{-12}

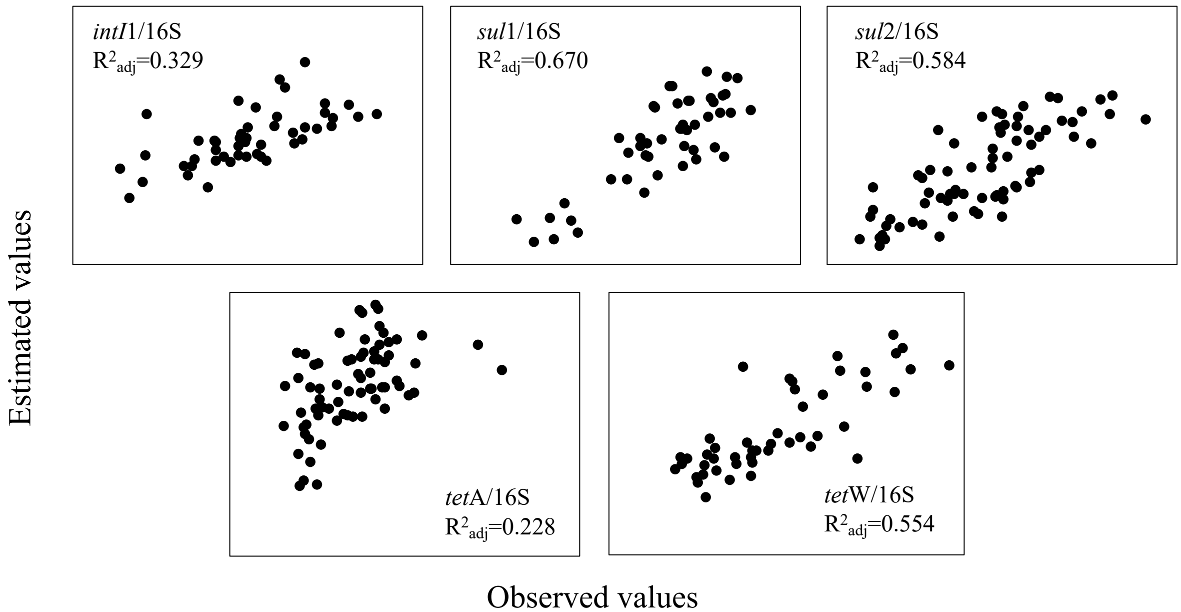

Figure 21. Multiple linear regression between observed and estimated values of relative abundances of *sul*1, *sul*2, *tet*A, *tet*W, and *intI*1 genes.

The association between heavy metals and AR has been identified in many environmental compartments for decades. Our results from this study indicate the co-occurrence of ARGs, heavy metals, and soil properties in urban biofilters over three time periods. However, long-term monitoring might be necessary since heavy metals are highly persistent and can pose long-term selective pressure on ARGs. Moreover, no information on characteristics of surface runoff in addition to the outflow from the biofilters to account for removal efficiencies of both ARGs and heavy metals exists. Indeed, knowing concentrations of heavy metals and ARGs in surface runoff into biofilters would indicate whether stormwater significantly contributes to ARG and heavy metal reservoirs. High removal efficiencies of heavy metals may explain the amount of leached heavy metals from surface soil. Yet, we still cannot preclude the possibility that elevated levels of heavy metals promote AR proliferation in biofilters. More efforts focusing on AR in urban landscapes, including metagenomics and linking ARGs with hosts, are required to provide more insights into this finding as they are currently not fully understood.

4.4 Conclusions

Despite the restricted use of some key antibiotics, other factors still contribute to the spread of AR and need to be understood. Heavy metals have been extensively proven to exert selective pressure on environmental microbes. However, the link between heavy metals and AR in urban biofilters still remains poorly assessed. Our results indicate that both bioavailable and total metal(loid)s had significant correlations with both ARGs and MGE. It is noteworthy that even trace levels of heavy metals may co-select for ARGs. Other factors that may also likely contribute to the prevalence of AR were also considered in order to create multiple linear regression models with respect to different genes. To our knowledge, this work proposed the first multiple linear regression model with the inclusion of soil properties and metal(loid)s for ARGs in soil environments. Strong and significant regression coefficients were identified, implying additional stressors may govern the selection of AR. These results from biofilters are of importance for management strategies of urban biofilters to mitigate the spread of AR.

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CHAPTER 5. TRACKING ANTIBIOTIC RESISTANCE GENES THROUGH THE ENVIRONMENT NEAR A BIOSOLID SPREADING GROUND: RESISTOME CHANGES, TRANSPORT, AND METAL CO-SELECTION

5.1 Introduction

The global increase in antibiotic resistance (AR) is one of our greatest current challenges. Our ability to effectively treat infections is hindered by the proliferation of antibiotic resistance genes (ARGs), which encode various mechanisms conferring drug resistance. Human exposure to these microbial contaminants can occur in many ways, resulting in difficult-to-treat diseases (Snary et al., 2004). Growing evidence has shown that our environmental resistome is significantly affected by human activities and is increasingly recognized as a critical ARG hot spot through antibiotic use in medicine and agriculture, and other anthropogenic pollution (Martinez, 2009). Waste streams from humans and animals produced by wastewater treatment plants (WWTPs) are known to carry elevated ARG levels (Sui et al., 2016). For instance, land application of biosolids may be one of important human activities creating selective environments for the emergence and proliferation of ARGs in the environment.

Biosolids undergo various types of treatment before agricultural application, including dewatering (DW), gravity thickening (GT), anaerobic digestion (AD), and lime stabilization (LS). Biosolids treated with the advanced methods had lower levels of tetracycline- and sulfonamide-resistant bacteria; however, while lime stabilized biosolids had consistently lower ARG levels, AD-treated biosolids did not have reliably lower ARG levels (Munir et al., 2011a). Two Chinese WWTPs also showed a significant increase of 23 ARGs through dewatered sludge (Mao et al., 2015). However, among municipal wastewater treatment plants in the United States, less than 4% of those employ AD (Ma et al., 2015), including three WWTPs investigated in this study. Burch et al. (2016) investigated the persistence of ARGs in waste solids at various AD temperatures. ARG and class 1 integron-integrase gene (*intI*1) levels decreased at all temperatures, with greater removal at higher temperatures. In many cases, this removal was of two orders of magnitude, yet ARG concentrations remaining in treated biosolids were still high. This is reasonable since treatment conditions can be conductive to horizontal gene transfer (HGT).

Multiple studies have reported individual ARGs in waste sludge greater than 10^9 copies/mL (Auerbach et al., 2007; Mao et al., 2015; Munir et al., 2011b) or than 10^{10} copies/mL (Burch et al., 2016). Mao et al. (2015) showed that even though the effluent concentration was relatively low for twelve ARGs, the total loading when both effluent and waste sludge were considered was higher than the loading in the influent, indicating proliferation throughout the treatment processes.

More than 4 million dry metric tons of biosolids are applied to land in the United States in 2019 (USEPA, 2020). Biosolids used for land application are currently regulated under 40 CFT Part 503 to ensure levels of metals and pathogens pose minimal risk to human health. After initial treatment to reduce odor, biosolids are categorized into "Class A" and "Class B" with specified treatment requirements for pollutants, pathogens, and vector attraction reduction. Class A biosolids are commonly produced by AD, especially under thermophilic digestion (55°C), and can be used without restriction (Mclain et al., 2017). They must also meet the limit of either less than 3 *Salmonella* per 4g total solids or 100 MPN fecal coliforms per gram total solids. However, class B biosolids do not eliminate pathogens (< 2 million MPN fecal coliform per g total solids) and are restricted by certain regulations when applied to land (Zaleski et al., 2005). While current regulations were established on conservative risk assessment of soil- and food-transmitted enteric pathogens, the ARG distribution, diversity, and transport in soil microbiomes treated with biosolids still remain largely unknown. Therefore, the risk of disseminating ARGs into agricultural fields and products has yet to be considered (Mclain et al., 2017).

Agricultural land application is one of the most common practices for recycling and reuse of nutrients in biosolids. More than 60% of biosolids from WWTPs are applied to land in the United States (USEPA, 2000). Anaerobically digested biosolids are known to contain elevated levels of ARGs, yet the effect of soil biosolids application on the resistome has received little attention. Multiple studies have documented elevated levels of ARGs and/or antibiotic-resistant bacteria (ARB) in soils after either manure or biosolid application. Tang et al. (2015) showed that, in general, additions of manure to paddy soils resulted in increased ARG levels, and ARG concentration decreased with soil depth. Several studies have applied metagenomic techniques to characterize the impacts on the soil resistome after land application of waste solids. The diversity and prevalence of ARGs were found to increase after the addition of waste solids (Chen et al., 2017; Yang et al., 2018). After biosolid application, increased availability of plasmids encoding for resistance genes was shown using a model recipient for HGT (Riber et al., 2014). Fahrenfeld et al. (2014) also found that in fields treated with waste solids, ARG levels were higher than that expected based on the mass balance, implicating HGT. However, several studies have reported no significant effect of biosolid application on ARGs and ARB, as they did not persist in the receiving soil systems (Brooks et al., 2007; Munir and Xagoraraki, 2011; Zerzghi et al., 2010). Soil microbiomes before land application of biosolids may already contain high ARG levels (Bondarczuk et al., 2016; Brooks et al., 2007). Moreover, the occurrence, concentration, and spatial distribution of soil ARGs were also highly associated with land uses (Zhao et al., 2020).

In this study, the environmental impact of biosolid land application was assessed by analysis of ARGs and ARB in soils and their surrounding areas. In addition, the influence of geographical factors on the spatial distribution of soil ARGs was also evaluated. The objectives of the present study were to (1) determine the occurrence of ARGs and ARB in biosolids from WWTPs; (2) assess levels of ARB and ARG in biosolid-amended soils and nearby soil environments; (3) compare levels of ARB and ARG to those in agricultural soils, park soils from remote areas (controls), and pristine soils from hiking trails; (4) investigate potential relationships between ARG levels and geographical factors; (5) determine the co-selective effects of heavy metals on ARGs.

5.2 Materials and Methods

5.2.1 WWTP sample collection

A total of five biosolid samples were collected from three different WWTPs in California. Two biosolids were sampled (after AD and dewatering) individually in two sampling events (October 2018 and July 2019) from WWTP 1. A majority of biosolids produced from WWTP 1 were applied in the study area. In parallel, anaerobically digested sludge (before dewatering) was also collected at WWTP 2 in July 2019 for comparison purposes. In WWTP 3, both waste sludge (before AD) and biosolids (after AD and dewatering) were collected for ARB enumeration. All sludge samples were stored in 1 L pre-sterilized polypropylene bottles stored individually on ice (4°C) before arrival at the laboratory. Wet sludge samples (before dewatering) were centrifuged at 5,000 g for 1 min, and the supernatant was discarded. Both dewatered sludge from WWTP 1 and the pellets derived from wet sludge from WWTP 1 were stored at -20 °C for DNA extraction.

5.2.2 Soil sample collection

A total of 91 soil samples were collected in December 2019 and November 2020 within 36 km^2 of the study area within the biosolid-applied site and their surrounding areas (Figure 22). A grid soil sampling covering 900 km^2 of the study area was used. The study area was divided into thirty-six continuous 25-km^2grid cells. At each grid on the map, soil samples were collected in the vicinity of the middle of each grid cell. Based on land use types, soil samples were collected from vacant lands, agricultural sites, and open space (i.e., parks and recreation areas). Once the sampling location has been determined, a 1 m^2 plot was randomly selected from the area. Surface soil (0−10cm) was collected in acid-washed glass screw-top jars by randomly selecting ten points in each plot, yielding a composite sample representative of the 1 m^2 plot. Rocks and grass were avoided or removed using sterilized plastic scoops. Soil samples were kept on ice (4°C) until transported to the laboratory prior to processing.

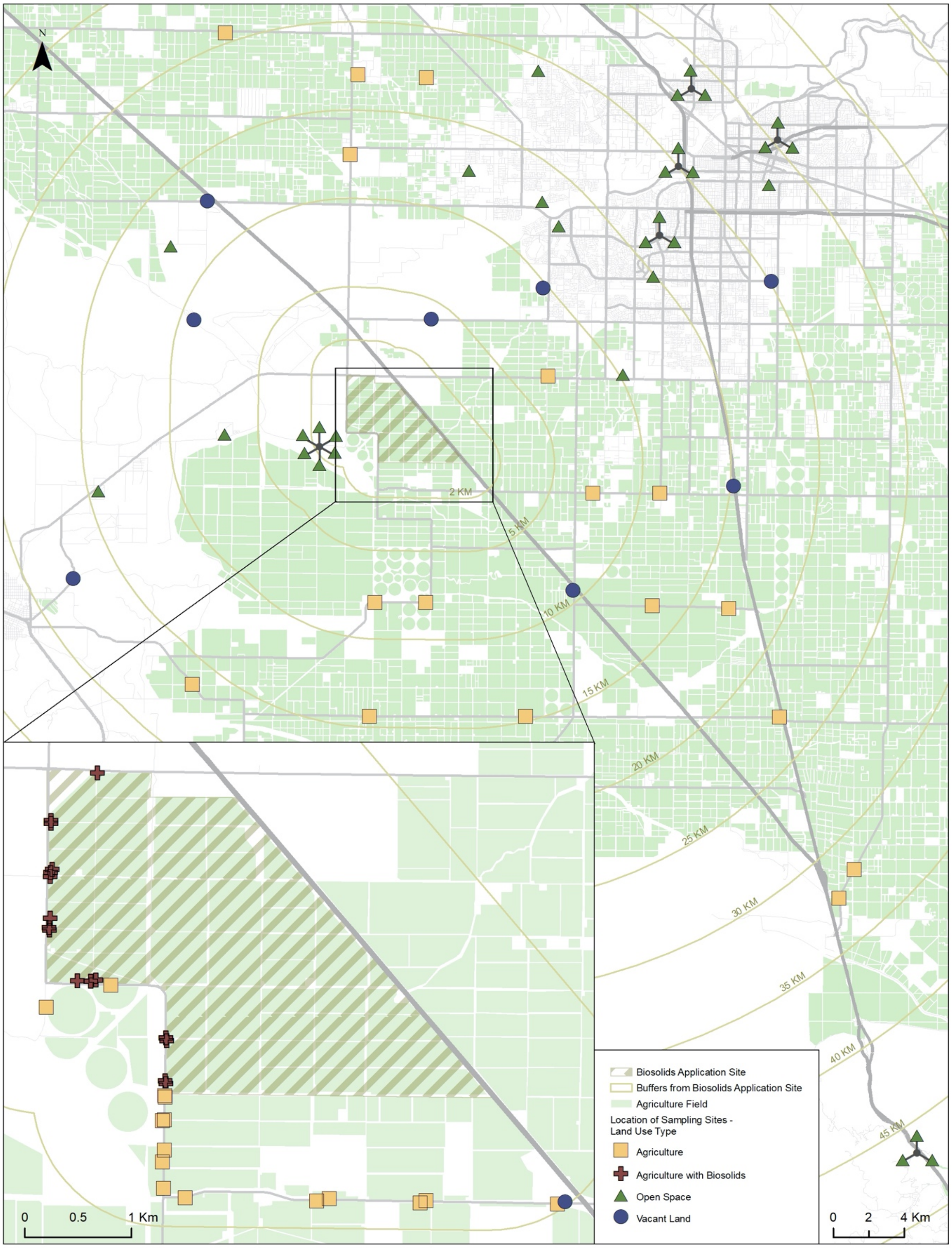

Figure 22. Map of the study area and sampling locations subject to different land uses.

5.2.3 DNA extraction and real-time qPCR of ARGs

Both soil and biosolid DNAs were extracted from all samples using DNeasy PowerSoil Kits (Qiagen, Valencia, CA, USA) following the manufacture's guidelines. The final DNA extracts were store at −20 °C for subsequent real-time qPCR analysis. In parallel, the purity and quantity of total DNA extracts were determined using UV absorption by a Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA). DNA extracts were considered relatively free of contamination if the A260/280 ratio was above 1.8, per manufacturer's instructions.

All samples were analyzed for ARGs (*erm*B, bla_{SHV}, *sul*1, *sul*2, *tet*A, and *tet*W), class 1 integron-integrase gene (*intI*1), and 16S rRNA (a proxy for total cells) gene abundances. Quantitative polymerase chain reaction (qPCR) was performed using PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) in the StepOne Plus qPCR system (Applied Biosystems). Primer concentrations (Table 17) and thermocycling conditions (

Table 18) were optimized as described in Echeverria-Palencia et al. (2017). DNA standards were designed using sequences from the National Center for Biotechnology Information (NCBI) database and obtained through Integrated DNA Technologies (IDT) (Coralville, IA, USA). Standard concentrations of the designed DNA fragments were analyzed alongside soil samples in triplicate. Soil DNA extracts were diluted to the levels where they had identical DNA concentrations to minimize the effects of qPCR inhibition.

Table 17. Primer sequences and concentrations of qPCR reactions. Abbreviations: F, Forward; R, Reverse.

Gene	Primer	Concentration (nM)	Sequence (5'-3')	Amplicon size (bp)	References
*sul*1	*sul*1-F	200	CGCACCGGAAACATCGCTGCAC	258	Pei et al., 2006
	*sul*1-R		TGAAGTTCCGCCGCAAGGCTCG		
*sul*2	*sul*2-F	200	CTCCGATGGAGGCCGGTAT	190	Luo et al., 2010
	*sul*2-R		GGGAATGCCATCTGCCTTGA		
*tet*A	*tet*A-F	200	GCTACATCCTGCTTGCCTTC	250	Ng et al., 2001
	*tet*A-R		CATAGATCGCCGTGAAGAGG		
*tet*W	*tet*W-F	200	GAGAGCCTGCTATATGCCAGC	210	Aminov et al., 2001
	*tet*W-R		GGGCGTATCCACAATGTTAAC		
*erm*B	*erm*B-F	200	AAAACTTACCCGCCATACCA	139	Echeverria-Palencia et al., 2017
	*erm*F-R		TTTGGCGTGTTTCATTGCTT		
bla$_{SHV}$	*bla*$_{SHV}$ -F	200	TGATTTATCTGCGGGATACG	215	Echeverria-Palencia et al., 2017
	bla$_{SHV}$ -R		TGATTTATCTGCGGGATACG		
*intI*1	*intI*1-F	200	GGCTTCGTGATGCCTGCTT	424	Luo et al., 2010
	*intI*1-R		CATTCCTGGCCGTGGTTCT		
16S rRNA	16S rRNA-F	500	ATGGCTGTCGTCAGCT	351	Pan et al., 2018
	16S rRNA-R		ACGGGCGGTGTGTAC		

Table 18. Thermocycling conditions of real-time quantitative PCR.

Gene	Holding		Denaturation		Annealing		Extension		R^2	Efficiency (%)
	Temp (°C)	Time (min)	Temp (°C)	Time (sec)	Temp (°C)	Time (sec)	Temp (°C)	Time (sec)		
*sul*1	95	10	95	15	65	30	72	30	0.998 – 1	88.8 – 92.4
*sul*2	95	10	95	30	60	30	72	30	0.993 – 1	85.2 – 90.4
*tet*A	95	4	95	5	55	30	72	30	0.990 – 1	85.5 – 91.8
*tet*W	95	4	95	30	60	30	72	30	0.991 – 1	91.6 – 100
*erm*B	95	10	94	20	60	60	-	-	0.996 – 1	91.0 – 97.5
bla_{SHV}	95	10	94	20	55	60	76	30	1	86.6 – 91.0
*intI*1	95	10	95	15	55	30	72	30	0.992 – 1	88.4 – 98.4
16S rRNA	94	4	94	40	60	45	72	60	0.997 – 1	87.6 – 95.5

5.2.4 Enumeration of ARB

Standard plating techniques were used to screen for antibiotic resistance levels on antibiotic-selective media in randomly selected soil samples. One gram (wet weight) of each soil sample was transferred individually into 9 mL of sterilized phosphate-buffered saline (PBS, pH = 7.4), mixed by vortexing for 5 s followed by shaking on a wrist action shaker (Model 95, Burrell Scientific, Pittsburgh, PA) for 40 mins at maximum speed. Soil samples were serially diluted with PBS (pH = 7.4) onto 25% Luria-Bertani growth media (LB Broth, Miller, Fisher BioReagents, Pittsburgh, PA) agar plates amended with tetracycline (20 ppm) (MP biomedicals, Santa Ana, CA), erythromycin (10 ppm) (MP biomedicals), ciprofloxacin (4 ppm) (MP biomedicals), and sulfamethoxazole (50.4 ppm) (MP biomedicals) to achieve the desired final concentrations (Gao et al., 2012). A dilution volume of 100 μL was spread on LB agar plates. Plates were incubated at 30°C for 3 days colonies were counted (Negreanu et al., 2012). For each assay, plate counts were carried out in duplicate and averaged with two different dilutions for each sample.

5.2.5 Determination of heavy metals in soils

All soil samples except for biosolid samples were screened with a Bruker S1 Titan portable X-ray fluorescence spectrometer (pXRF, Bruker, Kennewick, WA, USA) for metal(loid)s following EPA method 6200 and the instrumental protocol. In brief, soil samples were oven-dried (105°C) overnight, ground with a mortar, and passed through a 60-mesh sieve to achieve homogenized particle size. Soil samples and standard material (NIST 2710a–Montana I Soil) were loaded onto polyethylene sample cups (3 cm diameter) and covered with a 2.5 um Mylar film prior to pXRF analysis. The instrument contains an Rh tube (4 W, 15–50 keV, and 5–100 μA) and a silicon drift detector with a resolution < 145 eV. Each soil sample was scanned in triplicate in soil mode with each measurement performed for 60 s. Metal(loid) concentrations were automatically produced from the pXRF spectra according to the internal factory–installed calibration procedure. Mean heavy metal concentrations were obtained by averaging triplicate data from each scanned soil sample. Reference material was analyzed every twenty samples to calibrate the instrument readings as part of the quality control protocol.

5.2.6 Statistical analysis and geographical analyses

Site locations and their associated land uses were entered and digitalized into ArcMap Version 10.7 (ESRI, Redlands, CA, USA) to generate the sampling map surrounding the land application site. Statistical analysis and graphical outputs were performed by RStudio Version 1.3 (RStudio, Inc., Boston, MA, USA) and SPSS Version 23 (IBM Co., Armonk, NY, USA). Statistical comparisons of ARG levels in soils among land uses were performed using the Mann−Whitney U test. Spearman's correlation was utilized to identify correlations among ARGs and metal(loid) concentrations. The p-values were adjusted according to Benjamini–Hochberg method in consideration of false discovery rate (Benjamini and Hochberg, 1995). Both are nonparametric methods that do not assume a normal distribution. Significance was assigned at a p-value of < 0.05 was set to be significant. Metal(loid) concentrations below the detection limit (BDL) were manually designated a value of half the detection limits (Helsel and Gilloom, 1986) for further analysis.

5.3 Results and Discussion

5.3.1 Occurrence of ARGs in biosolids in WWTPs

Both ARGs and *intI*1 genes were quantified to characterize their abundances in biosolids. Gene abundances of *erm*B, *bla*$_{SHV}$, *sul*1, *sul*2, *tet*A, *tet*W, and *intI*1 normalized to the quantity of bacterial 16S rRNA genes (hereafter abbreviated as genes/16S) in biosolids (after dewatering) from WWTP 1 are shown in Figure 23. Biosolids were anaerobically digested under thermophilic conditions (55°C) in WWTP 1 and mesophilic conditions (35°C) in WWTP 2. The majority of genes were detected in sludge samples with the exclusion of *bla*$_{SHV}$ genes in WWTP 1. The mean relative abundances of *erm*B and *tet*A genes were the highest, averaging 1.77×10^{-1} ($\pm 1.52 \times 10^{-2}$) genes/16S in WWTP 1 and 4.10×10^{-1} ($\pm 4.81 \times 10^{-2}$) genes/16S in WWTP 2, respectively. The average relative abundances of the *bla*$_{SHV}$ gene were the lowest in both WWTPs, with no detection in any biosolids in WWTP 1. The average relative abundances of sulfonamide resistance (*sul*1 and *sul*2), *intI*1, *tet*A, and *tet*W genes in WWTP 1 were of the same order of magnitude as those in WWTP 2. However, there was an order of magnitude difference between the average relative abundances of the *erm*B gene between WWTP 1 (1.77×10^{-1} genes/16S) and WWTP 2 (1.51×10^{-2} genes/16S).

Both thermophilic and mesophilic AD have been studied for ARG removal in treated biosolids and compared to the effectiveness of other conventional treatment methods (Munir and Xagoraraki, 2011; Sui et al., 2016). Overall findings suggested that thermophilic conditions were generally more favorable for ARG removals (Jang et al., 2017; Zhang et al., 2015). However, the results were inconsistent in comparing thermophilic and mesophilic conditions. Based on different locations and WWTP design, each ARG type may adapt differently to AD conditions (Jang et al., 2017). Although ARGs responses to AD conditions are highly variable, many ARGs in AD-treated biosolids in WWTPs become more abundant. Researchers found that biosolids had significantly higher ARG loads than those in the treated effluent (Munir et al., 2011b). In Michigan, the relative abundances of *sul*1 and *tet*W genes in anaerobically digested biosolids ranged from 10^{-4} to 10^{-3} and 10^{-4} to 10^{-3} genes/16S, respectively (Munir and Xagoraraki, 2011). Relative abundances of *intI*1, *sul*1 *sul*2, *tet*A, and *tet*W genes in Scottish biosolids ranged from 10^{-2} to 10^{-1}, 10^{-3} to 10^{-2}, 10^{-4} to 10^{-3}, 10^{-3} to 10^{-2}, and 10^{-3} to 10^{-2} genes/16S, respectively. Relative ARG abundances in the present study were at least an order of magnitude higher in anaerobically digested biosolids than in aforementioned studies that excluded *intI*1 and *tet*W. Consequently, the elevated relative abundances of ARGs in anaerobically digested biosolids are more likely to increase the burden of ARGs on soil bacteria via land application.

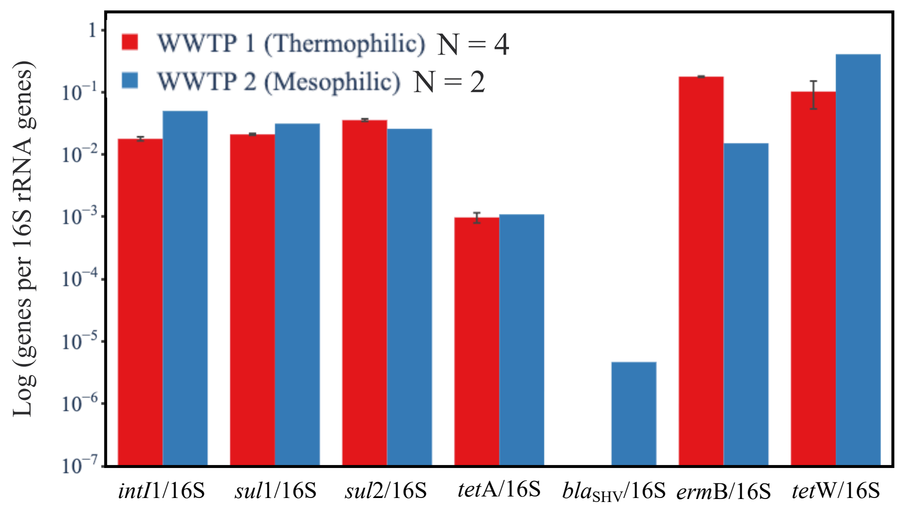

Figure 23. Mean relative abundances of seven targeted genes (*erm*B, bla_{SHV}, *sul*1, *sul*2, *tet*A, *tet*W, and *intI*1) in biosolids from WWTP 1 and WWTP 2. Error bars represent the standard deviation of their means.

5.3.2 Occurrence of ARGs in soils inside and outside the spreading ground

Soil samples from the spreading ground and sites representing different land-use types outside the spreading ground were extracted and analyzed for selected ARGs, MGE (*intI*1), and 16S rRNA (a surrogate of total bacteria). The numbers of gene copies were all normalized to 16S rRNA genes as relative gene abundances to account for the size of the microbial community (Ji et al., 2012). All selected genes were detected in soil samples except for the bla_{SHV} gene, which was not detected in most soil samples. In addition, bla_{SHV} genes within each land use type were mostly below the detection limits except for those in WWTP biosolid samples. The relative gene abundances of selected ARGs and *int*I*1* ranged from 10^{-7} to 10^{-1} genes per 16S rRNA gene copies, meaning *intI*1 and ARGs represented roughly 0.00001% to 10% of the total bacteria. The mean relative abundances of sulfonamide resistance genes (*sul*1 and *sul*2) were generally the highest across all land use types (Figure 24), followed by *intI*1, tetracycline resistance (*tet*W and *tet*A), *erm*B, and bla_{SHV} genes.

To further compare gene levels in different soil environments, log-transformed relative gene abundances in different soils are shown in Figure 24. Log-transformed gene abundances based on total mass inputs are also reported in Figure 25. The mean and median relative genes abundances in soils were generally 2–5 logs lower than those in biosolids except for the bla_{SHV} genes. The mean relative gene abundances of sulfonamide resistance genes (*sul*1 and *sul*2) and *intI*1 were the highest in soils of vacant lands, followed by biosolid-amended soils, agricultural soils (without biosolids), and park soils. A similar trend was also observed in the relative gene abundance of bla_{SHV}, yet most were below the detection limits. The trends of the mean relative gene abundances of *tet*A and *erm*B in different soils were as follows: agricultural soils (without biosolids) $\approx$ soils from vacant lands $\approx$ biosolid-amended soils > park soils. The following trend was observed for mean relative gene abundances of *tet*W in sites with different land uses: agricultural soils (without biosolids) > biosolid-amended soils $\approx$ soils from vacant lands > park soils.

In this study, even though soils before the land application of biosolids were not available, it may still be possible to evaluate the effects on spoil ARGs after land application of biosolids. Sulfonamide and tetracycline resistance genes were the most frequently detected ARGs in agricultural soils (Lin et al., 2019). In the present work, the mean relative gene abundances of *sul*1, *sul*2, and *intI*1 were significantly higher in biosolid-amended soils than in non-amended agricultural soils from the same area ($p < 0.05$). This additional introduction of ARGs from biosolids into the soil environments compared to adjacent soils is consistent with data from many previous studies (Lin et al., 2019; Munir and Xagoraraki, 2011; Tang et al., 2015). Furthermore, in Michigan, the average absolute abundances of *sul*1 and *tet*W genes in biosolid-amended soils were approximately 10^5 and 10^4 copies/g soil, respectively. Lin et al. (2019) in the United Kingdom assessed ARG and *intI*1 gene abundances in soils over time after land application of biosolids. In brief, relative gene abundances of *tet*A and *intI*1 ranged from 10^{-5} to 10^{-4} (gene copies/16S), while relative gene abundances of *tet*W, *sul*1, and *sul*2 fluctuated from 10^{-6} to 10^{-4} (gene copies/16S). These reported values (relative and absolute gene abundances in each area) were generally at least an order of magnitude lower than those reported in the current study.

It is evident that anaerobically digested biosolids contain elevated levels of ARGs, yet the effect of incorporating land-applied biosolids to soils on the resistome is still not clear. In this study, biosolid application appeared to favor the proliferation of certain ARGs in biosolid amended soils. A previous study reported substantial variability at two different sites after biosolids application in Michigan: one site showed significant increases of all absolute ARG abundances (*tet*W, *tet*O, and *sul*1), while the other site no significant increases of all absolute ARG abundances of these genes (Munir and Xagoraraki, 2011). Soil microbiomes without biosolids application may already contain high ARG abundances due to geographical differences and diverse ARG pools in the soil environment. In this case, the effects of ARGs addition related to biosolids application tended to be minimal, which is consistent with the data of Brooks et al. (2007) and Munir and Xagoraraki (2011).

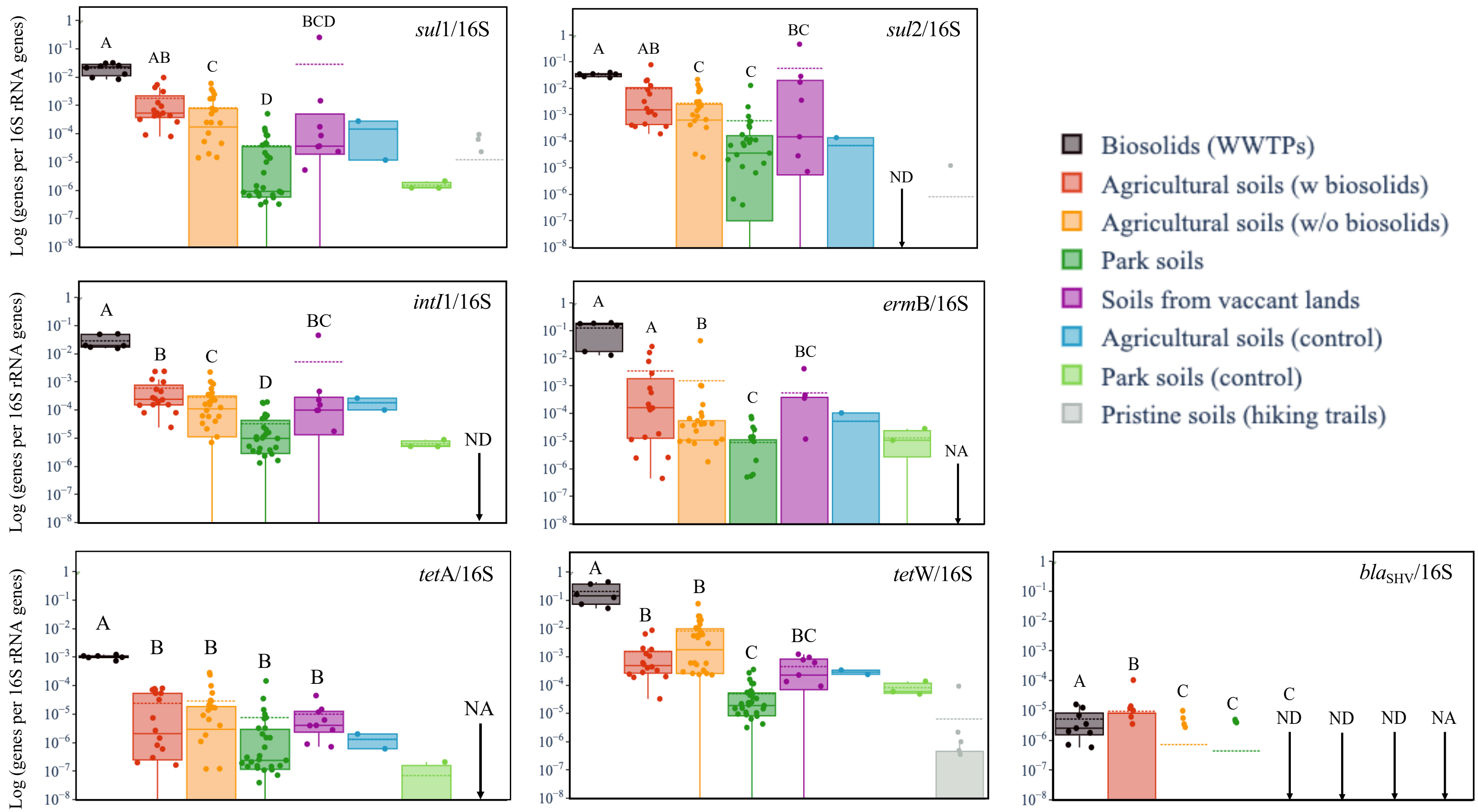

Figure 24. Relative ARG abundances by type of land use. Statistical comparisons of ARG levels in soils among land uses were performed using the Mann–Whitney U test. Boxes containing the same letters denote statistical differences (adjusted p value < 0.05). Abbreviations: ND, Not Detected; NA, Not Available.

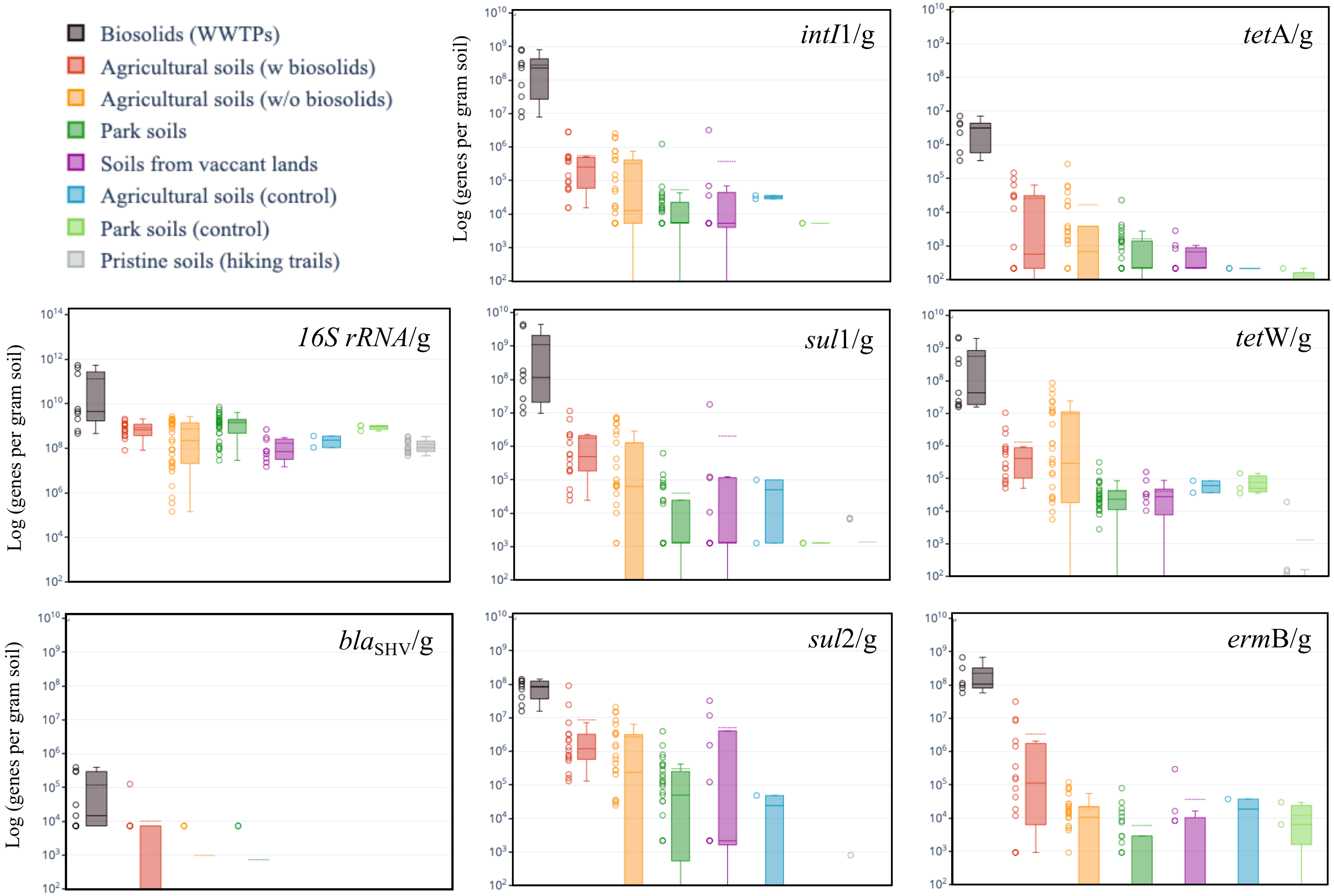

Figure 25. Absolute ARG abundances by type of land use. Abbreviations: ND, Not Detected.

5.3.3 ARG comparison with pristine and remote agricultural soils

Ranges of relative abundances of ARG and *intI*1 in collected soil samples from different land uses were compared to those found in control park soils and control agricultural soils (approximately 35 km from the biosolid applied site). These control park soils were collected from the state park (approximately 45 km from the biosolid applied site). A previous study assessing ARGs in pristine soils from the Santa Monica Mountains provided a baseline for ARG in soils with minimal human impacts (Cira et al., n.d.). Identical methods were used for gene detection in all soil samples, and results are shown in Figure 26 and Figure 27. From this analysis, all mean relative abundances of selected genes (*intI*1, *sul*1, *sul*2, and *tet*W) in pristine soils (hiking trails)were significantly lower than those detected in soils of vacant lands, biosolid-amended soils, agricultural soils (without biosolids), and park soils ($p < 0.05$). Although large differences in ARGs existed between pristine and other soil types, some agricultural soils were found to contain even less ARGs than those in control agricultural soils, meaning control agricultural soils may already have greater diversity and abundance of ARGs than agricultural soils. This finding is consistent with results from a Nebraskan study in which tetracycline efflux genes were more frequently detected in pristine soils (Cadena et al., 2018).

ARG and *intI*1 quantities in remote agricultural soils were also included for comparison as farming may contain similar AR anthropogenic inputs. Surprisingly, relative abundances of all selected genes in remote agricultural soils were not statistically different from those in biosolid-amended soils and agricultural soils without biosolids. These insignificant differences imply that agricultural soils still contained certain levels of ARGs due to human activities. Specifically, relative gene abundances of *sul*1 and *intI*1 genes were still high in remote agricultural soils. These findings are consistent with results from previous studies as *sul*1 and *intI*1 have often been considered proxies for anthropogenic pollution (Gillings et al., 2015; Pruden et al., 2006).

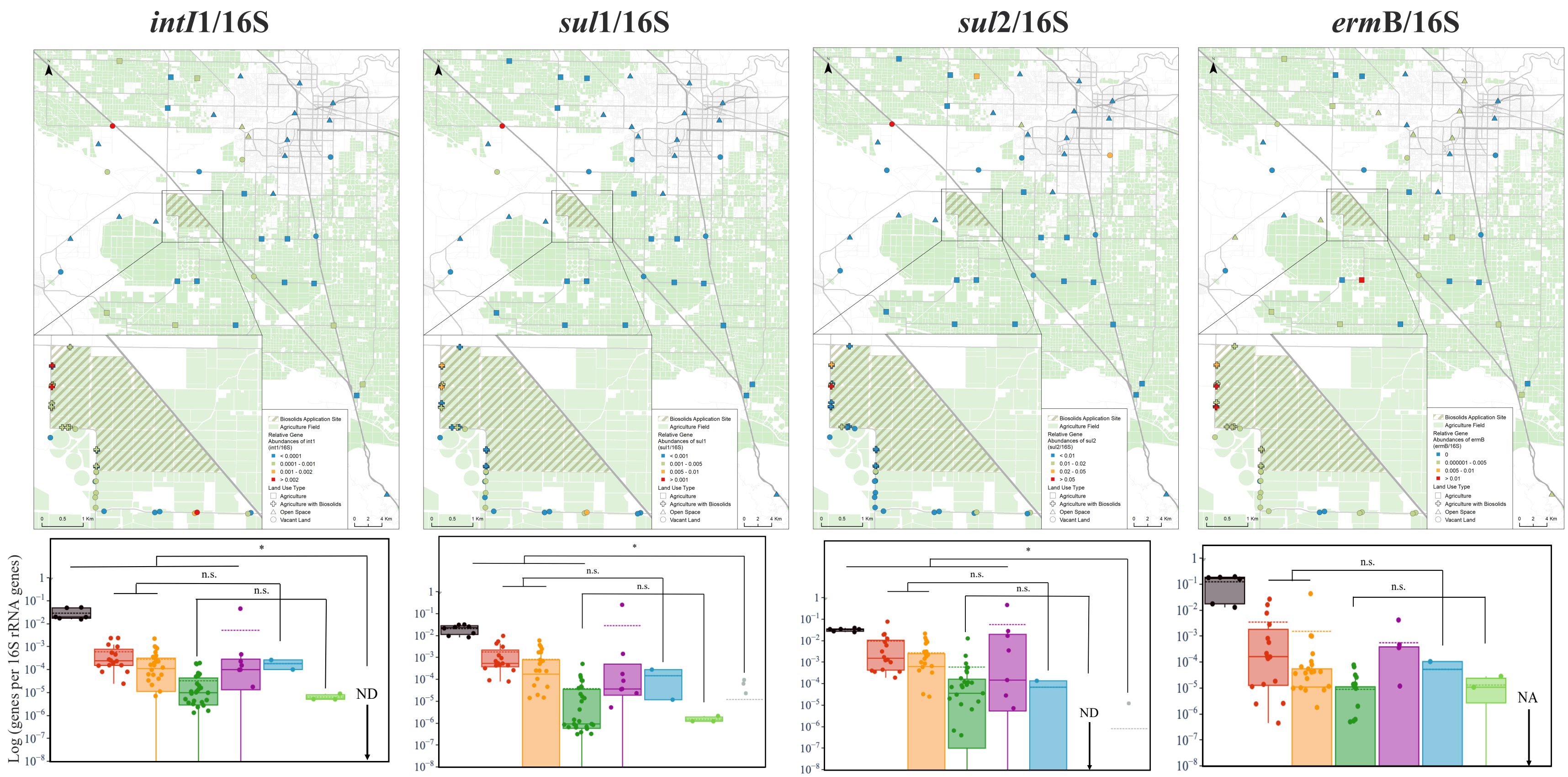

Figure 26. Spatial distribution of relative gene abundances of *intI*1, *sul*1, *sul*2, and *erm*B in soils and their comparison with control agricultural and park soils and pristine soils. Abbreviation: n.s., not significant; NA, Not Available; ND, Not Detected.

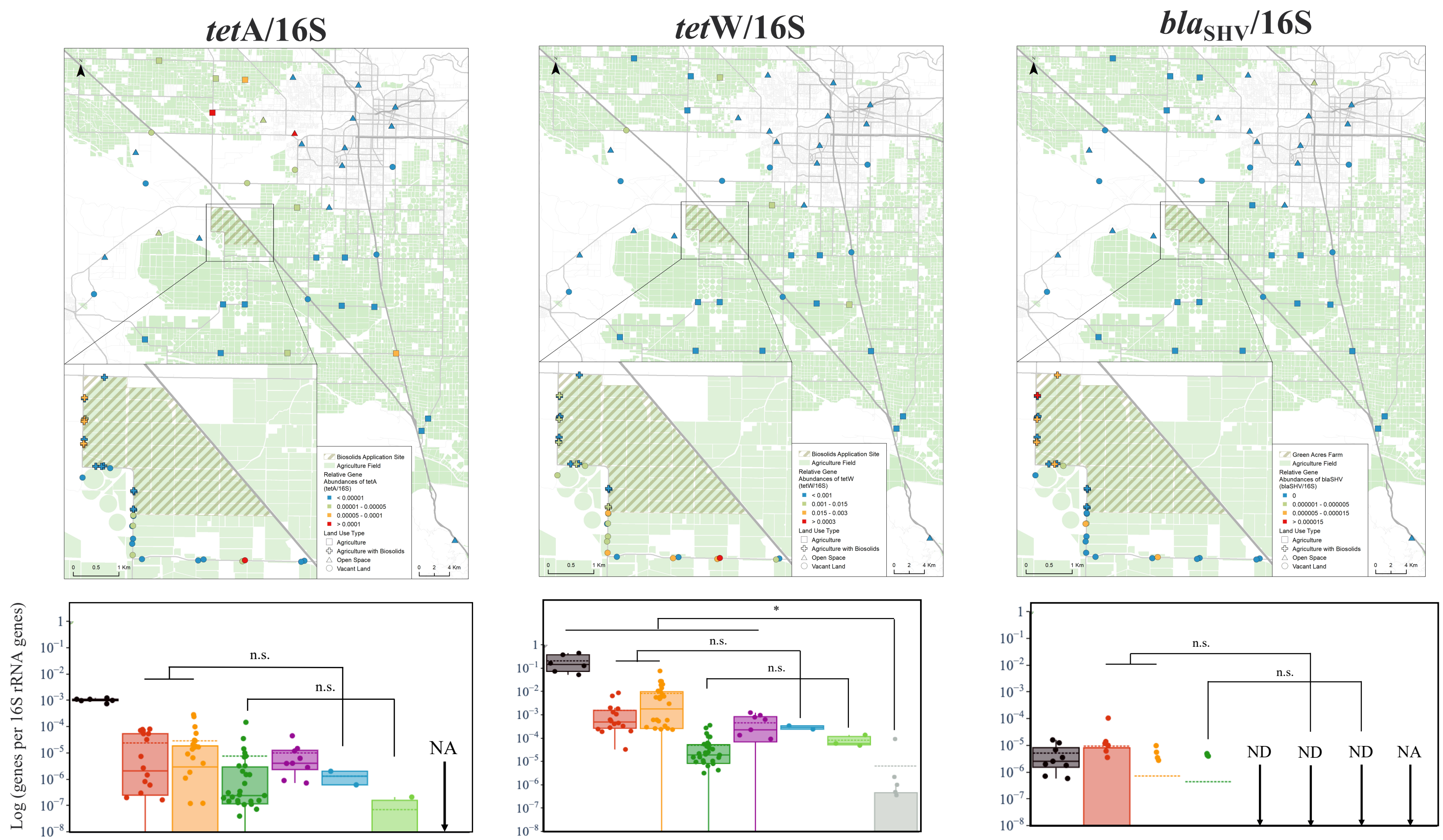

Figure 27. Spatial distribution of relative gene abundances of *tet*A, *tet*W, and *bla*$_{SHV}$ in soils and their comparison with control agricultural and park soils and pristine soils. Abbreviation: n.s., not significant; NA, Not Available; ND, Not Detected.

5.3.4 Geographical factors impacting ARG levels in agricultural soils

We performed a large-scale geographical analysis of ARG abundances in agricultural soils as a function of distance from farms where biosolids were applied. Soil samples based on the distance to the spreading ground were divided into three groups: 0 km, 0–2 km, and > 2 km (Ma et al., 2018). The influence of land application of biosolids on the distribution of ARGs in soils was evaluated by comparing average relative genes in each group (Figure 24). Average relative abundances of *intI*1, *sul*1, and *sul*2 genes at the biosolid spreading site were significantly higher than those in agricultural soils further than 2 km from the biosolid site ($p < 0.01$). However, the average relative abundances of selected genes were largely affected by the number of samples below the detection limits. Ignoring ARG quantities in agricultural soils, all selected genes were less frequently detected in agricultural soils as their distances to the biosolid site increased.

Although the effect of biosolid application on the resistome of soils that received biosolids has been evaluated in many studies (Brooks et al., 2007; Munir and Xagoraraki, 2011), the soil resistome as ARB and ARGs in the vicinity of biosolid application sites remain largely unknown. The comprehensive and regional investigation on the spatial variability ARGs in soils is shown in Figure 28. Soils in the vicinity of the biosolid application site appeared to contain elevated levels of ARGs. Atmospheric dust, aerosols, and particulates are known to carry antibiotics (Hamscher et al., 2003; Paez-Rubio et al., 2007), ARB, and ARGs (Ouyang et al., 2020). Sanchez et al. (2016) also demonstrated that airborne ARB and ARG levels were elevated in the vicinity of cattle farms where antibiotics are used relative to organic farms. Therefore, atmospheric transport is likely to be a major transport pathway for ARGs from the land application site into adjacent soil systems. However, ARGs can still enter soils from various human activities, such as fertilizers application and irrigation with treated wastewater effluent. Numerous researchers have also revealed that the spatial distribution of soil antibiotics can be highly associated with land uses (Zhao et al., 2020).

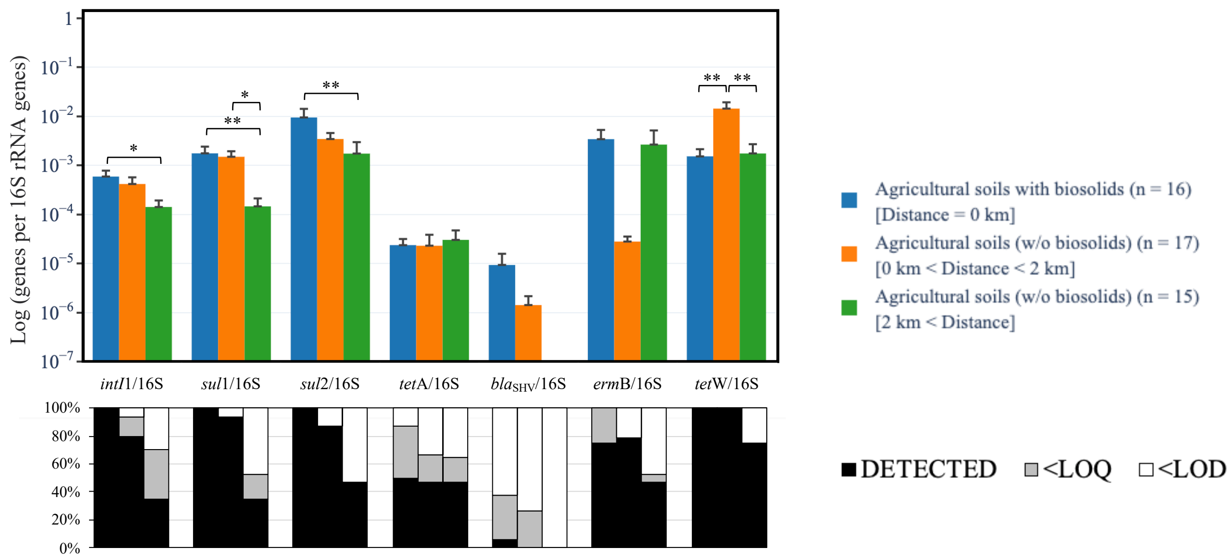

Figure 28. Relative abundances of ARGs (*erm*B, bla_{SHV}, *sul*1, *sul*2, *tet*A, *tet*W, and *intI*1) in agricultural soils based on their distances to the biosolid applied site. Distances were divided into three groups: 0 km, 0–2 km, and >2 km.

5.3.5 ARB in biosolids, biosolid-amended soils, and nearby and remote soils

Log-transformed enumerations of ciprofloxacin-, erythromycin-, sulfamethoxazole-, and tetracycline-resistant culturable bacteria in soil samples from different soil environments are shown in Figure 29. These environments include at or near the land application site, sludges (before AD), and biosolids (after AD and dewatering) from WWTP 3. Both sludge (before AD) and biosolids (after AD and dewatering system) from WWTPs have been frequently recognized as significant reservoirs of numerous ARB (Brooks et al., 2007; Gao et al., 2012). The highest average concentrations of culturable ciprofloxacin-, erythromycin-, sulfamethoxazole-, and tetracycline-resistant bacteria in sludges were 2.16×10^7, 2.17×10^7, 1.62×10^6, and 4.07

$\times 10^6$ CFU/g, respectively. Waste sludges reported by Gao et al. (2012) in Michigan had relatively higher concentrations of culturable sulfamethoxazole-resistant bacteria (range: 10^7–10^9 CFU/g) but similar concentrations of tetracycline-resistant bacteria (range: 10^5–10^7 CFU/g). In this work, ARB levels in biosolids (after AD) were approximately 1–2 logs less than in sludges (before AD). Furthermore, in this study, we found lower average concentrations (2-3 logs) of culturable ciprofloxacin- and tetracycline-resistant bacteria than in a previous study of biosolids from four different states (Brooks et al., 2007).

There were variations between ARB concentrations in biosolid-amended soils and those in biosolids in the present study. Levels of erythromycin- and sulfamethoxazole-resistant bacteria were significantly different from those in biosolid-amended soils, while ciprofloxacin- and tetracycline-resistant bacterial concentrations were not statistically different between biosolids and biosolid-amended soils. In addition, ARB concentrations in biosolid-amended soils were generally similar to or lower than those in adjacent agricultural soils and park soils (< 2 km and without biosolid application). ARB soil concentrations in remote agricultural soils and park soils, however, were significantly lower than those in biosolid-applied soils and their adjacent soil environments ($p < 0.01$). Our results are consistent with those of previous studies in which long-term biosolid application does not increase soil ARB concentrations (Brooks et al., 2012; Zerzghi et al., 2010). Negreanu et al. (2012) also found that the ARB concentrations in soil environments irrigated with treated wastewater were significantly decreased. ARB concentrations in soils following biosolid applications appeared to dissipate rapidly over time (Riber et al., 2014). Overall, biosolids have no observable effects on ARB concentrations in the land-applied field compared to its adjacent soils in this study. However, ARB may have widely spread through airborne particulates and become prevalent around the biosolid application site (Ouyang et al., 2020). Moreover, many environmental factors, such as selective pressure, that may contribute to ARB proliferation must be considered. Since many environmental microorganisms remained unculturable, there is no doubt that advanced metagenomics to determine non-culturable microorganisms will shed light on characterizing biosolid impacted soils.

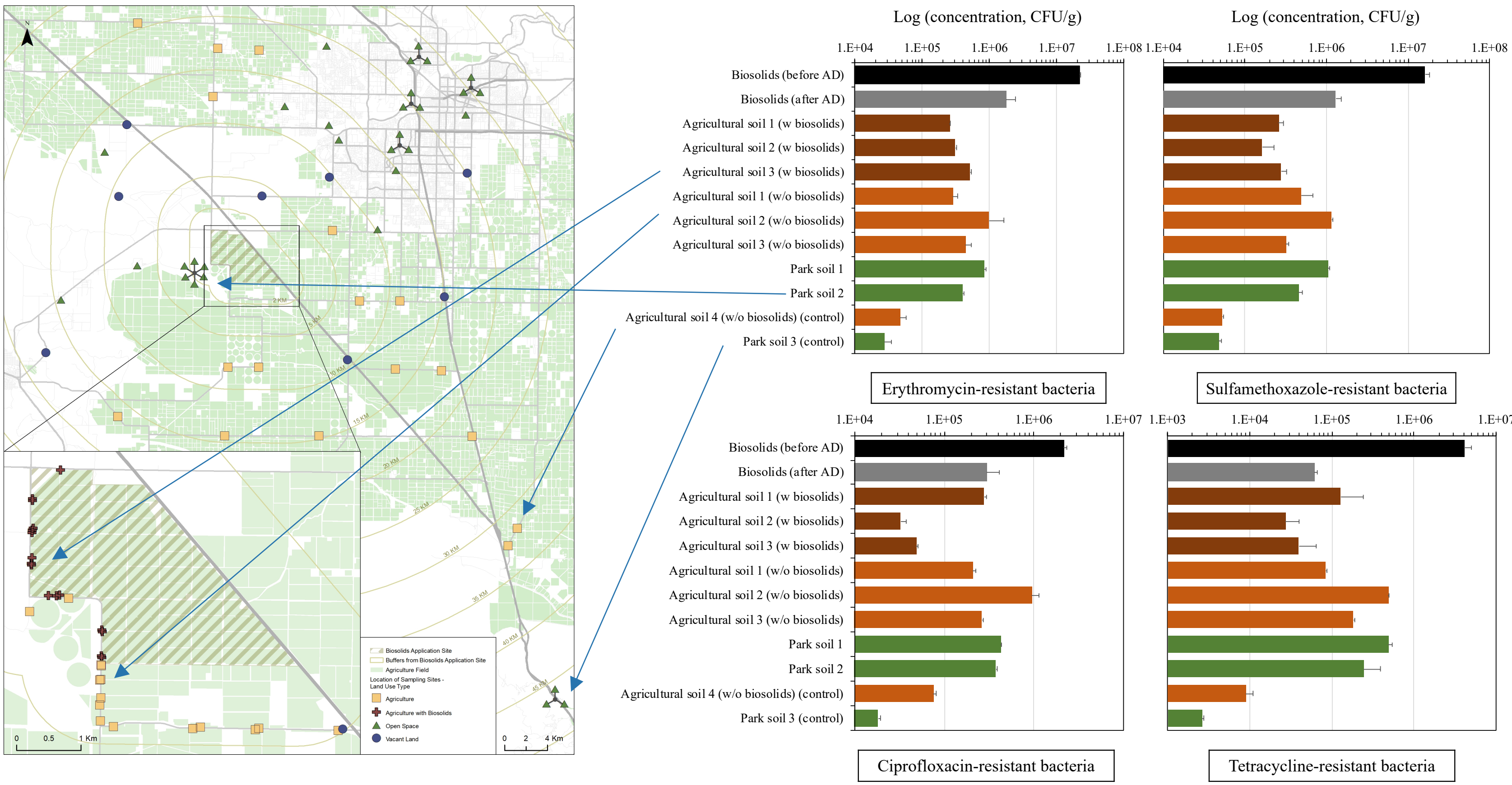

Figure 29. Log-transformed mean concentrations of ciprofloxacin- (4 ppm), erythromycin- (10 ppm), sulfamethoxazole- (50.4 ppm), and tetracycline (20 ppm) -resistant culturable bacteria in soils and biosolids (25% Luria-Bertani growth media). Error bars represent the standard deviation of their means.

5.3.6 Correlations between ARB, ARGs, and heavy metal concentrations

Coefficients of Spearman's (ρ) correlations between total metal(loid) concentrations and ARGs in collected soil samples are shown in Figure 30. Many significant correlations were found between ARGs and total metal(loid) concentrations. Levels of Cu, Ni, and Fe were individually found to be significantly positively correlated with relative gene abundances of *intI*1 (ρ = 0.28–0.41, adjusted $p < 0.05$), *sul*1 (ρ = 0.39–0.56, adjusted $p < 0.01$), and *tet*W (ρ = 0.33–0.44, adjusted $p < 0.05$). In addition, total concentrations of Cu were significantly correlated with *sul*2/16S ($\rho = 0.41$, adjusted $p < 0.01$), *erm*B/16S ($\rho = 0.39$, adjusted $p < 0.01$), and bla_{SHV}/16S ($\rho = 0.34$, adjusted $p < 0.01$). However, Pb was significantly negatively correlated with *intI*1/16S ($\rho = -0.34$, adjusted $p < 0.05$) and *tet*W/16S ($\rho = -0.31$, adjusted $p < 0.05$), respectively. While most heavy metals showed positive correlations with gene abundances normalized to cell population, most correlations appeared weak. Notably, multiple correlations of heavy metals with gene abundances were also found.

Even in the absence of AR-associated compounds from biosolids, agricultural soils can still foster the proliferation of ARGs. One reason is that antibiotics and metals are frequent co-contaminants and select for resistance in the microbial community. ARGs have increased in agricultural soils during the same period in which we have seen a dramatic increase in human and veterinary antibiotic use (Knapp et al., 2010). Furthermore, levels of ARGs in agricultural soils were shown to correlate with metal concentrations (Knapp et al., 2011), indicating metal co-selection (Baker-Austin et al., 2006). A meta-analysis indicated that Hg, Cu, Cd, and Zn are the heavy metals most likely to exert selective effects for antibiotic resistance in agricultural soils (Seiler and Berendonk, 2012), consistent with our results.

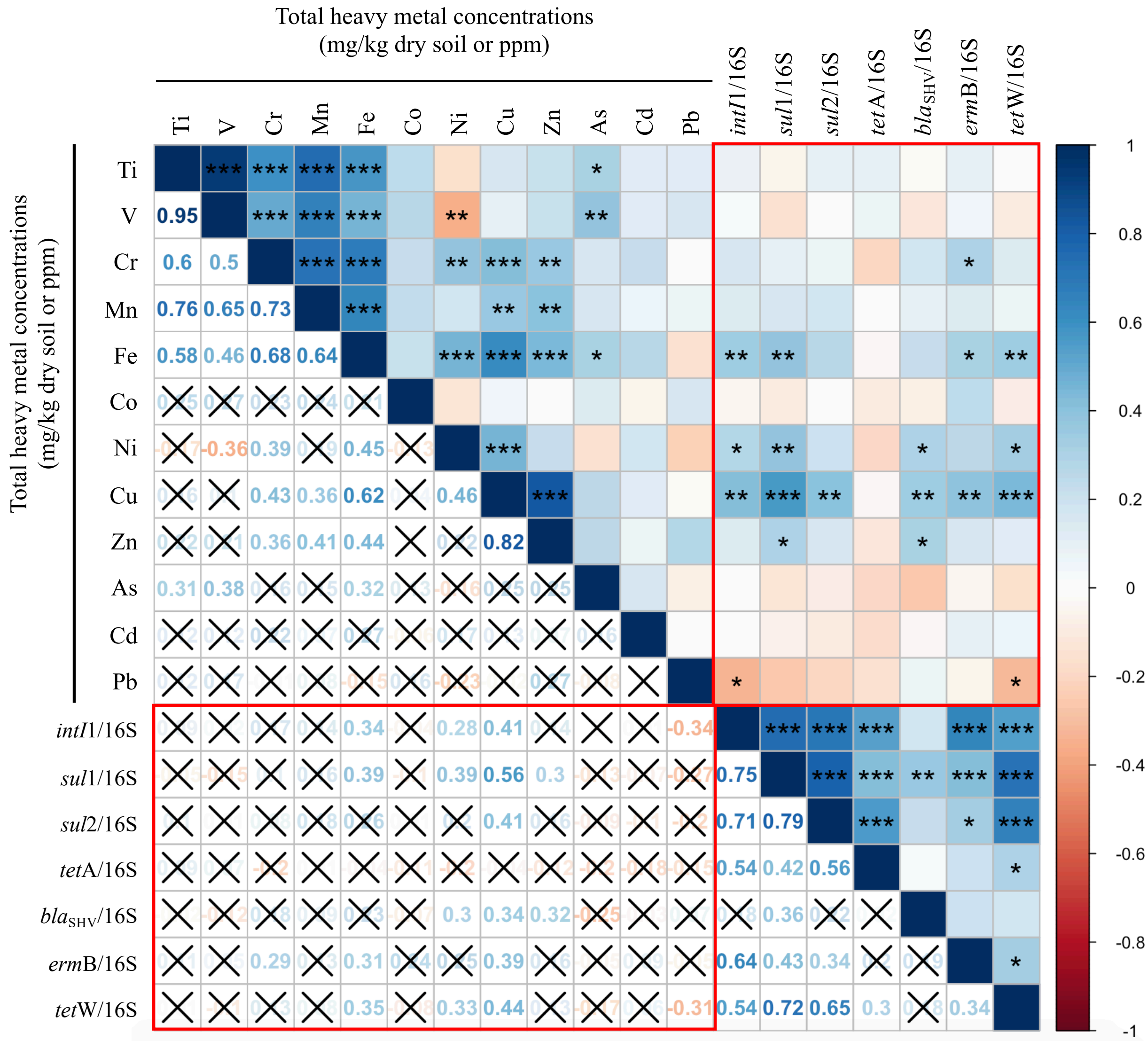

Figure 30. Spearman's correlation coefficients between targeted genes and total metal(loid)s (Positive = blue, negative = red, X = not significant). Correlations significant at $p < 0.001$, $p < 0.01$, and $p < 0.05$ are marked with ***, **, and *, respectively.

5.4 Conclusions

The general effect of biosolid application on the resistome of biosolid-amended soils has been investigated in several studies. However, soil resistomes, as determined by ARB and ARGs in the vicinity of the biosolids application site, are largely uncharacterized. Relative abundances of ARGs were found to be elevated in biosolid treated soils compared to those in the surrounding areas and generally at least an order of magnitude higher than the values reported in other regional studies. The mean relative abundances of *intI*1, *sul*1, *sul*2, and *erm*B genes in biosolid-amended soils were significantly higher than those in surrounding agricultural soils. It is also suggested that the spatial distribution of soil ARG levels were statistically associated with land use. Mean relative abundances of selected genes (*intI*1, *sul*1, *sul*2, and *tet*W) in pristine soils from the hiking trails were significantly lower than those detected in most other soil environments ($p < 0.05$). In addition, the mean relative abundances of *intI*1, *sul*1, and *sul*2 genes at the biosolid site were significantly higher than those in agricultural soils that were 2 km away from the biosolid site. Biosolids played an insignificant role in culturable ARB concentrations on the land application site as they were similar to those in the surrounding soil. This shift in the resistome implies pathogens at sites with specific land uses have a greater chance of acquiring resistance genes. Lastly, total concentrations of Cu, Ni, and Fe were individually significantly positively correlated with relative gene abundances of *intI*1, *sul*1, and *tet*W ($p < 0.05$). By investigating ARG quantities and trends in soils, this study brings attention to the need to redefine our antimicrobial standards in soils in terms of public health.

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CHAPTER 6. CONCLUSIONS AND FUTURE WORK

6.1 Summary

Los Angeles has a long history of lead (Pb) contamination due to automobile emissions, industrial processes, and Pb-containing paint. Although the amount of Pb coming from these sources has been reduced or eliminated, exposure to Pb-contaminated soil is still a concern. Contaminated soil in urban parks may constitute an important exposure route, since children are particularly vulnerable to the toxic effects of Pb. While the current EPA clean up level is 400 parts per million (ppm) in recreational and residential areas, the Office of Environmental Health Hazard Assessment (OEHHA) of California revised the soil-screening level to 80 ppm, based on a 1 μg/dL increase of blood level that children experience. In this work, top soil samples in triplicate were taken from each of 100 urban parks in Los Angeles and analyzed to determine Pb concentrations and soil properties. Results show that the average Pb concentration in park soil was 65.5 ppm (range: 0.969–363 ppm), with 35 parks exceeding the California guideline of 80 ppm based on the average Pb concentration measured at each park. Pb concentrations in 42 parks exceeded the background value of 19.7 ppm (by a factor of 3 to 17). However, variation in measured levels of Pb within most parks was high (>10% RSD in 89 parks). Parks containing elevated Pb concentrations in soil were distributed across many areas of Los Angeles, including central Los Angeles, Beverly Hills, Inglewood, and Central Alameda. Children might potentially suffer from an estimated 1–4 μg/dL increase of blood Pb level according to LeadSpread model. Pb concentrations did not significantly correlate with most soil properties, except for silt ($\rho = 0.226$, p value = 0.012). Additionally, soil from sandy loam contained significantly higher Pb concentrations as compared to soil from loamy sand (p value = 0.008). Pb concentrations were significantly correlated with park age ($\rho = -0.319$, $p = 0.033$) and were generally lower in areas located away from major roads. Monitoring of parks with high levels of Pb as well as investigation of unexamined regions in Los Angeles are needed.

In addition to urban park soils that accumulate Pb, stormwater biofilters built as natural treatment systems have also become increasingly popular in urban landscapes. While biofilters have been widely and effectively used to capture chemical pollutants from surface runoff, the effect of biofilters on both heavy metals and ARGs has been relatively understudied. The co-occurrence of heavy metals and ARGs is important because of known metal(loid) co-selection in environmental compartments. Surface soil samples from six biofilters and bioswales in Southern California over three time periods were analyzed for ARGs (*sul*1*, sul*2*, tet*A*, tet*W*, and erm*F), mobile genetic element (*intI*1), and 16S rRNA (proxy for total bacterial load). The impact of soil properties and the co-selective effect of nine heavy metals (both bioavailable fraction and total) on ARG levels in the biofilters were also investigated. Both relative *sul*1 and *intI*1 levels in biofilters were statistically greater than those detected in pristine soils ($p < 0.01$). Total concentrations of arsenic, lead, vanadium, and zinc exhibited significant correlations individually with relative abundances of *sul*1, *sul*2, and *tet*W ($p < 0.05$). Soil organic matter, total nitrogen, total carbon, and the percentage of sand and silt within biofilters appeared to be significantly associated with absolute gene abundances of *sul*1, *sul*2, and *tet*W. Stronger relationships were found using a multiple linear regression model, suggesting multiple effects of soil properties, in addition to bioavailable and total heavy metals on the microorganisms within biofilters. This work will enhance our knowledge of ARG baseline levels for risk assessment and better strategery developments in urban biofilters.

Rising levels of antibiotic resistance are also a critical public health concern in agriculture. Recent research has identified wastewater treatment plants (WWTPs) as important reservoirs of

ARGs. Hence, the application of urban WWTP products to agricultural environments may contribute to widespread antibiotic resistance. Multiple studies have documented elevated ARG levels in soils after biosolid application, possibly increasing at field sites due to horizontal gene transfer (HGT) and co-selective pressures. Biosolids from two WWTPs, treated with anaerobic digestion, were spread in this study area per the regulations governing this practice. Quantitative polymerase chain reaction (qPCR) was performed for detection of a suite of genes, including several ARGs, the gene for the integrase of class 1 integrons (*intI*1), and total bacterial 16S ribosomal RNA (16S rRNA). Our data show that samples of these biosolids contained elevated levels of several ARGs. The average relative gene abundances of *sul*1, *sul*2, and *intI*1 were significantly higher in biosolid-amended soils than nearby control agricultural soils ($p < 0.05$). However, concentrations of culturable antibiotic-resistant bacteria (ARB) at the land application site were similar to those in the surrounding soils. Levels of *intI*1, *sul*1, and *sul*2 copies/16S in soils significantly decreased with increasing distance from the biosolid spreading ground, highlighting possible airborne spread. Many metal(loid)s (Cu, Ni, and Fe) were found to be significantly and positively correlated with relative gene abundances of *intI*1, *sul*1, and *tet*W ($p < 0.05$). This study will inform the future evaluation of antibiotic resistance in soils impacted by biosolid application.

6.2 Significance

This work comprehensively examined the prevalence and fate of heavy metals and antibiotic resistance in urban and agricultural soils on a regional basis. The significant aspects of this dissertation address major gaps in knowledge as follows:

1. Legacy of Pb emissions in Los Angeles continued to affect soil Pb concentrations in public parks. These were shown to be high, with 35 parks exceeding the CalEPA guideline of 80 ppm. Children may suffer from an estimated 1–4 μg/dL increase of blood Pb level according to EPA LeadSpread model. Metals such as Pb accumulating in the urban soils may trigger co-selection on AR.
2. A comprehensive survey of seasonal and regional ARGs and metal(loid)s in urban biofilters enhanced our knowledges on their baseline levels for risk assessment and better strategery developments for urban biofilters. To our knowledge, we proposed the first multiple linear regression model with the inclusion of soil properties, total metal(loid)s, and bioavailable metal(loid)s on ARGs in urban biofilters.
3. Effect of land application of biosolids on soil ARG concentrations was significant as they were higher in biosolids-amended soils than those surrounding agricultural soils without receiving biosolids.
4. Regional spatial patterns of ARGs in soils provided an insightful understanding of the distribution of ARGs and their associations with land uses, ultimately leading to better managed soil ARGs.
5. The influence of heavy metals on selection for antibiotic resistance in different soil environments provided valuable data for determining minimum co-selection concentrations that contribute to ARG persistence.

6.3 Future Research

6.3.1 Role of airborne ARGs and ARB in transport across a region

ARGs are unique emerging pollutants in that they are frequently observed to increase rather than decrease through downstream environments. While dynamics in water and soil environments have been extensively studied for these emerging contaminants, their fate and transport in air is just beginning to be understood. Our previous work has revealed significant air transport of ARB and ARGs downwind of agricultural facilities in CA (Sanchez et al., 2016). Previous study has hypothesized inhaling airborne ARGs could possibly induce the disequilibrium of respiratory tract bacterial community and thus affect the immune system if up-taken and further expressed by human-borne bacteria (Li et al., 2018). Czajkowski (2010) found some statistical correlation between illness and proximity to permitted biosolids fields in Ohio, but it did not show a cause and effect relationship between biosolids applications to farm fields and illnesses.

This new knowledge will inform safe land application of urban biosolids and address the stigma frequently associated with its use. This monitoring approach will also inspire a more thorough global assessment and reporting of ARGs in air and soils due to biosolids being spread, creating a larger database on ARG quantities as well as co-contaminants. In doing so, this study would also call attention to the need of redeveloping our current air and soil quality standards in terms of public health and construct better antibiotic-safe environments.

6.3.2 Importance of the transfer of ARGs and ARB from the environment to human populations

While the environment is recognized as an important reservoir for ARB and ARGs, its role in transmission still remains largely unknown. While a few papers have shown geographical trends in antibiogram data, there is a paucity of research linking differences to environmental factors. A major innovative facet of this work is the use of hospital antibiograms as a tool for investigating possible regional differences in antibiotic resistance due to disparate environmental ARG levels. Data on antibiotic resistance in clinical isolates are organized into antibiograms. However, inconsistences in methodology confound the use of antibiograms for detecting regional trends in resistance. So far there are relatively few studies comparing antibiograms from geographically diverse areas. A recent study (Tamma et al., 2013) surveyed hospitals and grouped them into four geographical regions across the US. The conducted a regional comparison for four different organisms and several antibiotics. There are several statistically significant findings; the most notable was that the *Enterococcus Faecium* has higher susceptibility to ampicillin and vancomycin in the South of the U.S. A compilation of antibiograms from twelve Virginia hospitals (Var et al., 2015) observed trends between five geographical areas. Penicillin susceptibility in *Streptococcus pneumoniae* varied a great deal between the regions. *Enterobacter cloacae* showed differences in susceptibility to aztreonam, moxifloxacin, and piperacillin/tazobactam. *Acinetobacter baumannii* showed large variability across regions in susceptibility to cefepime, meropenem, and piperacillin/tazobactam. Other work (Munson et al., 2016) looked at regions of Wisconsin and compared antibiotic resistance data. The major results were seen for *P. mirabilis*, *P. aeruginosa*, MRSA, and VRE. Silvaggio et al. (2016) and the Los Angeles County Department of Public Health (LADPH) examined 70 hospitals, demonstrating over 20% burden of carbapenem resistance among *Klebsiella spp*. Importantly, the burden of carbapenem resistance ranged from 4% to over 31% within Los Angeles County. In contrast, data from San Diego suggests that carbapenem resistance among *Klebsiella* spp is less than 3%. These findings regarding geographical trends in antibiotic resistance have not yet been linked to environmental factors including proximity to sources or co-contaminants.

6.4 References

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