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Disparate Antibiotic Resistance Gene Quantities Revealed across 4 Major Cities in California: A Survey in Drinking Water, Air, and Soil at 24 Public Parks

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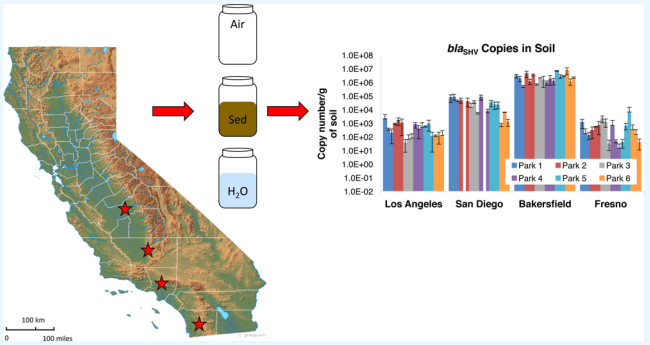
Supporting Information

ABSTRACT: Widespread prevalence of multidrug and pandrug-resistant bacteria has prompted substantial concern over the global dissemination of antibiotic resistance genes (ARGs). Environmental compartments can behave as genetic reservoirs and hotspots, wherein resistance genes can accumulate and be laterally transferred to clinically relevant pathogens. In this work, we explore the ARG copy quantities in three environmental media distributed across four cities in California and demonstrate that there exist city-to-city disparities in soil and drinking water ARGs. Statistically significant differences in ARGs were identified in soil, where differences in bla_{SHV} gene copies were the most striking; the highest copy numbers were observed in Bakersfield (6.0×10^{-2} copies/16S-rRNA gene copies and 2.6×10^{6} copies/g of soil), followed by San Diego (1.8×10^{-3} copies/16S-rRNA gene copies and 3.0×10^{4} copies/g of soil), Fresno (1.8×10^{-5} copies/16S-rRNA gene copies and 8.5×10^{2} copies/g of soil), and Los Angeles (5.8×10^{-6} copies/16S-rRNA gene copies and 5.6×10^{2} copies/g of soil). In addition, ARG copy numbers in the air, water, and soil of each city are contextualized in relation to globally reported quantities and illustrate that individual genes are not necessarily predictors for the environmental resistome as a whole.



INTRODUCTION

A sustained rise in antimicrobial resistance is predicted to lead to 10 million deaths per year by 2050, with the current resistance accounting for a minimum of 700 000 lives lost per year.[1,2] Concurrently, recent cases of multi- and pandrug-resistant bacteria have raised questions about the future of the current last line of defense antibiotics and, by extension, the future treatability of common bacterial infections.[3,4]

Emergence of antibiotic-resistant pathogenic bacteria has directed substantial attention toward antibiotic resistance genes (ARGs), which confer resistance and can be transferred among bacteria through horizontal gene transfer (HGT).[5] Additionally, environmental co-selective pressures can drive antibiotic-resistant strain development, promote persistence of ARGs, and result in ARG abundance in the absence of antibiotic selective pressure.[6−8] Considered as environmental contaminants of emerging concern, ARG abundance in environmental compartments warrants increased documentation.[9−12] Indeed, recent work has pointed to the importance of environmental routes for disease transmission,[13−15] with several examples of ARGs in clinical isolates believed to be derived from environmental bacteria.[15]

Possible ARG sources to the environment, including wastewater treatment plants,[16−18] confined animal feeding operations,[19,20] manure-fertilized fields,[21] and medical waste streams,[22,23] comprise an intricate system that consistently inputs ARGs into the environment. However, although these sources continue to be explored, the current volume of studies on the extent of dissemination of ARGs in environmental compartments is lacking.

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Table 1. *P*-Values from Welch's *T*-Test Comparisons between Soils in California Cities

Soil: bla_{SHV}	LA (copies/g)	SD (copies/g)	Bks (copies/g)	F (copies/g)
Los Angeles		0.024	0.003	0.340
San Diego			0.003	0.025
Bakersfield				0.003
Fresno				

Soil: bla_{SHV}	LA (copies /16S)	SD (copies /16S)	Bks (copies /16S)	F (copies /16S)
Los Angeles		0.017	0.003	0.055
San Diego			0.003	0.017
Bakersfield				0.003
Fresno				

Soil: *sul*1	LA (copies/g)	SD (copies/g)	Bks (copies/g)	F (copies/g)
Los Angeles		0.321	0.313	0.309
San Diego			0.186	0.063
Bakersfield				0.115
Fresno				

Soil: *sul*1	LA (copies /16S)	SD (copies /16S)	Bks (copies /16S)	F (copies /16S)
Los Angeles		0.369	0.284	0.287
San Diego			0.05	0.06
Bakersfield				0.85
Fresno				

Key: 0.05<P-value≤0.1 | 0.01<P-value≤0.05 | P-value≤001

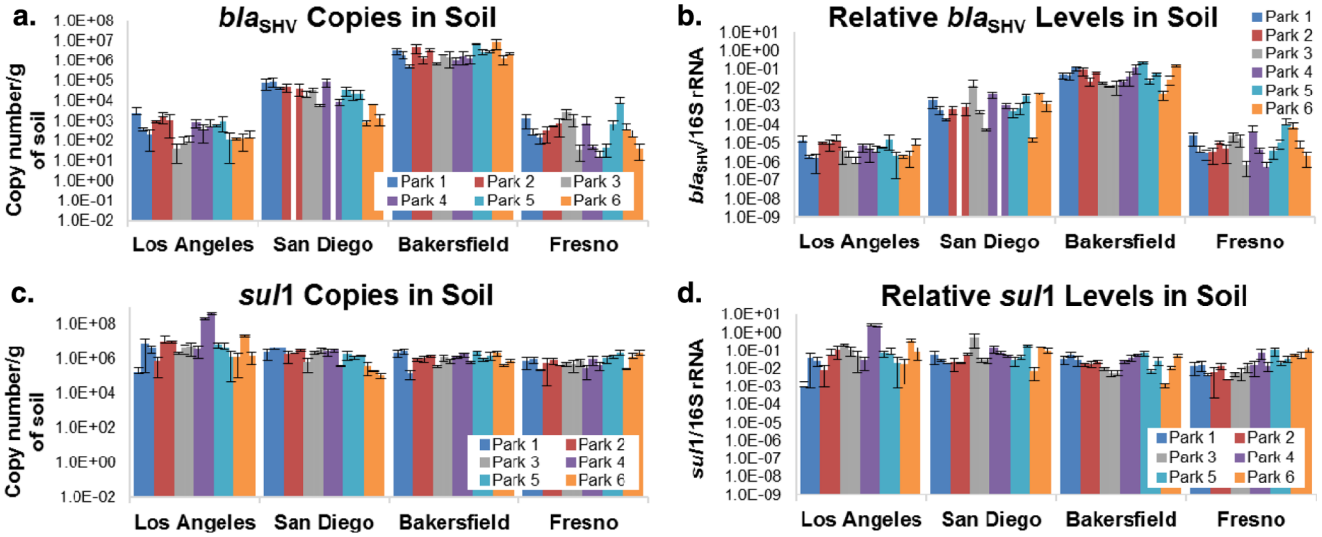

Figure 1. bla_{SHV} and *sul*1 ARG quantities in California soils. Soil ARG gene copy numbers across 24 parks in 4 California cities. Error bars denote intrapark variability when averaged over three sampling triplicates. (a) bla_{SHV} copy numbers normalized to per gram of soil, (b) bla_{SHV} copies per 16S-rRNA gene copies, (c) *sul*1 copy numbers normalized to per gram of soil, and (d) *sul*1 copies per 16S-rRNA gene copies.

Background ARG quantities in soils have been confirmed as altered and increasing due to anthropogenic antibiotic use,[19,21,24] but there is little research quantitatively reporting them. Instead, data often focus on fold increases due to a particular human activity, such as manure application[21,25−27] or reclaimed water use,[28−30] making site-to-site comparisons difficult. Studies on waterborne ARG quantities are more extensively available and have included surface,[31,32] ground[20] and coastal waters,[33] drinking water effluent,[34,35] and tap water,[36,37] but the large majority of investigation has not involved water systems that directly interact with general populations, leaving open questions concerning ARG exposure. Air is by far the least studied environmental compartment with the majority of research addressing only antibiotic-resistant bacteria (ARB).[38−40] Some quantitative ARG values have been reported; however, these are typically in response to and downwind of a suspected source, rarely concerning ARGs affecting populations outside of immediate feedlot vicinities.[41−43]

When available, studies reporting ARG quantities in environmental compartments are often difficult or impossible to compare, with researchers reporting results in different units, as well as reporting different genes. Moreover, a broad resistance profile of a particular site is rare, with many studies focused solely on a single environmental compartment. Several public databases and global surveillance projects exist; however, these focus on clinical isolates, rather than ARGs in the environment.

In this study, ARGs were assessed in 26 public parks across 4 major California cities and three environmental media: soil, drinking water, and air. It was hypothesized that unique ARG profiles exist across medium, genes and location, such that when comparing cities within California, city-to-city differences would be identified. Air, water, and soil from each site was analyzed for two ARGs, *sul*1 and bla_{SHV}, as well as the bacterial 16S-rRNA gene. Additionally, all soil samples were analyzed for the *erm*F and *erm*B genes. *sul*1 gene was selected for inclusion in this study due to its proposed use as an urbanization marker,[44] its propensity for persisting in the environment,[45,46]

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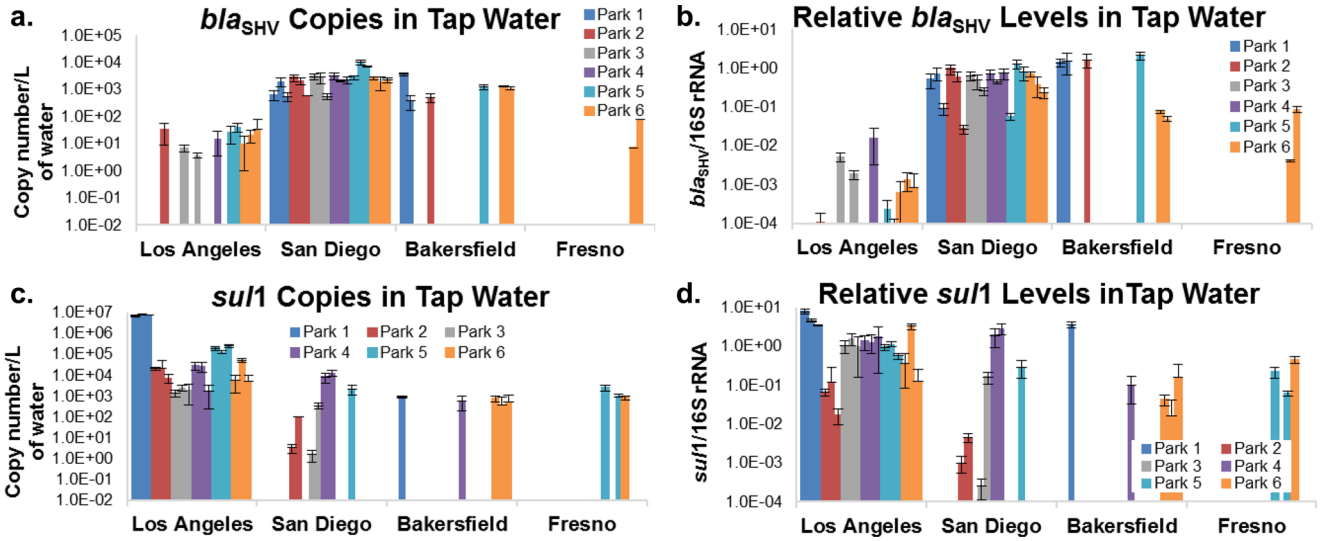

Figure 2. bla_{SHV} and *sul*1 ARG quantities in California tap water. Tap water ARG gene copy numbers across 24 parks in 4 California cities. Error bars denote intrapark variability when averaged over three sampling triplicates. (a) bla_{SHV} copy numbers normalized to per liter of water, (b) bla_{SHV} copies per 16S-rRNA gene copies, (c) *sul*1 copy numbers normalized to per liter of water, and (d) *sul*1 copies per 16S-rRNA gene copies.

Table 2. *P*-Values from Welch's *T*-Test Comparisons between Tap Water in California Cities

Tap Water: bla_{SHV}	LA (copies/L)	SD (copies/L)	Bks (copies/L)	F (copies/L)
Los Angeles		0.019	0.096	0.370
San Diego			0.035	0.019
Bakersfield				0.093
Fresno				

Tap Water: bla_{SHV}	LA (copies /16S)	SD (copies /16S)	Bks (copies /16S)	F (copies /16S)
Los Angeles		8.74E-05	0.083	0.288
San Diego			0.444	8.56E-05
Bakersfield				0.086
Fresno				

Tap Water: *sul*1	LA (copies/L)	SD (copies/L)	Bks (copies/L)	F (copies/L)
Los Angeles		0.347	0.346	0.346
San Diego			0.363	0.396
Bakersfield				0.739
Fresno				

Tap Water: *sul*1	LA (copies /16S)	SD (copies /16S)	Bks (copies /16S)	F (copies /16S)
Los Angeles		0.126	0.111	0.078
San Diego			0.869	0.382
Bakersfield				0.409
Fresno				

Key: 0.05<P-value≤0.1 | 0.01<P-value≤0.05 | P-value≤001

and a high degree of previously recorded data, which allows for ARG quantity contextualization. The bla_{SHV} gene was included in this study due to its close relationship to the genes suggested for environmental monitoring.[47] Additionally, along with *erm* genes, bla_{SHV} gene copy quantities had been shown to be increasing in soils.[24] *erm*B and *erm*F have both been proposed as indicators for assessing the antibiotic resistance status of a particular environment.[47] Together, *erm*B and *erm*F can shed light on possible differences in genes that are closely related and operate via similar resistance mechanisms.

Through this work, we sought to provide a more comprehensive approach to documenting ARG exposure across a given region by introducing a study design that has not been utilized in conjunction with ARG monitoring. Additionally, this study is to our knowledge, the first to contextualize reported ARG quantities in relation to those reported globally.

RESULTS

bla_{SHV}. Gene copies of bla_{SHV}, with gene copies defined as the quantity of the target gene present in sample as determined via qPCR, were regularly detected in all soil samples. For bla_{SHV} gene copy numbers measured per gram of soil, all city-to-city comparisons, with the exception of the Los Angeles−Fresno comparison, revealed statistically significant differences (Welch's *t*-test $p < 0.05$, comparing two cities at a time each with $n = 24$) (Table 1), with ARG copy numbers highest in Bakersfield (5.1×10^5 to 6.9×10^6 copies/g) followed by San Diego (7.7×10^2 to 9.0×10^4 copies/g when detected) and tailed by both Los Angeles and Fresno (3.5×10^1 to 2.1×10^3 and 1.5×10^1 to 6.7×10^3 copies/g respectively), as summarized in Figure 1. When normalized by 16S-rRNA gene copies, city-to-city differences persisted (Table 1), with all relationships now found to statistically differ ($p < 0.1$). City quantities for bla_{SHV} gene copies from highest to lowest were: Bakersfield (1.9×10^{-2} to 2.2×10^{-1} copies/16S-rRNA gene copies), San Diego (1.5×10^{-5} to 1.2×10^{-2} copies/16S-rRNA gene copies), Fresno (5.1×10^{-7} to 6.5×10^{-5} copies/16S-rRNA gene copies), and Los Angeles (8.2×10^{-7} to 1.6×10^{-5} copies/16S-rRNA gene copies), as summarized in Figure 1.

Detection of bla_{SHV} fluctuated in drinking water samples collected from Los Angeles, Bakersfield, and Fresno. In contrast, bla_{SHV} was detected in all San Diego drinking water

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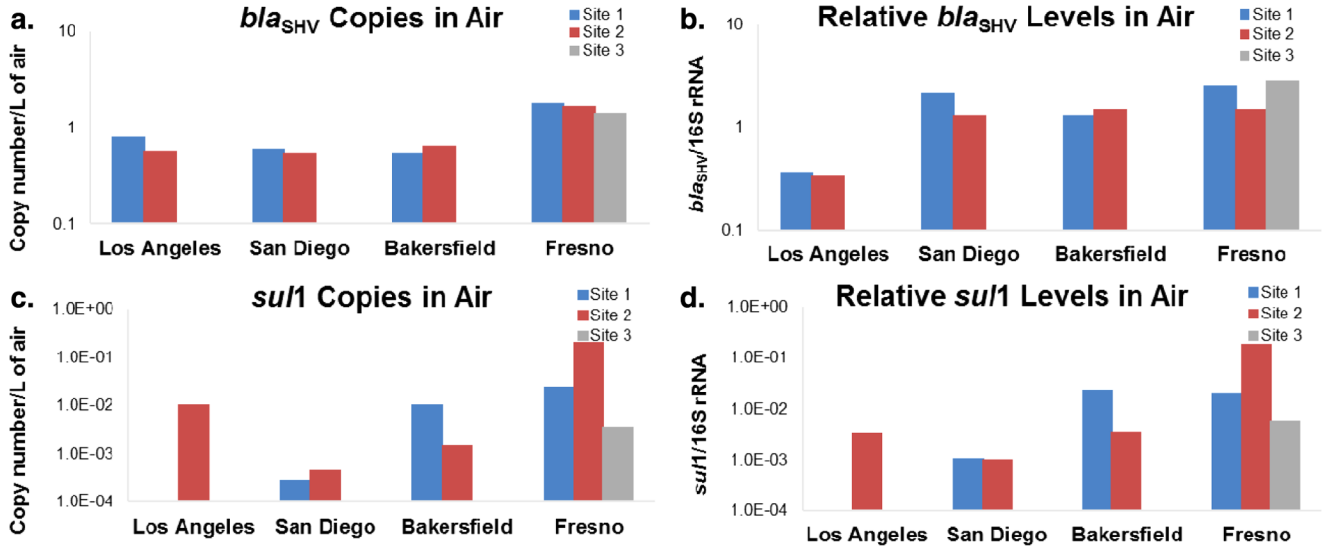

Figure 3. bla_{SHV} and *sul*1 ARG quantities in California air. ARG gene copy numbers in air across four California cities: (a) bla_{SHV} copy numbers normalized to per liter of air, (b) bla_{SHV} copies per 16S-rRNA gene copies, (c) *sul*1 copy numbers normalized to per liter of air, and (d) *sul*1 copies per 16S-rRNA gene copies.

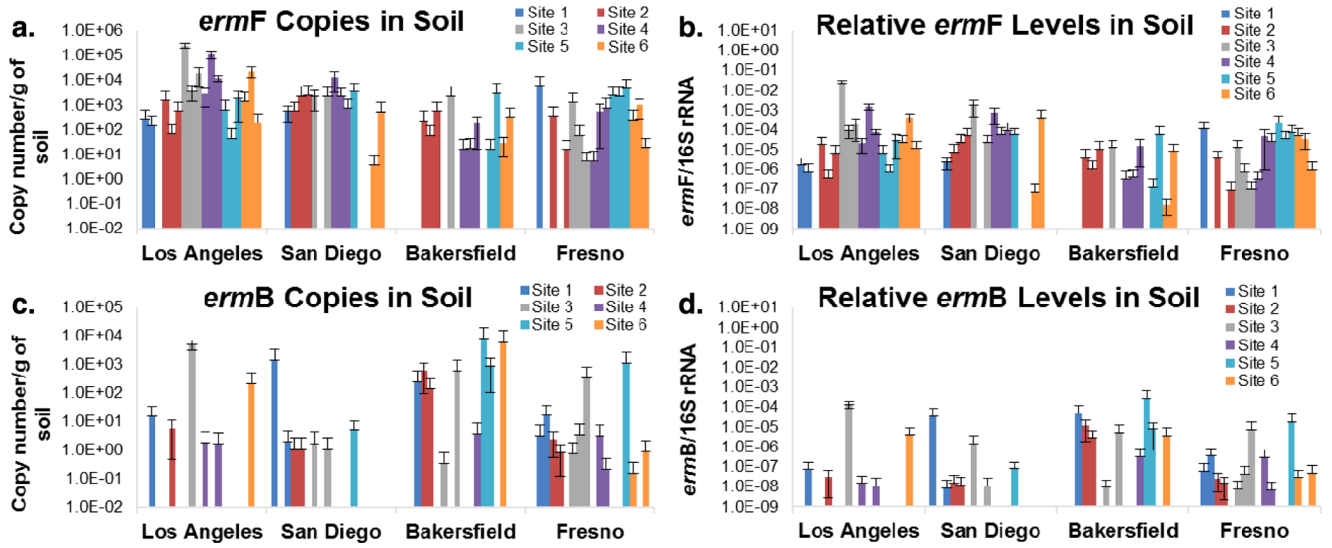

Figure 4. *erm*F and *erm*B ARG quantities in California soils. Soil ARG gene copy numbers across 24 parks in 4 California cities. Error bars denote intrapark variability when averaged over three sampling triplicates. (a) *erm*F copy numbers normalized to per gram of soil, (b) *erm*F copies per 16S-rRNA gene copies, (c) *erm*B copy numbers normalized to per gram of soil, and (d) *erm*B copies per 16S-rRNA gene copies.

samples, as shown in Figure 2 (gene copy numbers ranged from 5.5×10^2 to 10.0×10^3 copies/L). Gene copy numbers normalized to per liter of drinking water were statistically different when comparing each city ($p < 0.1$), with the exception of an LA−Fresno comparison (Table 2). Overall, the ranking of gene copy number from highest to lowest was found to be: San Diego, Bakersfield, and equivalent lower relative ranking for Los Angeles and Fresno. When gene copy numbers were compared per 16S-rRNA gene copy number (Figure 2), all city differences were statistically significant ($p < 0.1$), with the exception of the LA versus Fresno and San Diego versus Bakersfield comparisons (Table 2). When viewed in relation to 16S-rRNA gene, two tiers were seen, with San Diego and Bakersfield both showing higher gene copy numbers (2.7×10^{-2} to 1.3 and 5.0×10^{-2} to 2.1 copies/16S-rRNA gene copies respectively) than those of Los Angeles and Fresno (9.4×10^{-5} to 5.1×10^{-3} and 4.0×10^{-3} to 8.6×10^{-2} copies/16S-rRNA gene copies, respectively).

Because of sampling constraints, air was sampled at only two sites per city, with each site sampled in replicates of three to five. The exception to this was Fresno, where three sites were sampled. bla_{SHV} copy numbers per liter of air sampled revealed higher bla_{SHV} gene copy numbers in Fresno (0.19−600 copies/m^3), with the gene copy numbers in Bakersfield, Los Angeles, and San Diego all found to be lower than the Fresno gene copy numbers by approximately 50% but comparable to each other (Figure 3). When analyzed per 16S-rRNA gene copy number, Los Angeles gene copy numbers were the lowest (3.8×10^{-4} to 9.3×10^{-3} copies/16S-rRNA gene copies), with significant overlaps among the Fresno, Bakersfield, and San Diego sites (Figure 3).

***sul*1.** *sul*1 was consistently detected in soil across all parks and cities studied, with some variability observed to occur

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within any given park. City-to-city comparisons for *sul*1 gene copy numbers were found to be statistically different when comparing San Diego parks to those in Fresno (Table 1), with higher *sul*1 gene copy numbers measured in San Diego (p = 0.06). When normalized by 16S-rRNA gene copy number, San Diego was found to have a higher gene presence than both Fresno and Bakersfield ($p < 0.06$), with similar gene copy quantities observed in Fresno and Bakersfield (Table 1). Los Angeles exhibited the most park-to-park variability (ranging 1.5 × 10^5 to 4.0 × 10^8 copies/g and 9.0 × 10^{-4} to 2.7 copies/16S-rRNA gene copies), and ARG quantities did not prove to be significantly different from any of the other three cities, regardless of the normalization approach. *sul*1 values in soil were found to vary from 1.1 × 10^5 to 2.2 × 10^7 copies/g to 9.0 × 10^{-4} to 5.1 × 10^{-1} copies/16S-rRNA gene copies, with two LA park values yielding particularly high values of 2.0 × 10^8 and 4.0 × 10^8 copies/g, and 2.6 and 2.7 copies/16S-rRNA gene copies (Figure 1).

The presence of *sul*1 per liter of water sampled was highly variable in San Diego (detected in 30% samples), Bakersfield (detected in 21% of samples), and Fresno (detected in 13% samples), with Fresno having the lowest frequency of gene detection. In contrast, *sul*1 was detected in 100% (n = 18) of Los Angeles drinking water samples. Absolute gene copy numbers of *sul*1 in San Diego, Bakersfield, and Fresno proved too variable to result in statistically significant city-to-city differences when compared to those of Los Angeles and to each other, as shown in Figure 2 and Table 2. When analyzing gene copies per copies of 16S-rRNA gene (Figure 2), only the LA−Fresno comparison exhibited a statistically significant city difference, with higher gene copy numbers observed in Los Angeles than those in Fresno (p = 0.08).

*sul*1 gene copy numbers in air were found to vary widely within individual cities with Fresno air measurements ranging from 1.5 × 10^1 to 5.3 × 10^3 copies/m^3, Bakersfield ranging from 1.4 × 10^2 to 7.2 × 10^2 copies/m^3, Los Angeles ranging from 1.6 × 10^1 to 1.6 × 10^2 copies/m^3, and San Diego ranging from 1.7 × 10^{-2} to 2 copies/ m^3. Absolute copy numbers per liter of air indicate a higher incidence of *sul*1 gene found in Fresno site 2 when compared to that found in the other three cities (Figure 3). After normalizing by 16S-rRNA gene copy number, Fresno site 2 gene copy numbers remained highest, followed by Bakersfield site 1 as a distant second (Figure 3).

***ermF* and *ermB*.** Gene copy measurements for both *erm*F and *erm*B were obtained for soil samples, with both genes greatly fluctuating when detectable (Figure 4); *erm*F fluctuated between 3.9 × 10^0 and 2.6 × 10^5 copies/g and between 1.7 × 10^{-8} copies/16S-rRNA gene and 2.7 × 10^{-2} copies/16S-rRNA gene, whereas ermB fluctuated from 1.1 × 10^0 and 8.4 × 10^3 copies/g, and between 7.0 × 10^{-9} copies/16S-rRNA gene and 2.7 × 10^{-4} copies/16S-rRNA gene (Figure 4). In general, the *erm*F gene had a higher frequency of detection than that of *erm*B. *erm*F was consistently identified in Los Angeles park soils, with only one triplicate from site 1 failing to amplify via qPCR. All other cities contained at least one park with confirmed ARG readings obtained for only one out of three park samples. Overall, *erm*F detection frequency decreased in the order: Los Angeles, Fresno, San Diego, and finally Bakersfield. *erm*B was identified much less frequently, with the frequency of detection decreasing in the order: Fresno, Bakersfield, San Diego, and finally Los Angeles. No statistically different city-to-city relationships could be determined for either gene, regardless of the normalization technique. These results place *erm*F as per the above reported values and *erm*B as well below those currently shown in the literature.

California ARG Quantities in Global Context. Previous reports of *sul*1 per 16S-rRNA gene copies in archived soils, lake, and river sediments and environmental soil prior to manure or wastewater application allow this study to place average California soil quantities as generally above those currently documented in Germany,[21] along the Poudre River in Colorado,[31] and in a cornfield in Virginia.[26] However, the range of values seen in this study is wide enough to be inclusive of many values reported in the aforementioned studies. The highest values previously published are identified as those in sediment from Lake Geneva, Switzerland (approx. up to 0.08 copies/16S-rRNA gene copies).[48] The higher spectrum of values reported from the Lake Geneva site are found to be comparable to the quantities found in this study, although readings for San Diego and Los Angeles exceed the largest reported lake sediment values at several sampling sites. To our knowledge, these values place California *sul*1 gene copy numbers in soil above or comparable to the highest reported values. Absolute copy number was found to rarely be reported for *sul*1 in the soil compartment, with absolute values identified having also been reported as per 16S-rRNA gene copies.[28]

Gene copy numbers of *sul*1 normalized to per 16S-rRNA gene copies in aquatic environmental compartments have been documented in rivers, seas, lakes, bays, reservoirs, reclaimed water, and to a scarce extent, in drinking water. Placed within the context of these studies, it can be determined that drinking water ARG gene copy numbers observed in the California cities studied are generally higher than those recorded in lakes, rivers, and bays in locations ranging from Manila[49] to China[33] and Colorado[31] among others. Water bodies similar in order of magnitude to cities in California have been recorded in an urban and industrial river,[50] sites along the Baltic Sea,[51] Swiss lakes,[52] and in drinking water treated from Lake Taihu, China.[53] However, nearly all drinking water samples obtained from Los Angeles, CA, exceed these reported values by nearly an order of magnitude.

Relative to reporting for *sul*1, information on bla_{SHV} is infrequent, making global contextualization difficult. bla_{SHV} per 16S-rRNA gene copy numbers have been reported in sediments in Cuba as well as in archived Scottish soils.[7,54] ARG quantities in Cuban sediment were found to generally be comparable to soil values obtained for Fresno and Los Angeles, with Scottish soil values falling above those in Fresno and Los Angeles but below those in Bakersfield and San Diego. Absolute bla_{SHV} values were only found reported once in Cuban sediments, with values found to be comparable to San Diego.

Although there exists scarce reporting of the bla_{SHV} gene copy numbers in water bodies, this study is able to place bla_{SHV} drinking water quantities in Los Angeles and Fresno as below those reported in the Baltic Sea[51] and along the Almendares River in Cuba.[54] However, several Bakersfield and nearly all San Diego samples were found to fall within or above the range reported for the Baltic Sea (approx. 500−1000 copies/L). These two cities fell below the grand mean reported in the Almandres River in Cuba (approx. 10^5 copies/L), but Los Angeles gene copy numbers were generally within its range of seasonal variation (approx. 1.3 × 10^3 to 3.2 × 10^5).

*erm*F copies per 16S-rRNA gene copies were found to occur in the literature in archived Scottish soils,[7] with values far lower than the values frequently identified in this study. Other studies reporting *erm*F report nondetection of readings.[26] *erm*B values

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have been reported in sediments in Cuba ($10^{3.7}$−$10^{5.4}$ copies/g),[54] with these falling well above those identified in all California cities studied.

Intergene Observations. When analyzing drinking water samples, we observe that San Diego dominates in bla_{SHV} presence across the cities studied, with the dominant city shifting to Los Angeles when analyzing the *sul*1 gene. This implies that it is ineffective to test for a small number of genes and assume results to be representative of ARG abundance in general. This is further observed when observing *erm*F and *erm*B soil data. Both genes provide resistance to erythromycin, acting as adenine *N*-6-methyltransferases and keeping erythromycin from inhibiting aminoacyl translocation. Despite having a similar function and conferring resistance to the same drug, city-to-city detection comparisons reveal that one gene is not necessarily a predictor for the other. This indicates that even genes closely related in function may not be able to behave as indicators for each other.

Statistical Analysis. Linear regression and correlation matrices between each gene and population, population density, and distance from the nearest feedlot operation revealed no direct relationships in either environmental compartment. ARGs across each environmental compartment could not be largely attributed to a particular factor. Instead, there exists a multifaceted set of influences that make ARG quantity prediction complex. In addition, statistical analysis between genes across environmental compartments additionally yielded no direct relationship between genes.

Environment and Health. A study of the resistome in human and environmental samples in low-income human habitats indicated ARG sharing between human, animal, and environmental microbial communities, reporting a positive correlation between the proportion of ARG contigs with a mobile genetic element (MGE) and the number of habitats encoding antibiotic resistance proteins, indicating a role for MGEs in environmental transfer.[13]

It is when analyzing bla_{SHV} in soils that we see the highest indicator of disparities in ARG quantities across cities. This is pertinent, as it has been suggested that bla_{SHV} be given a REScon1 risk categorization, indicating that there exists published evidence that bla_{SHV} poses a substantial risk for resistance dissemination and treatment failure.[55] This is further alarming when considered in conjunction with the lack of current reporting for this gene, necessitating the re-evaluation of current approaches in reporting.

To conclude, this work amalgamates and extends observations reported globally on the quantity of ARGs in drinking water, soil, and air. Our results indicate that there exists a disparity in ARG quantities in the environment across four major cities within California. Additionally, this disparity is observed to be distinct within each environmental compartment as well as for each gene measured, highlighting the importance of testing a collection of ARGs to effectively identify environmental reservoirs and hinting at a complicated set of factors that dictate environmental ARG presence. These results were found to be consistent when gene copy numbers were analyzed by two different and widely used approaches, quantitative gene copy number per mass or volume of compartment as well as per 16S-rRNA gene copy. Future work should include follow-up studies to determine whether significant reservoirs for ARGs play the dual role of also being hotspots for HGT.

This article presents a monitoring approach that if standardized, prompts a more thorough global assessment and reporting of ARG quantities in environmental compartments. Moving forward, future regional assessments should focus on genes categorized as posing a higher risk for being acquired by and conferring resistance to human pathogens and should include data on co-contaminants, soil characterization, MGEs, and microbial community, where possible to create a larger database for statistical analysis. In doing so, further trends that might aid our understanding of antibiotic resistance proliferation and the dissemination of ARGs will be more easily identified.

MATERIALS AND METHODS

Site Selection. Los Angeles, San Diego, Fresno, and Bakersfield were chosen to provide a balance of land uses as well as a range of urbanization. For each city, three of the parks chosen were in the heart of the city, whereas the other three were positioned toward the outskirts. Proximity to major highways, where mixing of environmental influence and vehicular pollution was most likely, was avoided where possible. Priorities for inclusion in the study included accessible soil, presence of a water source, and access to a safe and stable air-sampling location. A catalog of parks chosen for inclusion in this study along with population, population density, zip code, degree of urbanization, and distance from the nearest feedlot can be found in Table 1 of the Supporting Information.

Park urbanization was categorized as high, mid, or low on the basis of the number of roads and buildings located within a 500 m radius of the park. The parks whose 500 m radius included ≥75 buildings and ≥15 roads were categorized as highly urban, whereas the parks whose radius included ≤50 buildings and ≤10 roads were categorized as having low urbanization. Any park that fell between these designations was denoted as having midlevel urbanization. Buildings that fell on the circumference line were included in the count if over half of the building fell within the designated area of interest. This urbanization scheme is a modified urban intensity index as defined by the U.S. Environmental Protection Agency (EPA). A summary of urbanization categorization for each site can be found in Table 1.

Sample Collection. *Soils.* Three meter-squared plots were randomly selected at each park for soil sampling. Top soil was collected in 50 mL sterile falcon tubes by randomly selecting ten points in each plot and dipping the falcon tube to gather top soil (0−2.5 cm), yielding a composite sample representative of the meter-squared plot. This was repeated for the remaining two plots, yielding three falcon tubes that would be taken as representative of the site. Rocks and grass were avoided or removed using sterilized plastic scoops. Samples were kept on ice until transported to the laboratory, where tubes were stored at 4 °C prior to processing. For processing, soil tubes were extensively shaken to homogenize the ten points of collection and 0.25 g of each soil was weighed into individual 2 mL screw cap tubes preloaded with 1 ± 0.05 g, 0.7 mm garnet beads (MO BIO Laboratories, Inc., Carlsbad, CA) before being stored at −20 °C to await DNA extraction.[56] The remaining soil was used to conduct a moisture content, hydrolysis (soil composition), and loss on ignition analysis (see Table 2). All of the soil was processed within a week of sampling. Three samples were collected from each of the 24 parks, resulting in a total of n = 72 soil samples.

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Tap Water. At each site, tap water was collected from either drinking fountains or exterior spigots. All water sources used were publicly accessible, and drinking fountains were prioritized whenever possible. Polypropylene plastic bottles used for sampling were washed and treated with 1.2 N HCl overnight before being rinsed three times with milliQ water immediately prior to sample collection. A total of 10 L of sample was collected at each park just prior to returning to the laboratory. Samples took several minutes to obtain, and the resulting 10 L were a composite of water initially exiting each fountain (no flushing) as well as after several minutes of flushing. Samples were kept on ice for the duration of transport and stored at 4 °C until the time of processing. Processing consisted of vacuum-filtering 3 L of sample water through a 47 mm diameter, 0.4 μm pore size polycarbonate filter (EMD Millipore, Billerica, MA). After filtration, filters were folded and placed into 2 mL screw cap tubes preloaded with 1 ± 0.05 g of 0.7 mm diameter garnet beads (MO BIO Laboratories, Inc., Carlsbad, CA), as required for DNA extraction. Tubes were subsequently stored at −20 °C, awaiting DNA extraction. All processing was performed in triplicate and all samples were processed within 48 h of collection.

Air. Air was collected in as close a proximity to parks as possible and not directly in parks due to considerations including park hours and duration of the sampling trip. Because of time constraints inherent to air sampling, not every park was sampled for air, yielding a smaller *n* for air than that for soil and tap water. Air was collected at two sites per city, except for Fresno, where samples were collected from three sites. All sampling occurred in the months of January through March, 2015 to ensure that all cities were sampled within the same season, minimizing seasonal variation as a possible cause for city ARG disparities.[57] In total, 24 samples were obtained for tap water and soil and 9 samples were obtained for air. Summary information for the sampling duration and sampling window can be found in Table S3.

Air sampling was conducted using three to five simultaneously operating personal pumps (SKC 224-PCXR4 Aircheck Sampler, SKC Inc. Eighty Four, PA). Operation was set to a flow rate of 2 L/min for at least 4 h, and air samples were obtained from a height of approximately 1 m. Longer sampling times were preferable if possible and variances between sampling duration were corrected for during data analysis. The use of 7 mm diameter, 0.4 μm pore size polycarbonate filters (EMD Millipore, Billerica, MA) and 45.72 mm, 1.6 μm glass fiber filters (EMD Millipore, Billerica, MA) was interchangeable, with glass fiber filters manually cut in a sterile environment to a size of 7 mm. A short preliminary experiment conducted prior to sampling concluded that both filter types yielded a comparable quantity of DNA for the same sampling duration upon extraction. Upon sampling completion, all filters were immediately folded and placed in 2 mL screw cap tubes preloaded with 1 ± 0.05 g of 0.7 mm diameter garnet beads (MO BIO Laboratories, Inc., Carlsbad, CA) as specified for DNA extraction. Sample tubes were placed on ice and kept at 4 °C to the extent possible before transport to the laboratory, where they were stored at −20 °C prior to DNA extraction.

DNA Extraction. All drinking water and soil DNA extractions were completed using the MoBio PowerFecal DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) within 1 week of collection. Air samples were extracted within 3 months of collection. Extractions proceeded as per the manufacturer's guidelines except for the cell lysis step, where bead-beating for 2 min via a BioSpec MiniBeadbeater-8 (BioSpec Products, Bartlesville, OK) was used in place of vortexing. An additional 2 mL screw cap tube preloaded with 1 ± 0.05 g of 0.7 mm diameter garnet beads (MO BIO Laboratories, Inc., Carlsbad, CA) and without sample (extraction blank) was included in every extraction to identify the contamination incurred during the extraction process, should it occur. Eluted DNA was aliquoted and stored at −20 °C, awaiting qPCR analysis. The total DNA concentration was determined using UV absorption via a Nanodrop 2000 C (Thermo Scientific, Waltham, MA), as were 260/280 absorbance ratios. Gene recovery for samples of similar soil composition extracted, as described in this study, was calculated via a matrix spike and found to be 83%. For matrix spike methods, see Supporting Information.

qPCR. All samples were analyzed for ARG abundance of *sul*1, bla_{SHV}, and the 16S-rRNA gene, (a total bacteria surrogate measure) via qPCR. Soil samples were additionally analyzed for *erm*B and *erm*F genes. All assays utilized SYBR Green Master Mix and entailed a 25 μL of reaction volume consisting of 12.5 μL of 2X SYBR Green Master Mix (Life Technologies, Grand Island, NY), 1.25 μL of each primer, forward and reverse primer, and at least 2 μL of the template DNA, with molecular-grade water comprising any remaining reaction volume. Primers used were as developed and validated previously in the literature[24,58] and can be found in Table S4, along with primer concentrations. Each assay run included a 7-point standard curve positive control, all applicable extraction blanks, and a negative control of molecular-grade water, with each sample plated in triplicate. All assays were performed in 96-well reaction plates using StepOne Plus (Applied Biosystems). The temperature cycles used can be found in Table S3 and were as reported previously in literature.[24,58]

Both soil and water samples were diluted to a concentration of 0.25 ng/μL prior to qPCR to offset inhibition effects, as confirmed by well spike and inhibition dilution. Although the *sul*1 gene assay was found to amplify smoothly for a total mass of 0.5 ng DNA, the bla_{SHV}, *erm*F, *erm*B, and 16S-rRNA gene assays were modified to contain 2.5 ng to properly amplify. Template DNA and molecular-grade water volumes were varied as was necessary for each assay. Dilution factors were back-calculated during data analysis to reverse this effect and obtain per volume or per mass quantification. This dilution process was not found to be necessary for air samples. For well spike and inhibition dilution methods, see Supporting Information.

Target-containing DNA fragments to serve as positive controls were designed using Geneious coupled with NCBI database information and ordered through IDT Technologies. Known concentrations of the designed DNA fragment were run alongside environmental samples, yielding a seven-point standard curve and allowing for quantitation of gene copies. Melt curves were used to further verify correct target gene amplification.

Cataloging Globally Reported ARG Quantities. When assessing reported ARG quantities, only peer-reviewed articles were considered. Literature was confined to those reporting genes corresponding to the present study, where qPCR was used to determine ARG in environmental samples. Studies assessing gene quantities in subsets, that is, ARB that were cultured and subsequently analyzed via qPCR, were excluded. Included studies reported values for gene count per volume or mass, or a ratio of gene of interest per 16S-rRNA gene. Values used for comparison to the present data set were estimated

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from plots where necessary, although directly reported values were prioritized when available. Values considered did not include quantities reported following an input of interest, that is, intentional antibiotic enrichment, reclaimed water use, metals enrichment, and so forth. It is important to note that due to limited reporting values presented here are compared to values across different years.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.7b00118.

Detailed tables for study locations, soil characterization results, air sampling details, and qPCR primer sequences (PDF)

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The authors declare no competing financial interest.

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