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Tracking antibiotic resistance through the environment near a biosolid spreading ground: Resistome changes, distribution, and metal(loid) co-selection

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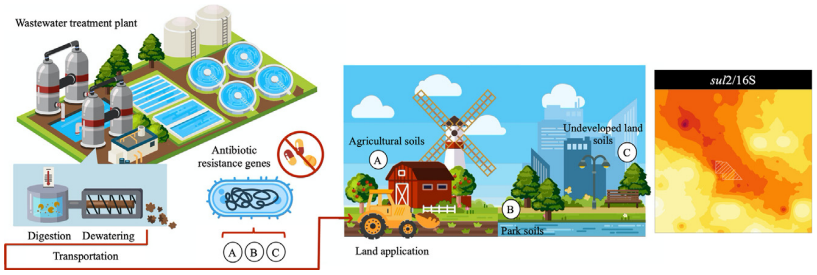
HIGHLIGHTS

- The effect of biosolid application on soil ARGs, ARB, and metal(loid)s was studied.
- *IntI*1 and selected ARGs were higher in biosolid-added soils than agricultural soils.
- ARGs were negatively correlated with distance to the biosolid spreading ground.
- Soil ARGs distribution was consistent with the dominant wind direction in winter.
- This study informs the evaluation strategies for long-term biosolids application.

GRAPHICAL ABSTRACT



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ABSTRACT

The application of urban wastewater treatment plants (WWTPs) products to agricultural lands has contributed to the rising level of antibiotic resistance and drawn a critical public health concern. It has not been thoroughly investigated at which spatial scales a biosolid applied area as a potentially predominant source affects surrounding soil resistomes. This study investigated distribution and impact of WWTP biosolids treated with anaerobic digestion on an agricultural area. Heterotrophic plate counts (HPCs) and quantitative polymerase chain reaction (qPCR) were performed for detection of selected antibiotic-resistant bacteria (ARB), selected antibiotic resistance genes (ARGs), *intI*1 genes, and 16S rRNA genes. Biosolid samples contained significantly higher levels of selected ARGs than the raw agricultural soils ($p < 0.05$). The average relative abundances of *intI*1, *sul*1, bla_{SHV}, and *erm*B genes were significantly higher in biosolid-amended soils than nearby agricultural soils ($p < 0.05$). Spatial interpolation analysis of relative gene abundances of *intI*1, *sul*1, *sul*2, and *tet*W across the studied area further indicated directional trends towards the northwest and southeast directions, highlighting possible airborne spread. Concentrations of Co, Cu, Ni, and Fe were found to be significantly and positively correlated with relative abundances of *intI*1, *sul*1, and *tet*W genes ($p < 0.05$). The resistance ratios of culturable antibiotic-resistant bacteria in agricultural soils with biosolid amendments were generally identical to those without biosolid amendments. This study will advance the understanding of the antibiotic resistome in agricultural soils impacted by long-term waste reuse and inform the evaluation strategies for future biosolids application and management.

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1. Introduction

The global increase in antibiotic resistance (AR) is one of society's greatest public health challenges. Our ability to effectively treat infections is hindered by the proliferation of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB), 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 agriculture and medicine, along with other anthropogenic pollution (Martinez, 2009). Waste streams from humans and animals produced by wastewater treatment plants (WWTPs) are known to carry elevated ARG levels, which are largely accumulated in solid waste (Sui et al., 2016).

Agricultural land application is one of the most common practices for recycling and reuse of nutrients in biosolids. Biosolids undergo various types of treatment before land application, such as dewatering (DW), gravity thickening, anaerobic digestion (AD), and lime stabilization (Munir et al., 2011). Biosolids treated with these methods had lower levels of tetracycline- and sulfonamide-resistant bacteria, while ARG abundances in biosolids varied after treatments (Munir et al., 2011). Two WWTPs in northern China showed a significant increase of 23 ARGs through DW-treated sludge (Mao et al., 2015). AD-treated in waste solid obtained from southern Minnesota WWTP indicated that abundances of ARGs and class 1 integron-integrase gene (*intI*1) decreased at all temperatures, with greater removal at higher temperatures (Burch et al., 2016). In many cases, the removal efficiency of ARGs and *intI*1 abundances was of two orders of magnitude, yet ARG concentrations remaining in treated biosolids were relatively high. 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., 2011) or in one case even more 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. Therefore, land application of biosolids may be one of the detrimental human activities creating selective conditions for the emergence and proliferation of ARGs in the receiving environment.

More than 4 million dry metric tons of biosolids are applied to land in the United States in 2019 (USEPA, 2019). Biosolids used for land application are currently regulated under 40 CFT Part 503 to ensure levels of metal(loid)s and pathogens pose minimal risk to human health. After initial treatment to reduce odor, biosolids are categorized into “Class A” 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* CFU per 4 g total solids or 100 MPN fecal coliforms per gram total solids. While studies have highlighted land application as an important source of ARGs to the environment (He et al., 2020), the risk of disseminating ARGs into agricultural soils from regulatory aspects is not yet fully understood (Chee-Sanford et al., 2009). Further, the abundance, distribution, diversity, and potential transport of ARGs and ARB in soils treated with biosolids and their surrounding soils are still not fully understood.

Among municipal WWTPs in the United States, less than 10% of those employ AD (Ma et al., 2015), including three WWTPs investigated in this study. AD-treated biosolids were known to contain elevated levels of ARGs, yet the reported effect of soil biosolid application on the resistome remains uncertain. Multiple studies have documented elevated levels of ARGs and/or antibiotic-resistant bacteria 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), and increased availability of plasmids encoding for resistance genes was shown using a model recipient for horizontal gene transfer (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 due to 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). Although the effect of soil biosolid application on the resistome varies between studies, the occurrence, concentration, and spatiotemporal distribution of soil ARGs and ARB in the surroundings of the biosolid applied area has not been comprehensively investigated. It is currently unknown at which spatial scales a biosolid applied area as a potentially predominant source for AR dissemination affects surrounding soils.

In this study, the environmental impact of biosolid land application was assessed by analyzing ARGs and ARB in receiving soils and their surrounding environments. 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) assess and compare abundances of ARB and ARGs in WWTP biosolids, biosolid-amended soils, as well as surrounding and remote soil environments (e.g., agricultural soils and park soils); (2) investigate potential relationships between ARG levels and geographical factors; and (3) evaluate the co-selective effects of metal(loid)s on ARGs distribution.

2. Materials and methods

2.1. Biosolid sample collection

Biosolid samples were collected from three different WWTPs in California with treatment methods summarized in Table 1 and schematic diagram of the AD process shown in Fig. S1. Each of three WWTPs was sampled once between July 2019 to October 2020. Briefly, two dewatered anaerobically-digested biosolids (anaerobic cake) were sampled in July 2019 from WWTP 1. A majority of biosolids produced from WWTP 1 were land-applied in the study area with approximately 650 metric tons per day. In parallel, another anaerobically-digested biosolid sample (anaerobic slurry) was also collected at WWTP 2 in December 2019 for comparison purposes. For WWTP 3, untreated sludge and anaerobically-digested biosolids (anaerobic slurry) were obtained for ARB enumeration in October 2020. All biosolid samples were stored in 1 L pre-sterilized polypropylene bottles individually on ice (4 °C) before immediate processing at the UCLA laboratory. If dewatered biosolids (i.e., anaerobic cake) were collected, approximately 250 mg of subsamples in triplicate were stored at −20 °C and used to extract DNA. In the case of liquid biosolid samples (i.e. anaerobic slurry), approximately 100 mL of samples were concentrated by centrifuging at 5000 g for 10 min under ambient condition (Yang et al., 2014). The supernatant was discarded and up to 250 mg of pellets derived from liquid biosolids in triplicate were stored at −20 °C for DNA extraction.

2.2. Soil sample collection

A total of 90 surface soil samples were collected in December 2019 and November 2020 within 900 km^2 of the study area (Fig. S2) using a systematic grid random sampling approach (U.S. Environmental Protection Agency, 2002). Soil was sampled at much higher densities within 36 km^2 of the biosolid-applied field and their surrounding areas. The surrounding areas were divided into thirty-six continuous 25-km^2grid cells. At each uniform grid on the map, soil samples were collected in the vicinity of the center of each grid cell (Hung et al., 2018). Based on land use types, these areas contained undeveloped lands, agricultural fields, and open space (i.e., parks and recreation areas). Once the sampling location has been determined, a 1

Table 1
Summarized biosolid treatment methods in WWTPs.

	WWTP 1	WWTP 2	WWTP 3
Biosolid sample type	Cake	Slurry	Slurry
Sludge treatment	Anaerobic digestion (thermophilic)	Anaerobic digestion (mesophilic)	Anaerobic digestion (mesophilic)
Temperature (°C)	55–57	35–37	35–37
Sludge disposal	Agricultural land application	Landfill	Landfill
Annual production (metric tons/wet weight)	264,000	488,516	126,000
Analysis in this study	ARG quantification	ARG quantification	ARB enumeration
Sample collection date	July 2019	December 2019	October 2020
Sample size (n)	n = 3	n = 3	*n* = 3

m^2 plot was randomly selected from the area. Remote park soils (approximately 45 km from the biosolid application site) and agricultural soils (approximately 35 km from the biosolid application site) were collected as controls.

Surface soil (0–10 cm) 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 UCLA laboratory prior to processing.

2.3. DNA extraction and quantification of ARGs from biosolids and soils

Three biosolid and soil subsamples of 0.25 ± 0.01 g from each glass screw-top jar and each 1-L pre-sterilized polypropylene bottle were measured into sterile 2 mL screwcap tubes preloaded with garnet beads and bead solutions (Qiagen, Valencia, CA, USA). Screwcap tubes were stored (−20 °C) until DNA extraction. DNA was extracted from archived and thawed biosolid and soil samples using DNeasy PowerSoil Kits (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The final DNA extracts were stored at −20 °C for quantitative polymerase chain reaction (qPCR). The purity and quantity of total DNA extracts were examined using UV absorption by a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA extracts were considered as relatively free of contamination from reagents used during extraction as the A260/280 ratio was above 1.8 per the instrument manual.

DNA extracts were analyzed in triplicate for selected ARGs (genes conferring resistance to β-lactamases (bla_{SHV}), macrolides (*erm*B), sulfonamides (*sul*1 and *sul*2), and tetracyclines (*tet*A, and *tet*W), a class 1 integron-integrase gene (*intI*1), and 16S rRNA gene (a proxy for total cells) abundances in 96-well reaction plates using StepOne Plus qPCR system (Applied Biosystems, Foster City, CA, USA). These ARGs were chosen as representatives of resistance mechanisms to β-lactamases, sulfonamides, tetracycline, and macrolides, respectively, due to the frequent detection in municipal wastewater biosolids (Kimbell et al., 2018). For both soil and biosolid samples, each qPCR reaction was conducted in a final reaction volume of 25 μL, containing 1.25 μL of each primer, 12.5 μL of PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 2 μL of soil DNA extracts (approximately 5–100 ng DNA), and 8 μL of molecular-grade water (Thermo Fisher Scientific, Waltham, MA, USA). Soil and biosolid DNA extracts were diluted to contain appropriate concentrations prior for proper DNA amplification (Echeverria-Palencia et al., 2017). Primer concentrations (Table S1) and thermocycling conditions (Table S2) were optimized as described previously (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 curves of the designed DNA fragments were analyzed in triplicate in addition to soil and biosolid extracts, with the correlation coefficients and amplification efficiencies of the standard curves ranging from 0.990 to 1 and from 85.2% to 100%, respectively (Table S2). No-template controls (molecular-grade water) were included with each qPCR assay to test false-positive results. The limit of the quantification for each selected gene (Table S2) were determined following MIQE guidelines (Bustin et al., 2009). Furthermore, soil and biosolid DNA extracts were spiked with known concentrations (10^3 copies/μL) of targeted DNA standards to examine inhibition effects (Echeverria-Palencia et al., 2017). The specificity of amplified DNA products was further confirmed by melt-curve analysis.

2.4. Enumeration of ARB

The total heterotrophic plate counts (HPC) (ISO 6222, 1999) were used to screen for antibiotic resistance levels on antibiotic-selective media in collected soil samples. Soil samples within each soil type were randomly selected from each soil type, including WWTP biosolids (n = 2), biosolid-amended agricultural soils (n = 3), agricultural soils without biosolids amendment (n = 3), remote agricultural soils (n = 1), park soils near the spread ground (n = 2) and remote park soils (n = 1). 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, USA) for 40 min at maximum speed. The samples were serially diluted with PBS (pH = 7.4) onto 25% Luria-Bertani growth media (LB Broth, Miller, Fisher BioReagents, Pittsburgh, PA, USA) agar plates amended with four different antibiotics, respectively: (1) tetracycline (20 ppm) (MP biomedicals, Irvine, CA, USA), (2) erythromycin (10 ppm) (MP biomedicals, Irvine, CA, USA), (3) ciprofloxacin (4 ppm) (MP biomedicals, Irvine, CA, USA), and (4) sulfamethoxazole (50.4 ppm) (MP biomedicals, Irvine, CA, USA). The desired final concentrations of aforementioned antibiotics were adopted as reported elsewhere (Brooks et al., 2007; Gao et al., 2012; Negreanu et al., 2012; Pei et al., 2006). A dilution volume of 100 μL was spread on LB agar plates. Plates were incubated at 30 °C for 3 days before 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. The fraction of colonies growing on a particular amended plate divided by the colonies growing on the unamended plate (total heterotrophic plate culturable bacterial population) for that treatment is referred to relative concentrations or resistance ratio (RR) of cultural ARB. However, this RR based on the sensitivity or resistance of microbiomes to antibiotics could be adjusted cording to standard test with gradient concentrations of antibiotics in the future study. A RR close to 1 or 0 indicates a trend of resistance or susceptibility, respectively.

2.5. Determination of metal(loid)s 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. Briefly, soil samples were oven-dried (105 °C) overnight, grounded 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 including arsenic (As), chromium (Cr), cadmium (Cd), cobalt (Co), copper (Cu), iron (Fe), Manganese (Mn), nickel (Ni), lead (Pb), titanium (Ti), vanadium (V), and zinc (Zn) were

automatically produced from the pXRF spectra according to the internal factory–installed calibration procedure. Average metal(loid) 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. The limit of the detection and calibration range for each element was also determined according to the manufacturer's manual (Table S3).

2.6. Data analysis and visualizations

Gene abundances of *erm*B, bla_{SHV}, *sul*1, *sul*2, *tet*A, *tet*W, and *intI*1 were normalized to the quantity of bacterial 16S rRNA genes as relative gene abundances (hereafter abbreviated as genes/16S) and normalized to the total mass of wet soil as absolute gene abundances (genes/g soil). The limit of quantification for qPCR were set as described previously (Bustin et al., 2009; Marti et al., 2014). In particular, the limit of quantification was determined at the lowest dilution of standards with triplicate positive results following the linearity range of standards. When the targeted gene was detected but below the limit of quantification, it was reported as detected but not quantifiable (DNQ). Non-detected gene targets were annotated as below the limit of detection (BDL). In cases where gene targets were DNQ and BDL, gene copy numbers were adjusted to the limit of quantification and zero (Klymus et al., 2020; Lau et al., 2017) for correlation analyses and non-parametric Mann-Whitney *U* test. Similarly, metal(loid) concentrations below the limit of detection were manually designated a value of half the detection limits (Helsel and Gilloom, 1986) to facilitate correlation analysis.

Statistical analysis and graphical outputs were performed in R software version 1.4.1 (RStudio Team, 2020). Specifically, correlation matrices were performed using the “Hmisc” and “corrplot” packages. Whisker box plots and scatter plots were generated with “devtools”, “ggplot2”, “gridExtra”, “dplyr”, and “ggpubr” packages. Statistical comparisons of ARG abundances in soils among various types of locations were performed using the Kruskal-Wallis test followed by Mann-Whitney *U* test for multiple comparisons. Spearman's correlation was used to identify correlations between selected ARGs and metal(loid) concentrations since our data were mostly not normally distributed as tested by the Kolmogorov-Smirnov method ($p < 0.05$). The *p*-values were adjusted according to Benjamini–Hochberg method in consideration of false discovery rate (Benjamini and Hochberg, 1995). Significance was assessed at $p < 0.05$.

Site locations and their associated land uses were entered and digitalized into ArcMap Version 10.7 (ESRI, Redlands, CA, USA). Spatial interpolation maps were created using the Inverse Distance Weighted (IDW) interpolation method with standard neighborhood type and optimized power function ranging from 1 to 10.

3. Results

3.1. Overall ARGs in biosolids and soil samples

All selected ARGs (*erm*B, bla_{SHV}, *sul*1, *sul*2, *tet*A, and *tet*W) and class 1 integron-integrase gene (*intI*1) were detected in biosolid samples from WWTP 1 and WWTP 2 (Fig. 1). The average relative abundances of *erm*B and *tet*W genes were the highest among detected genes, averaging 1.8×10^{-1} ($\pm 1.5 \times 10^{-2}$) genes/16S in WWTP 1 and 4.1×10^{-1} ($\pm 4.8 \times 10^{-2}$) genes/16S in WWTP 2, respectively. In contrast, the average relative abundances of the bla_{SHV} gene were the lowest in both WWTPs. In addition, 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 approximately an order of magnitude difference between the average relative abundances of the *erm*B gene between WWTP 1 (1.8×10^{-1} genes/16S) and WWTP 2 (1.5×10^{-2} genes/16S).

In addition to biosolids, most selected genes in soil samples from the spreading ground and sites representing different land-use types outside the spreading ground were also detected. However, bla_{SHV} genes within each land use type were mostly non-detected or below the limit of quantification. Overall, the relative gene abundances of selected ARGs and *intI1* ranged from 10^{-7} to 10^{-1} genes per 16S rRNA gene copies. Since many bacteria contain more than one 16S rRNA gene copy that may lead to biased cell counts (Louca et al., 2018), *intI*1 gene and ARGs herein represented up to roughly 0.00001% to 10% of the total bacteria. The average relative abundances of sulfonamide resistance genes (*sul*1 and *sul*2) were generally the highest across all land use types (Fig. 1), followed by *intI*1, tetracycline resistance (*tet*W and *tet*A), *erm*B, and bla_{SHV} genes.

Log-transformed (base 10) relative gene abundances in different soils are shown to further compare gene levels in different soil environments (Fig. 2 and Fig. S3). The mean relative abundances of all selected genes in soils from different land uses were statistically 2–5 logs lower than those in biosolids (Mann-Whitney *U* test, adjusted $p < 0.01$) except for the bla_{SHV} genes. In addition, the average relative abundances of *sul*1, *intI*1, bla_{SHV}, and *erm*B genes in biosolid-amended agricultural soils were significantly higher than those in agricultural soils without biosolids amendment (adjusted $p < 0.05$) (Fig. 2). While both agricultural soils with and without biosolids amendment had relatively higher relative abundances of all genes than park soils (Mann-Whitney *U* test, adjusted $p < 0.05$) except for the bla_{SHV} genes, no significant differences were observed the relative abundances of all genes between agricultural soils with and without biosolids amendment. Excluding biosolids, *intI*1, *sul*1, and *erm*B genes were found dominant in soils of undeveloped lands and biosolid-amended agricultural soils, while the average relative abundances of *sul*2, *tet*A and *tet*W were in the trend of agricultural soils without biosolids amendment ≈ soils from undeveloped lands ≈ biosolid-amended agricultural soils > park soils (Fig. S3).

Compared to those in remote soils, relative abundances of all selected ARGs and *intI*1 in park soils were not significantly different from those detected in remote park soils (adjusted $p > 0.05$). Similarly, 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 (adjusted $p > 0.05$) (Fig. S4).

3.2. ARB in biosolids, biosolid-amended soils, and nearby and remote soils

The RRs of culturable ciprofloxacin-, erythromycin-, sulfamethoxazole-, and tetracycline-resistant bacteria in collected soil and biosolid samples from WWTP 3 were shown (Fig. 3). Overall, the resistance ratios of culturable erythromycin- and sulfamethoxazole-resistant bacteria were relatively higher than those of culturable ciprofloxacin- and tetracycline- resistant bacteria within each soil type. Among all culturable antibiotic-resistant bacteria in soils, park soils appeared to contain the highest resistance ratios. More specifically, the RRs of culturable erythromycin- and sulfamethoxazole-resistant bacteria in untreated sludge and dewatered anaerobically-digested biosolids were approximately 2–3 times higher than those in biosolid-amended soils and agricultural soils without biosolid amendments, while biosolids amendments had no significant impact on RRs of culturable ARB. Although the RRs in park soils were relative higher than those in remote park soils, the RRs in agricultural soils without biosolid amendments were generally identical to those in remote agricultural soils.

3.3. Spatial distribution of selected genes in soils

The influence of biosolids land application on the distribution of ARGs in soils was evaluated by relative gene abundances in agricultural soils as a function of distances to the biosolid spreading ground. Here, only agricultural soils were chosen assuming they have undergone similar anthropogenic activities that can contribute to AR. In sum, all relative abundances of selected ARGs herein exhibited a decreasing trend from the land application site into adjacent soil systems due to the effect of winds except for *tet*A/16S (Fig. 4). Relative abundances of *intI*1, *sul*1, bla_{SHV}, and *tet*W were significantly and negatively correlated with distance to the spreading ground (*intI*1/16S: $r = -0.39$, $p < 0.01$; *sul*1/16S: $r = -0.65$, $p < 0.01$; bla_{SHV}/

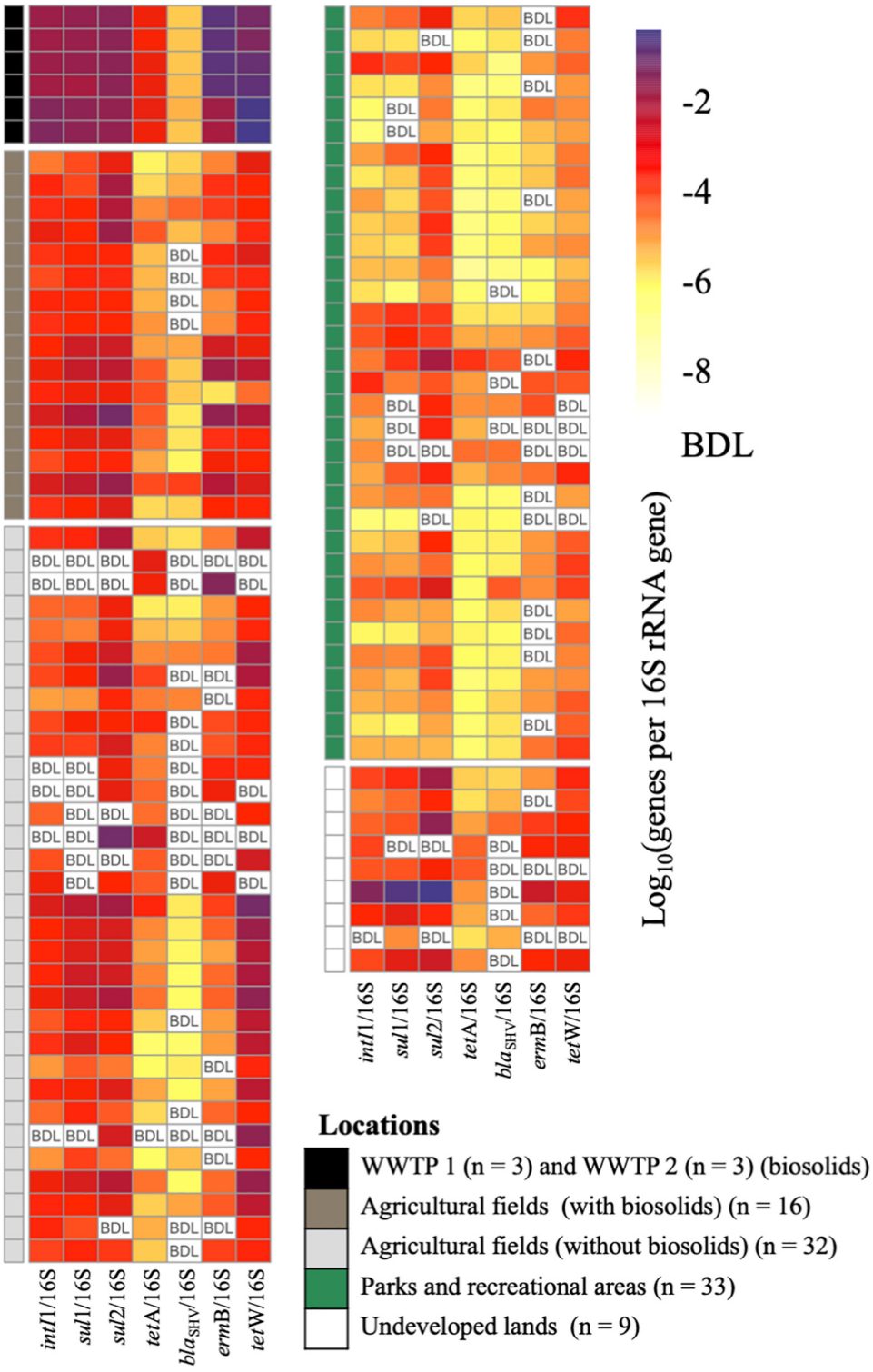

Fig. 1. Relative abundances of seven targeted genes (*intI*1, *sul*1, *sul*2, *tet*A, bla_{SHV}, *erm*B, and *tet*W) in soils from various types of locations. Abbreviation: BDL: Below Detection Limit; WWTP: Wastewater Treatment Plants.

16S: $r = -0.40$, $p < 0.01$; *tet*W/16S: $r = -0.45$, $p < 0.01$). In addition, average relative abundances of *sul*1 and *tet*W genes in agricultural soils within 2 km from the biosolid spreading site were significantly higher than those in agricultural soils further than 2 km from the biosolid spreading site ($p < 0.05$) (Fig. S5). All selected genes were less frequently detected (mostly BDL or DNQ) in agricultural soils as their distances to the biosolid site increased.

A spatial interpolation of measured soil ARG abundances in the present study was performed surrounding the farms where biosolids were applied (Fig. 5 and Fig. S6). The results indicated that soils in the vicinity of the

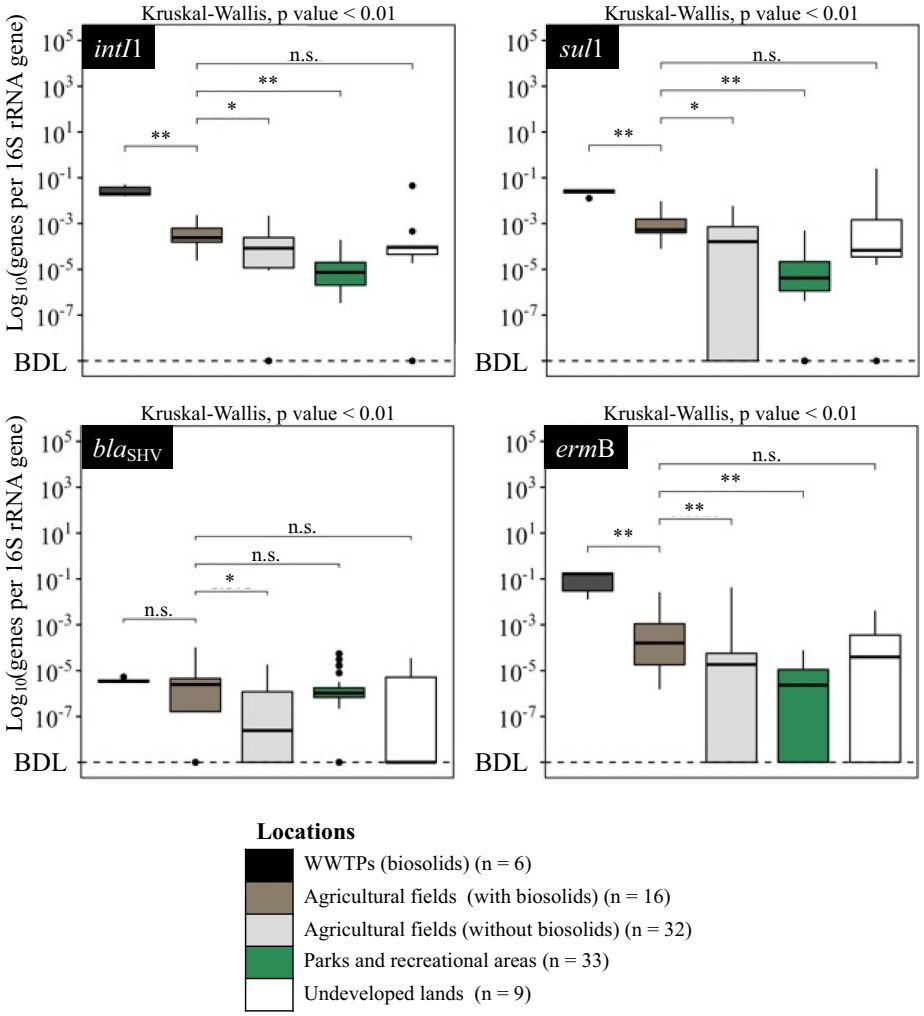

Fig. 2. Relative abundances of *intI*1, *sul*1, bla_{SHV}, and *erm*B genes among soils of various location types. The top and bottom boxes represent the 25th percentile and the 75th percentile. The whiskers exclude outliers and extend 1.5 times the interquartile range from both edges of the box. ** and * indicate statistical differences between soil types significant at $p < 0.01$ and 0.05. Abbreviation: BDL: Below Detection Limit; n.s.: Not significant ($p > 0.05$).

biosolid application site appeared to contain elevated levels of selected ARGs (Fig. 5 and Fig. S6). In particular, the heatmaps revealed a directionality of the genes transport in soils surrounding the biosolid land application site towards the northwest for relative abundances of *intI*1, *sul*1, *sul*2, which are consistent with the dominant wind direction in winter (Fig. S7). Relative gene abundances of *intI*1, *sul*1, *sul*2, and *tet*W genes were relatively low in the southwest and northeast regions from the biosolid application area (Fig. 5). In contrast, relative abundances of *erm*B, bla_{SHV}, and *tet*A genes from the spatial analysis did not clearly show a directional trend in soils around the biosolid land application site (Fig. S5).

3.4. Correlations between ARB, ARGs, and metal(loid)s

Metal(loid) concentrations in collected soil samples are summarized (Table S3). Significant correlations were found between selected gene abundances and total metal(loid) concentrations in collected soil samples from all types of land uses (Fig. 6). Levels of Co, Cu, Ni, and Fe were significantly positively correlated with relative gene abundances of *intI*1 (ρ = 0.24–0.40, adjusted $p < 0.05$), *sul*1 (ρ = 0.32–0.52, adjusted $p < 0.01$), and *tet*W (ρ = 0.29–0.40, adjusted $p < 0.05$). In addition, both total concentrations of Fe and Cu were significantly correlated with *sul*2/16S (Fe: ρ = 0.30, adjusted $p < 0.01$; Cu: ρ = 0.35, adjusted $p < 0.01$) and *erm*B/16S (Fe: ρ = 0.25, adjusted $p < 0.05$;Cu: ρ = 0.31, adjusted $p < 0.01$). However, Pb was significantly negatively correlated with *intI*1/16S ($\rho = -0.24$, adjusted $p < 0.05$) *tet*A/16S ($\rho = -0.40$, adjusted $p < 0.001$), and *tet*W/16S ($\rho = -0.24$, adjusted $p < 0.05$), respectively. While most metal(loid)s showed positive correlations with relative abundances of selected genes, most correlations appeared to be weak.

Significant correlations among ARB, selected gene abundances, and total metal(loid) concentrations in selected soil samples from all types of land uses were limited but high (Fig. S8 and Fig. S9). Fraction of sulfamethoxazole-resistant bacteria to total heterotrophic plate count was negatively correlated with total Ti ($\rho = -0.78$, adjusted $p < 0.05$)and Mn ($\rho = -0.87$, adjusted $p < 0.01$)concentrations. Fraction of tetracycline-resistant bacteria to total heterotrophic plate count was also negatively correlatively with total Zn ($\rho = -0.82$, adjusted $p < 0.05$). However, total As concentrations were positively significantly correlated with ciprofloxacin-resistant bacteria (ρ = 0.77, adjusted $p < 0.05$). Yet, no correlations were found between absolute and relative abundance of ARB and selected genes.

4. Discussion

4.1. WWTPs as significant reservoirs for ARGs

Both thermophilic and mesophilic AD have been studied for ARG removal in treated biosolids and compared to the effectiveness of other

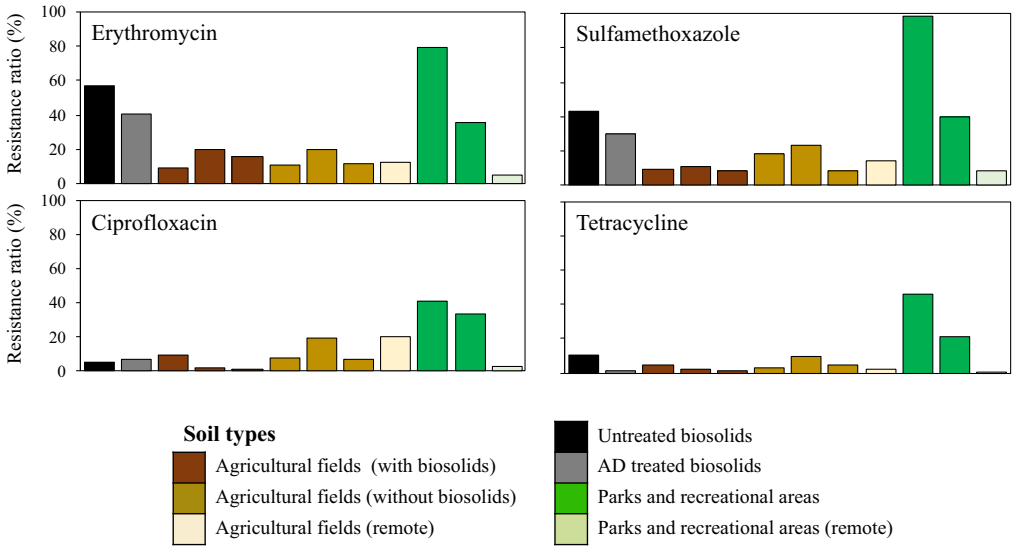

Fig. 3. Resistance ratios of culturable bacteria to different antibiotics (ciprofloxacin (4 ppm), erythromycin (10 ppm), sulfamethoxazole (50.4 ppm), and tetracycline (20 ppm)) in soils and biosolids. Error bars were not shown because plate counts were carried out in duplicate and averaged with two different dilutions for each sample. The fraction of colonies growing on a particular amended plate divided by the colonies growing on the unamended plate (total heterotrophic plate count) for that treatment is referred to resistance ratios.

conventional treatment methods (Munir and Xagoraraki, 2011; Sui et al., 2016). Previous studies suggested that thermophilic conditions were generally more favorable for ARG removals (Jang et al., 2017; Zhang et al., 2015). Furthermore, each ARG type may adapt differently to AD conditions depending on different locations and WWTP designs (Jang et al., 2017). For example, *tet*D and bla_{TEM} were found to be more persistent in AD-treated biosolids than other ARGs (Jang et al., 2017). Nonetheless, AD-treated biosolids from both thermophilic and mesophilic conditions can still contain high concentrations of ARGs and ARB compared to soil environments.

In the present study, relative abundances of ARGs and *intI*1 genes were at least an order of magnitude higher in AD-treated biosolids than those from other studies. The relative abundances of *sul*1 and *tet*W genes in anaerobically digested biosolids from seven WWTPs across Michigan ranged from 10^{-4} to 10^{-3} and 10^{-4} to 10^{-3} gene copies/16S, respectively (Munir and Xagoraraki, 2011). Relative abundances of *intI*1, *sul*1, *sul*2, *tet*A, and *tet*W genes in biosolids from Alloa, UK 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} gene copies/16S, respectively (Lin et al., 2019). Moreover, researchers

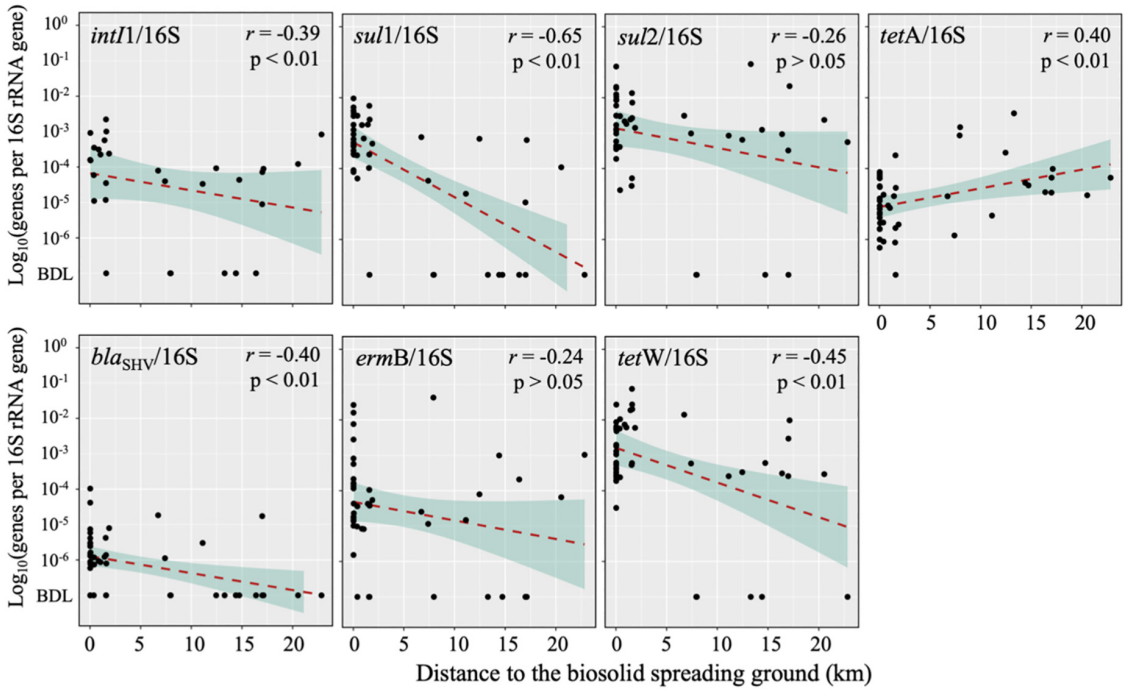

Fig. 4. The relationships between log-transformed relative abundances of selected genes (*intI*1 *sul*1, *sul*2, *tet*A, bla_{SHV}, *erm*B, and *tet*W) in agricultural soils and distances to the biosolid applied site. Abbreviation: BDL, Below the Detection Limits.

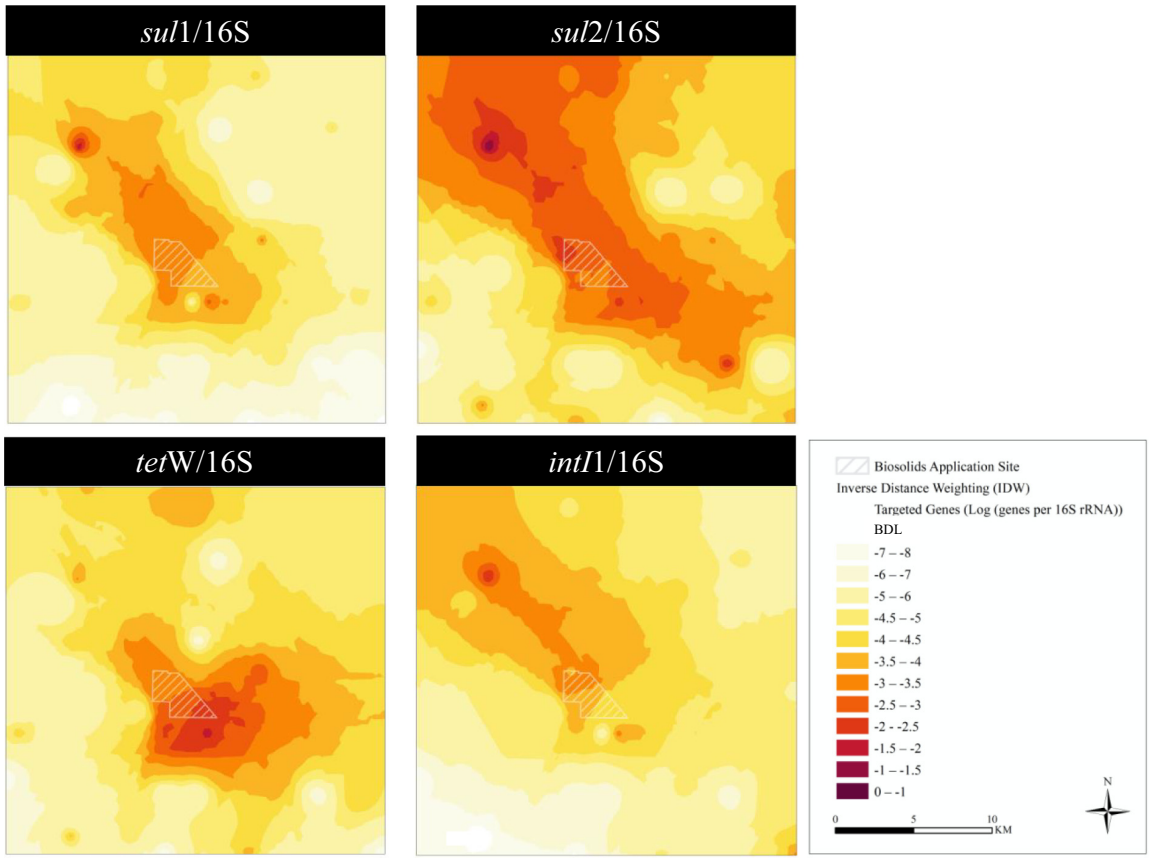

Fig. 5. Spatial interpolation of relative abundances of *intI*1, *sul*1, *sul*2, and *tet*W genes surrounding the biosolid land application site using spatial interpolation. Abbreviation: BDL, Below the Detection Limits.

found that biosolids had significantly higher ARG loads than those in the treated effluents (Munir et al., 2011). Therefore, this study confirmed the potential environmental health threats of AD-treated biosolids with high ARG abundances, which may contribute a significant burden of ARGs to soil environments if land-applied.

4.2. Effect of biosolids land application on agricultural soils

It is evident that AD-treated biosolids contain elevated levels of ARGs, yet the effect of incorporating land-applied biosolids to soils on the resistome is still not clear. While soils before the land application of biosolids were not available here, the effects of biosolid land application on ARGs were evaluated by comparing biosolid-amended soils to adjacent non-amended soils. The increased relative gene abundances of *sul*1, *sul*2, bla_{SHV}, and *ermB* in biosolid-amended soils were also demonstrated in previous studies where they were more abundant than soils before biosolid amendment (Lin et al., 2019; Munir and Xagoraraki, 2011; Tang et al., 2015). On a site in Michigan, the average relative abundances of *sul*1 and *tet*W genes in biosolid-amended soils were approximately 10^5 and 10^4 copies/g soil, respectively (Munir and Xagoraraki, 2011). Lin et al. (2019) assessed ARGs and *intI*1 genes in farm soils over time after land application of biosolids in northeast Scotland where 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). In comparison, ARG abundances reported herein (both relative and absolute gene abundances) were generally at least an order of magnitude higher than previously reported (Fig. S10).

Although biosolid application herein appeared to favor the proliferation of certain ARGs in biosolid amended soils, the effects of ARGs addition related to biosolids application appeared to be limited elsewhere (Brooks et al., 2007; Munir and Xagoraraki, 2011). The field site with different soil properties may show different responses to the biosolids application in terms of absolute ARG abundances (*tet*W, *tet*O, and *sul*1) (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. Previous studies have found that the occurrence, concentration, and spatial distribution of soil antibiotic contamination have close associations with land uses (Zhao et al., 2020). Sulfonamide and tetracycline resistance genes were the most frequently detected and abundant in agricultural soils compared to other ARGs (Lin et al., 2019). The effects of biosolid application on soil ARGs may be complicated as the background ARG abundances may have been influenced by aforementioned factors.

4.3. ARG comparison with native and remote soils

ARG and *intI*1 quantities in native, remote agricultural, and park soils were included for comparison as farming and gardening may exhibit AR anthropogenic inputs. Surprisingly, relative abundances of all targeted genes in remote agricultural and park soils were not statistically different from those in biosolid-amended soils and agricultural soils without biosolids and park soils, respectively. These insignificant differences imply that remote soils may already contain significantly abundant and diverse ARGs, due to the long-term human activities. Previous results also showed that sulfonamide resistance genes and tetracycline efflux genes were detected in 91% and 98% native prairie soils in Nebraska (Durso et al., 2016). In this study, relative gene abundances of *sul*1 and *intI*1 genes were still high in remote agricultural and park soils, which 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).

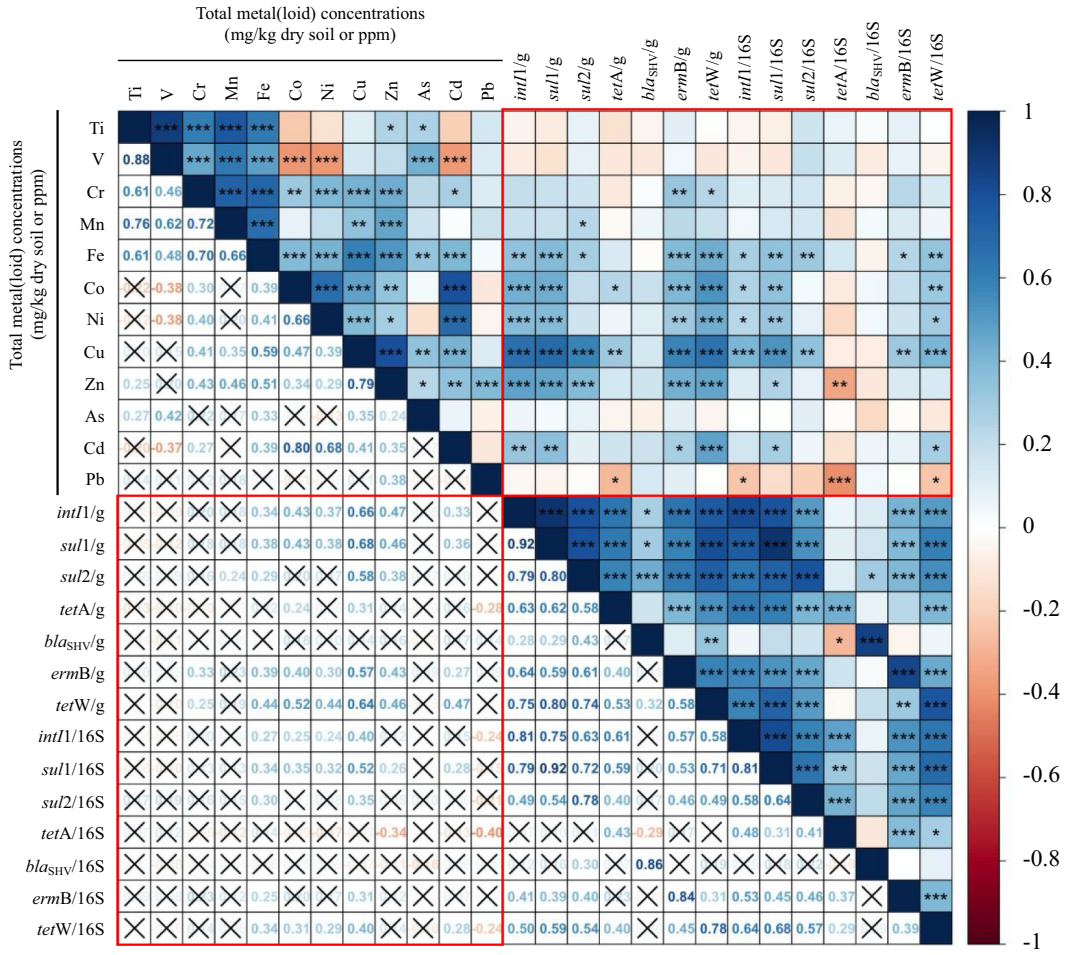

Fig. 6. Spearman's correlation analysis between targeted genes and total metal(loid)s (positive = blue, negative = red, X = not significant). Correlations significant at $p < 0.001$, $p < 0.01$, and $p < 0.05$ are marked with ***, **, and *, respectively.

Identifying and investigating the relatively pristine soil resistomes can be potentially challenging as human activities can impact the environment in many ways. Antibiotic resistance can occur naturally and their corresponding ARGs can still be found in natural soil environments. Antarctic and Tibetan soils, with hypothetically little to no human impact, have shown high abundances of ARGs (up to 3.2 genes/16S and 0.28 genes/16S) (Li et al., 2020; Wang et al., 2016). A previous study assessing ARGs in native soils from the Santa Monica Mountains were also included as soil ARG baselines with minimal human impacts for comparison (Cira et al., 2021). Average relative abundances of *intI*1, *sul*1, *sul*2, and *tet*W genes in native soils from the Santa Monica Mountains were significantly lower than those detected herein in undeveloped land soils, biosolid-amended soils, agricultural soils (without biosolids), and park soils (adjusted $p < 0.05$). This analysis suggested that ARGs in areas experiencing little anthropogenic impact may confer less resistance to commonly used antibiotics even if the total ARGs are high (Li et al., 2020).

4.4. ARB in biosolids, biosolid-amended soils, park, and remote soils

Both untreated sludge and anaerobically-digested biosolids from WWTP have been frequently recognized as significant reservoirs of numerous ARB (Brooks et al., 2007; Gao et al., 2012). Waste sludges reported by Gao et al. (2012) from East Lansing, Michigan had relatively higher absolute abundance of culturable sulfamethoxazole-resistant bacteria (range: 10^7–10^9 CFU/g) but similar absolute abundance of tetracycline-resistant bacteria (range: 10^5–10^7 CFU/g) compared to the present study (Fig. S11). In this work, ARB levels in dewatered anaerobically-digested biosolids were approximately 1–2 logs less than those in untreated sludge. Similarly, average absolute abundance of culturable ciprofloxacin- and tetracycline-resistant bacteria herein were found to be lower (approximately 2–3 logs) compared to those in biosolids from previous study across four different states (Fig. S11) (Brooks et al., 2007).

Overall, our results indicate that biosolids amendments had no significant impact on RRs of culturable ARB, suggesting that biosolids have relatively low or no long-term observable effects in the land-applied field compared to its adjacent soils in terms of absolute abundances and RRs of culturable ARB. It is likely that ARB abundances in soils following biosolid applications dissipate rapidly over time (Riber et al., 2014). Our results are consistent with those of previous studies in which long-term biosolid application does not increase absolute soil ARB concentrations (Brooks et al., 2012; Zerzghi et al., 2010). Negreanu et al. (2012) also found that the RRs of various culturable ARB in soil environments irrigated with treated wastewater were significantly decreased. However, the shift in the resistome implies pathogens at sites with specific land uses still have a greater chance of acquiring resistance genes. For example, RRs of ARB in park soils near the biosolid land application site were higher than those in agricultural soils regardless of receiving biosolids as well as remote park soils. One possible explanation is that ARB may have widely spread through airborne particulates and become prevalent around the biosolid application site (Ouyang et al., 2020).

4.5. Spatial distribution of selected genes in soils

Although the effect of biosolid application on the resistome of soils that received biosolids has been evaluated in several 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. Our interpolation results indicate a potential directionality of soil *sul*1, *sul*2, and *tet*W genes transport near the biosolid land application site. There is a significant potential for widespread distribution of ARGs from the land application site into adjacent soil systems due to atmospheric condition. Airborne transport of ARGs from surface soils is complicated, 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) demonstrated that airborne ARB and ARG levels were elevated in the vicinity of cattle farms where antibiotics are used relative to organic farms, which is consistent with the spatial observations of *intI*1, *sul*1, *sul*2, and *tet*W genes herein. Airborne particulate matter collected from downwind of feed yards contained more abundances of ARGs (i.e. tetracycline resistance genes), ARB, antibiotics, and distinct bacterial communities compared to upwind (McEachran et al., 2015). Meanwhile, PM_{10} concentrations at 3.5 km downwind of the boundary were observed to be approximately 8.5% of PM_{10} concentrations in the feed yard (Hiranuma et al., 2011), which agreed with the decreasing trend of ARG abundances with distance from the spreading ground.

Although our spatial interpolation results favor the hypothesis of the airborne transport ARGs from the biosolid application site, increasing sample size would warrant a more accurate interpolation result. The role of airborne ARGs transportation is needed for further study. In addition, many factors may govern soil ARG persistence and distribution, including HGT, metal(loid)s concentrations, and soil biochemical properties (e.g., pH, organic matter, and soil texture) (Hung et al., 2022). For example, relative abundances of the *intI*1 gene have been suggested as a good indicator of anthropogenic pollution and are commonly linked to genes conferring resistance to antibiotics and metal(loid)s (Gillings et al., 2015). In the present study, the relative gene abundances of *intI*1 were significantly correlated with relative gene abundances of *sul*1 ($r = 0.81$, adjusted $p < 0.001$), *sul*2 ($r = 0.58$, adjusted $p < 0.001$) and *tet*W ($r = 0.64$, adjusted $p < 0.001$). These findings suggested that the propagation of soil *sul*1, *sul*2, *tet*W may be facilitated by HGT of *intI*1 in soils, yielding different spatial distribution patterns.

4.6. Selective pressure for ARGs

Researchers have revealed that the spatial distribution of soil antibiotics was highly associated with land uses (Zhao et al., 2020). Plant microbiomes were found to carry diverse ARG profiles and significantly affect resistomes in peri-urban farmland soils (Xiang et al., 2020). Soil ARGs in different land-use types can also be affected by various human activities, such as fertilizers application and irrigation with treated wastewater effluents (Hung et al., 2022). As a result, many environmental factors, such as selective pressure discussed above, that may contribute to ARG proliferation must be considered. Even in the absence of AR-associated compounds from biosolids, agricultural soils can still foster the proliferation of ARGs. Antibiotics and metal(loid)s are frequent co-contaminants to accelerate the development of AR through co-selection of genes that protect both against antibiotics and metal(loid)s (Manaia et al., 2016). 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(loid) concentrations (Knapp et al., 2011), indicating metal co-selection (Baker-Austin et al., 2006). A meta-analysis indicated that Cu, Cd, and Zn are the metal(loid)s most likely to exert selective effects for antibiotic resistance in agricultural soils (Seiler and Berendonk, 2012), consistent with our results.

5. Conclusions

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 average relative abundances of *intI*1, *sul*1, bla_{SHV}, and *ermB* 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. Average relative abundances of selected genes (*intI*1, *sul*1, *sul*2, and *tet*W) in native soils were significantly lower than those detected in most other soil environments (adjusted $p < 0.05$). Most selected genes in agricultural soils decreased exponentially with distance to the spreading ground. Spatial interpolation patterns of soil relative abundances of *intI*1, *sul*1, and *sul*2 genes highly suggested a potential relation with dominant wind directions in winter. Culturable ARB concentrations did not vary significantly between biosolid-amended soil and surrounding soils. Lastly, total concentrations of Co, 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. The results also highlight the importance of considering relatively unstudied transmission routes, such as groundwater and air, when dealing with the current worldwide antibiotic resistance crisis.

CRediT authorship contribution statement

Wei-Cheng Hung: Conceptualization, Software, Data curation, Fund acquisition, Investigation, Methodology, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Yu Miao**: Investigation, Resources, Writing – review & editing, Visualization. **Nhi Truong**: Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Adriane Jones**: Conceptualization, Writing – review & editing. **Shaily Mahendra**: Conceptualization, Fund acquisition, Supervision, Writing – original draft, Writing – review & editing. **Jennifer Jay**: Conceptualization, Fund acquisition, Supervision, Project administration, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.153570.

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