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Methicillin-resistant Staphylococcus aureus in Southern California coastal waters: environmental exposure to humans and stormwater biofilters as a preventative solution

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Methicillin-resistant *Staphylococcus aureus* in Southern California coastal waters: environmental exposure to humans and stormwater biofilters as a preventative solution

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Civil Engineering

by

Megyn Brynna Rugh

2021

ABSTRACT OF THE DISSERTATION

Methicillin-resistant *Staphylococcus aureus* in Southern California coastal waters: environmental exposure to humans and stormwater biofilters as a preventative solution

by

Megyn Brynna Rugh

Doctor of Philosophy in Civil Engineering

University of California, Los Angeles, 2021

Professor Jennifer Jay, Chair

Antibiotic resistance is one of the greatest challenges to modern global health. Sources of antibiotic resistant bacteria (ARB) and antibiotic resistances genes (ARGs) to surface water include wastewater treatment plants, medical waste streams, and agricultural sites. Recent studies have identified stormwater as a source of ARGs, and often stormwater contains other contaminants such as heavy metals and antibiotics that select for antibiotic resistance. As an emerging contaminant, ARB and ARGs are not regulated or traditionally monitored in

recreational swimming waters that may receive stormwater runoff. This research is divided into two parts: 1) Chapter 1 is an epidemiological study on surfers' exposure to ARB in the ocean, and 2) Chapters 2 and 3 investigate the fate and transport of ARB, ARGs, and other pathogens and indicators in stormwater biofilters.

Exposure to sources of ARB has been associated with colonization and infection in human populations, and recent work suggests the environment serves as an open reservoir available for transferring human pathogens. Surfers are a unique population for evaluating the relationship between environmental exposure and ARB colonization. Surfing involves a high frequency of unanticipated head submersions, exposures of long duration, and surfers are in the ocean year-round, particularly in the winter when storms occur, resulting in poor water quality due to urban stormwater contaminants. Beginning Fall 2018, two Santa Monica Bay surfing beaches were monitored for Methicillin-resistant *Staphylococcus aureus* (MRSA). Nasal swab samples were concurrently taken from a group of surfers and a non-surfing control group to investigate how this pathogen colonizes humans. Presumptive MRSA was always detected in marine samples, with highly elevated levels observed after stormwater runoff events. Surfers that surfed during wet-weather events were over six times more likely to be colonized by MRSA compared to controls, and also over three times more likely to be colonized than dry-weather surfers. This research suggests that the ocean may be an important reservoir of MRSA and have a special role in pathogen transmission to humans.

Stormwater biofilters are a promising passive treatment solution for reducing microbial pollution in surface waters. 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. The

co-occurrence of heavy metals and ARGs is important because of known heavy metal co-selection in environmental compartments. Surface soil samples from six biofilters and bioswales in Southern California over three time periods were analyzed for ARGs, mobile genetic element (*int*I1), 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 *int*I1 levels in biofilters were statistically greater than those detected in pristine soils. Total concentrations of arsenic, copper, lead, vanadium, and zinc exhibited significant correlations individually with relative abundances of *sul*1, *sul*2, *tet*W, and *int*I1. 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.

While stormwater biofilters have been shown to remove chemical contaminants such as nutrients and heavy metals, their efficacy in removing microbial pathogens has been understudied. Full-scale biofilter studies are rare as most biofilter research has been conducted with laboratory-scale biofilters. Additionally, microbial removal is typically evaluated using traditional fecal indicator bacteria (FIB) as a proxy for pathogen removal, and it is not known if traditional indicators accurately reflect pathogen removal within biofilters. A pilot-scale biofilter located on the Glassell Public Works campus (Orange, CA) was synergistically studied for removal of conventional fecal indicators, bacterial and viral fecal source markers, antibiotic resistance genes, and bacterial and viral pathogens. Log reduction of fecal indicator bacteria (both genetic and culture-based) was high. Some of the pathogens tested were effectively

removed, while the biofilter itself served as a reservoir for two pathogens (*Campylobacter* and *Salmonella*). The removal of HF183 did not match FIB or pathogen removal, with no removal observed. Viral fecal source markers PMMoV and crAssphage had satisfactory log reductions that were more comparable to those observed in both FIB and some pathogens. ARGs and *intI*1 showed gene-specific log reduction. These findings suggest that FIB and fecal source markers may not adequately represent pathogen removal in stormwater biofilters.

This research will enhance our knowledge of the fate and transport of ARG and ARB in surface water and stormwater biofilter infrastructure. Additionally, it will serve to further our understanding of how the environment may transfer ARB to humans. The results of this research can illuminate appropriate public health responses and mitigation efforts for reducing antibiotic resistance in the environment.

The dissertation of Megyn Brynna Rugh is approved.

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Mathew Gutierrez performed DNA extractions while Marina Feraud and Jessica Kurylo collected soil samples from the biofilter. Sumant Avasarala performed extractions for bioavailable and total heavy metals. Nhi Troung assisted with creating figures and data analysis. Patricia Holden, Stanley Grant, Haizhou Liu, and Jennifer Jay were all the co-PIs of the entire collaboration.

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PRESENTATION

Rugh, M.B. "MRSA in Southern California Coastal Waters and its Impact on the Surfer Microbiome," *Global Wave Conference*, Gold Coast, Queensland Australia, February 12, 2020.

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Rugh, M.B. "MRSA in Southern CA Coastal Waters and its Impact on the Surfer Microbiome," *Water Microbiology Conference*, University of North Carolina at Chapel Hill, May 2019.

Rugh, M.B. "Antibiotic Resistant Genes and Copper Co-Selection in Stormwater Biofilter Microcosms," *Water Microbiology Conference*, University of North Carolina at Chapel Hill, May 2018.

Chapter 1: Colonization of methicillin-resistant *Staphylococcus aureus* in surfers following exposure to stormwater-impaired marine water in Southern California

Introduction

The proliferation of antibiotic resistance is a problem of increasing concern worldwide. It is estimated that 700,000 people die every year from antibiotic-resistant infections and this number is expected to rise to 10 million deaths by 2050 (IACG on Antimicrobial Resistance 2019; World Bank 2017). In the United States more than 2.8 million antibiotic-resistant infections occur annually, resulting in 35,000 deaths (CDC 2019). One particularly concerning antibiotic-resistant pathogen is methicillin-resistant *Staphylococcus aureus* (MRSA), categorized as a serious threat by the U.S. Center for Disease Control (CDC) (CDC 2019).

S. aureus is an opportunistic pathogen that can yield skin and soft tissue infections, invasive blood and heart infections, and lung infections all of which can lead to sepsis and death. (Stapleton and Taylor 2007; Klevens et al. 2007). *S. aureus* colonizes the nares and skin asymptomatically (Sakr et al. 2018), but is a serious risk factor for transmission or eventual infection (Paule et al. 2009). Strains of this organism are designated as MRSA if they are resistant to all beta-lactam antibiotics, including natural and synthetic penicillins (methicillin, oxacillin, ampicillin), cephalosporins, and carbapenems (Pandey and Cascella 2019; Pantosti and Venditti 2009). MRSA is responsible for 323,700 infections in hospitalized patients and 10,600 deaths in the U.S (CDC 2019). Approximately 32% of U.S. adults are colonized by antibiotic-susceptible *S. aureus* and one percent colonized by MRSA (P. L. Graham, Lin, and Larson 2006).

Historically considered a nosocomial disease, MRSA is increasingly ubiquitous in surface water (Akanbi et al. 2017; Fogarty et al. 2015; Soge et al. 2009; Roberts, Soge, and No 2013; Thapaliya et al. 2017). Sources of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) to the aquatic environment include wastewater treatment plants, medical waste streams, and agricultural runoff (Vikesland et al. 2017; Ya He et al. 2020). In Southern California both methicillin-susceptible *S. aureus* (MSSA) and MRSA were detected in seawater and beach sand in a 2012 study by Goodwin et al. (K.D. Goodwin & Pobuda, 2009; Goodwin et al., 2012). To our knowledge, MRSA in Southern California marine water has not been monitored in the 13 years since the previous study and it is unknown how prevalence has changed. Additionally, marine water samples were taken during dry summer months so there is no understanding of how wet weather events might impact MRSA distribution. The Southern California coast has precipitation events that can cause high loadings of fecal indicator bacteria (FIB) and other pathogens into local waters. Swimming near Los Angeles storm drain discharges and surfing during wet-weather events leads to higher incidences of gastrointestinal and respiratory illnesses, along with other ailments including infections and fever (Haile et al. 1999; Schiff et al. 2016; Soller et al. 2017; Arnold et al. 2017). Recent studies have also identified stormwater as a source of ARGs into urban creeks and lakes (Garner et al., 2017; Kawecki et al., 2017; Zhang et al., 2016; Ahmed et al 2018). It is plausible that Southern California wet-weather events could cause an increase in MRSA concurrent with elevated FIB levels.

While the relationship between illness and exposure to impaired water following wet-weather events has been explored, the potential for ambient exposure to antibiotic resistant bacteria (ARB) including MRSA in surface water needs to be investigated. There is currently a dire need for prospective studies on the link between antibiotic resistant bacteria (ARB) exposure

and the human microbiome. Exposure to sources of ARB been associated with colonization and infection in human populations; however, questions remain about the directionality of transfer and the complex fate of ARB in the environment. There are documented associations between exposure to ARB sources and colonization and infection in certain human populations. Recently it was proven that strains of multidrug-resistant *Escherichia coli* were transferred from grocery poultry products to humans, resulting in urinary tract infections (Liu 2018). Several studies have also established that livestock workers have occupational exposure to ARB and have a higher risk of MRSA colonization and carriage (Becker et al. 2017; Huang et al. 2014; Larsen et al. 2015; Nadimpalli et al. 2015; Rinsky et al. 2013; Ribeiro and Zeferino 2017; T. C. Smith 2015; Voss, Loeffen, and Bakker 2005; Ye et al. 2015).

Surfers are an ideal population for evaluating the relationship between environmental exposure to MRSA and subsequent colonization. Surfing involves a high frequency of unanticipated head submersions, exposures of long duration, and swallowing considerably more water than other beach users (Harding et al., 2015; Stone et al., 2008). Surfers are in the ocean year-round, particularly in the winter when storms occur, resulting in poor water quality due to urban stormwater contaminants (Arnold et al., 2017). Additionally, surfers will often either ignore or miss warning signs regarding water quality or beach closures. One survey reported that approximately 40% of surfers were not sure whether they surfed during a health advisory, while 28% reported that they proceeded to surf when they knew at the time a health advisory was posted (Harding et al. 2015). With regard to a link between antibiotic resistance and recreational exposure to degraded water quality, recent work found U.K. surfers were three times more likely to be fecal carriers of cefotaxime-resistant *Escherichia coli* than a control group that had very little exposure to seawater (Leonard et al. 2018). However, biological samples were collected

from surfers and the control group at just one time; thus, exposure followed by colonization could not be established.

This research was a controlled, prospective study of the impacts of recreational exposure to MRSA on surfers at beaches in the Santa Monica Bay from October 2018 to March 2020. Surfers and non-surfers were monitored at baseline in the fall and during wet weather when water quality was most degraded. Water samples and nasal swabs were monitored for MRSA and FIB to document temporal relationships between exposure and colonization. This work addresses the following hypotheses: 1) Wet weather events increase MRSA concentrations in recreational water due to stormwater runoff, 2) There is an association between MRSA colonization and exposure to impaired water quality, and 3) There is an association between adverse health outcomes in surfers and exposure to ocean water after rain events. The purpose of this research is to further the understanding of environmental exposures and transmission of MRSA to humans. It will inform the development of affordable monitoring techniques for ARB in environmental media that affect public health and generate new tools for assessing patterns and trends in antibiotic resistance proliferation.

Methods

Site selection

Southern California has a Mediterranean climate which is characterized by dry summers and mild, wet winters. Los Angeles County has an average of 16 inches of rain per year, which is 22 inches below the country's average. As an extremely urbanized county with 75 miles of coastline, stormwater runoff pollution poses a persisting issue. Lack of year-round rainfall causes a disproportionate amount of microbial and chemical pollution to enter the Santa Monica Bay

during the first major rain event, leading to extremely degraded water quality. Venice Beach (33.985393, -118.475363) and El Porto Beach (City of Manhattan Beach 33.9007136, -118.421606) were chosen as the two study sites due to their popularity amongst surfers and their impaired winter water quality (Figure 1) (Beach Report Card, 2020).

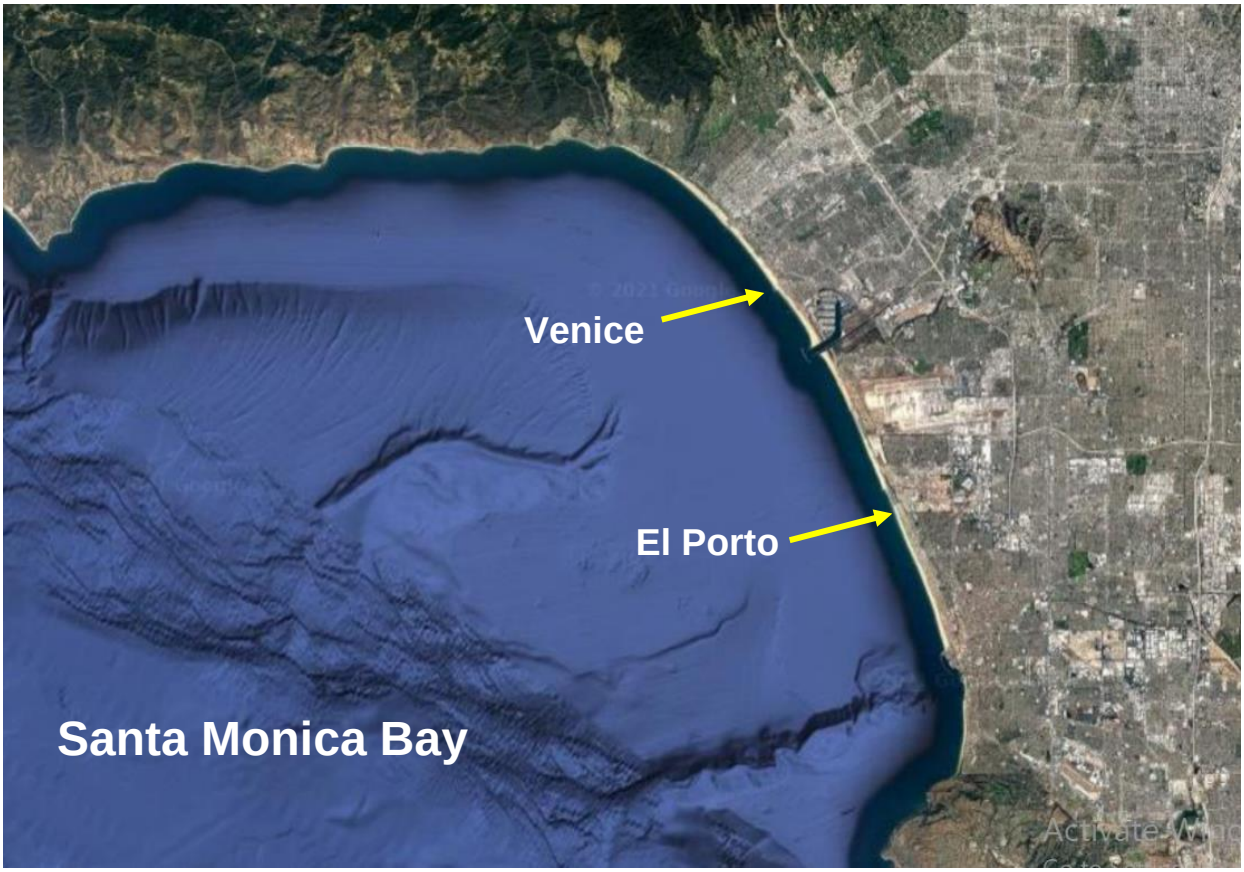

Figure 1: Los Angeles is the most populated county in the U.S. with over ten million people. Accordingly, most surf spots are extremely crowded with surfers.

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Quantification of MRSA and FIB in marine water

Marine water was collected from Venice Beach and El Porto Beach from October 1, 2018 to April 24, 2019 and September 25, 2019 to March 4, 2020. For the 2019 to 2020 monitoring season, a Hydrolab HL4 Multiparameter Sonde (OTT Hydromet, Loveland, Colorado) was used to collect seawater temperature, pH, turbidity and conductivity. Water samples were collected in collected in knee-deep water during an incoming wave between 6:45-7:45 AM. Sterile 2L Nalgene containers were first rinsed once with sample, filled completely, capped, and then placed on ice. Water samples were transported to the laboratory within three hours of collection. Upon laboratory arrival, water samples were processed within three hours.

MRSA was enumerated following an adapted protocol from previous studies that utilized culture-based methods to quantify MRSA in seawater (K.D. Goodwin and Pobuda 2009; Kelly D. Goodwin et al. 2012). with an antibiotic supplement was used to quantify MRSA. Membrane filtration was conducted with gridded 0.45 µM filter paper (EMD Millipore, Burlington, MA). A 20 µm nylon net (EMD Millipore, Burlington, Massachusetts) was stacked directly on the gridded filter to minimize difficulty counting colonies on turbid filters. The volume of seawater filtered varied according to appropriate volume necessary to yield 20 to 60 colonies. Seawater was preceded through the filter by a 20 mL phosphate buffer solution (PBS) prime and followed by a 20 mL PBS rinse. The filters were incubated on CHROMagar™MRSA (DRG International, Springfield, NJ) at 37°C for 18-24 hours. In addition to a CHROMagar™MRSA filter blank, a method filter blank was incubated on tryptic soy agar (TSA), which was made from Tryptic Soy Broth (BD Bacto, Fisher Scientific, Waltham, MA) and agar (Fisher Science, Hampton, NH). Each batch of CHROMagar™MRSA agar was tested with a positive (environmental MRSA strain containing *nuc*, *clf*A, and *mec*A gene and negative (ATCC® 25923) control strain of *S.*

aureus. MRSA morphology was identified following the recommendations of Goodwin & Pobuda (2009) and the CHROMagar™MRSA contained beta-lactam antibiotic supplement. Thus, all MRSA enumerated from marine samples are presumptive MRSA. Concentrations of MRSA were calculated in colony forming units (CFU) per 100 mL of water.

A subsample (n = 55) of MRSA colonies were streaked three times and underwent Kirby-Bauer disc diffusion to determine phenotypic antibiotic susceptibility to four other antibiotic classes: tetracycline (30 μg/disk), trimethoprim-sulfamethoxazole (25 μg/disk), erythromycin (15 μg/disk), and ciproflaxin (5 μg/disk). Preparation of disc diffusion plates was performed following the methodology of (Hudzicki 2012) and the zone of inhibition was interpreted following Clinical and Laboratory Standards, 30th edition (CLSI, 2020).

For the 2018 to 2019 monitoring season FIB data were downloaded from the California Waterboard's website (https://www.waterboards.ca.gov). This database compiles all the public water quality monitoring data occurring in the state of California. For the 2019 to 2020 monitoring season, total coliform, *E.coli* and *Enterococcus* were quantified by Colilert-18™ and Enterolert™ (IDEXX, Westbrook ME) following manufacturer's recommendations.

Participant recruitment

Surfers were recruited from El Porto and Venice Beach, both directly on the beach and by posting information on the Surfrider Los Angeles' social media account. The nonsurfer population was recruited from three local environmental nonprofits and UCLA. Participants qualified as surfers if they reported as surfing on average once a week or more. Nonsurfers, hereafter called "controls", were individuals that self-reported that they did not swim or submerge in the ocean during the winter months. All participants had to be aged 18 or older.

Prior to first sample collection, all participating individuals filled out both a consent form and an enrollment survey (IRB#18-001123). Returning participants filled out a separate, shorter follow-up survey. All surveys included questions pertaining to demographic, health, and behavioral data.

Detection of MRSA in surfers and controls

Human nasal swab samples were collected from surfers and controls from October 1, 2018 to April 24, 2019 and October 23, 2019 to March 20, 2020. The monitoring period was intended to end April 30, 2020 but was ended prematurely due to COVID-19 outbreak. Human nasal samples were collected from surfers at Venice Beach and El Porto Beach between 6:30AM and 8AM. Surfers were swabbed before they entered the ocean to surf to minimize the chance that incubating MRSA from a previous surf session would be rinsed out. Surfers were encouraged to provide multiple nasal swabs per month so that they could be monitored over time. However, due to the voluntary nature of the study, some surfers were swabbed just one time while other surfers chose to be swabbed multiple times. Control subjects were swabbed monthly during business hours. Similarly, some control subjects were swabbed just one time while other control subjects chose to be swabbed multiple times.

For each sampling event participants used one BBL Stuart Media swab (BD, Franklin Lakes, NJ). Participants were provided with medical gloves and swabbed themselves by placing the swab approximately half an inch up their nostril, rotating the swab five times counter-clockwise and then five times clockwise. After sample collection, the swab was placed back into its transport tube, immediately placed on ice, and transported to the laboratory for analysis within two hours. Upon arrival, the BBL swabs were inoculated into Tryptic Soy Broth (BD Bacto,

Fisher Scientific, Waltham, MA) and incubated at 37°C for 18-24 hours. An enrichment step is more sensitive for detecting MRSA than direct plating of the nasal swabs (Brown et al. 2005). After incubation 30 µL of the enriched broth was subsequently inoculated onto CHROMagar™MRSA media containing antibiotic supplement and incubated again at 37°C for 18-24 hours. Following manufacturer's guidelines, mauve colony growth was considered MRSA positive.

Up to five presumptive MRSA colonies were selected and streaked three times for isolation prior to secondary confirmation tests. DNA was extracted from the MRSA isolates with ZymoBIOMICS DNA Mini Kit (Zymo Research, Irvine, CA). Molecular confirmation of *Staphylococcus aureus* was conducted via PCR of the *nuc* gene using the forward primer 5'-GCGATTGATGGTGATACGGTI-3 and reverse primer 5'-AGCCAAGCCTTGACGAACTAAAGC-3' (Brakstad, et al., 1992). The PCR amplification was performed in a GeneAmp 9800 thermocycler (Life Technologies, Carlsbad, CA). The reaction occurred in a 20 µL volume and consisted of Invitrogen™ Platinum™ II Hot-Start PCR Master Mix (2X) (Life Technologies, Carlsbad, CA) and 200 nM of each primer. The reaction conditions are as follows: an initial denaturation at 94°C for one minute, followed by 37 cycles of denaturation at 94°C for one minute, annealing at 60°C for 30 seconds, and DNA extension at 72°C for 1.5 minutes. There was a final DNA extension at 72°C for 3.5 minutes and then PCR products were stored at 4°C prior to gel electrophoresis. The PCR product was analyzed by using a 1.2% agarose FlashGel™ Cassette on a FlashGel™ System (Lonza, Morristown, NJ). The DNA cassette was run for less than 10 minutes at 100V. Each cassette contained ATCC 25923 as a positive control and DNA free water as a negative control. Samples were identified as *S. aureus* if there was a band at 267-bp, which corresponded to the presence of the *nuc* gene.

Presumptive MRSA isolates underwent Kirby-Bauer disc diffusion to determine phenotypic antibiotic susceptibility to five other antibiotic classes: tetracycline (30 μg/disk), trimethoprim-sulfamethoxazole (25 μg/disk), erythromycin (15 μg/disk), ciproflaxin (5 μg/disk), and vancomycin (30 μg/disk). Preparation of disc diffusion plates was performed following the methodology of (Hudzicki 2012) and the zone of inhibition was interpreted following Clinical and Laboratory Standards, 30th edition (CLSI, 2020).

Data Analysis

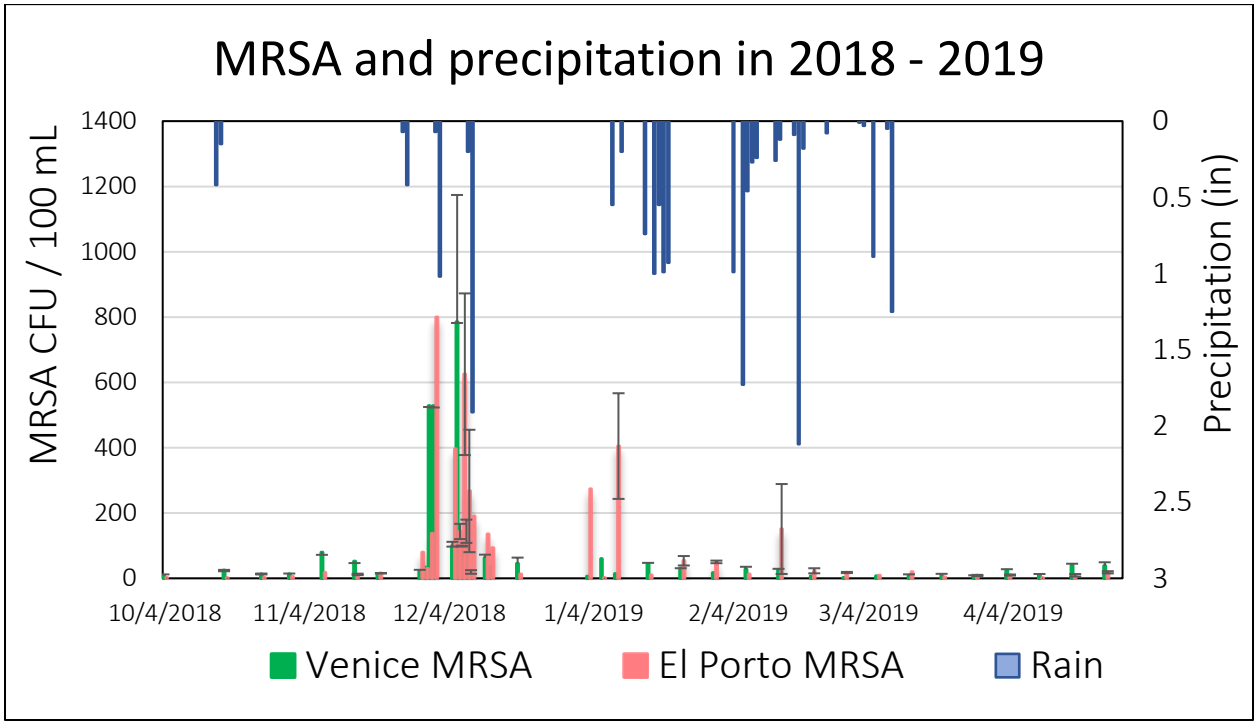
Marine samples were considered representative of wet-weather events if any precipitation occurred that day or in the following three days. Similarly, if surfers were swabbed during a week of rain greater than 0.10 inches, and self-reported either surfing the day it rained or in the following three days, that was considered a wet-weather event. If surfers self-reported that they had not surfed since the last swabbing event, that swab was excluded from analysis.

To determine if differences between two groups were statistically significant, the risk ratio, 95% confidence intervals, and p-value were calculated. In a comparative analysis, the precipitation data from the National Oceanic and Atmospheric Administration (NOAA) (https://www.weather.gov/lox/) were used to establish relationships between MRSA and rain events throughout the year. A Welch's unpaired two-tailed t-test was utilized to compare differences between MRSA concentrations. The concentrations were transformed logarithmically before performing the t-test so that the geometric mean could be used. Relationships between FIB, physical and chemical parameters, and MRSA were based on Spearman rank correlations.

Results

Detection of FIB and MRSA in seawater

Marine water samples were collected on 128 days throughout the two winter seasons. For Venice Beach 33 samples were collected during wet-weather and 30 during dry-weather. For El Porto 26 samples were collected during wet-weather and 39 during dry-weather. MRSA was always detected in El Porto (n = 65) and Venice (n = 63) marine water (Figure 2).



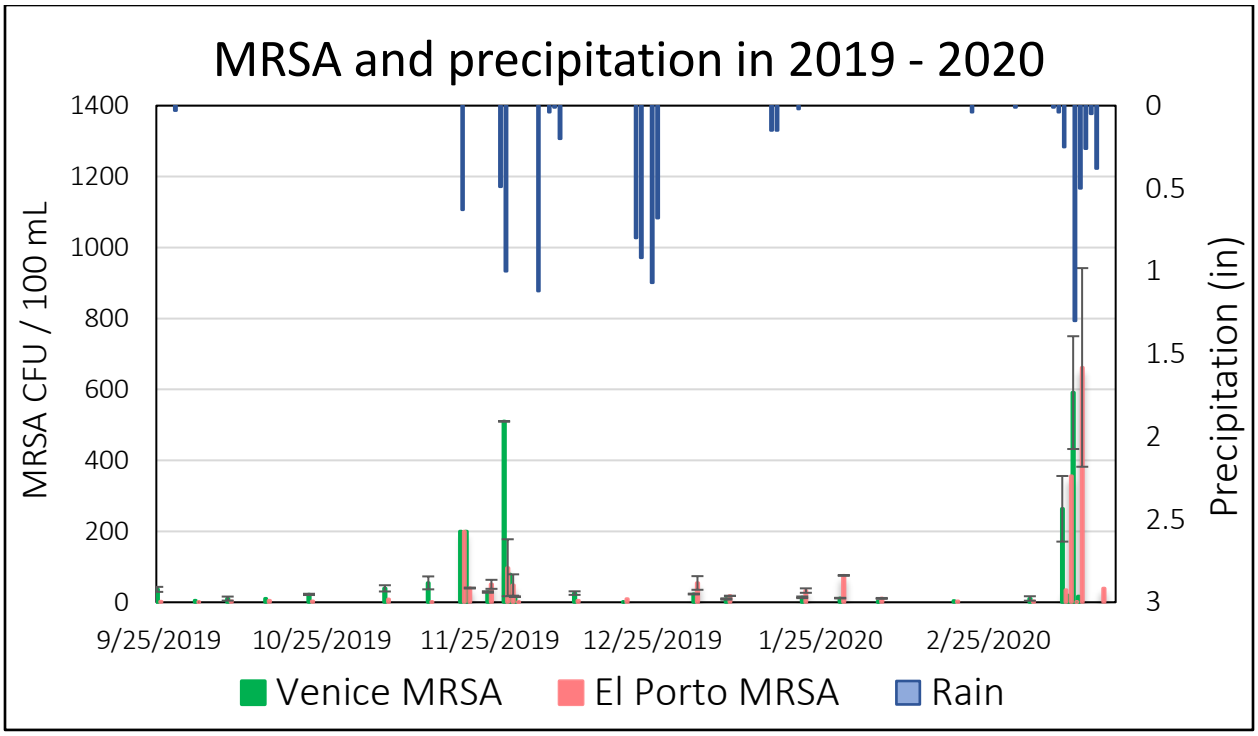


Figure 2: Red and green bars show measured MRSA levels in water at El Porto and Venice beaches, respectively, as a function of time through the 2018-19 wet season (top) and 2019-2020 wet season (bottom). Precipitation (inches of rain recorded) is depicted with blue lines from the upper x-axis.

For El Porto, MRSA concentrations during wet-weather events were significantly greater than during dry-weather events ($t = 4.05$, $p < 0.01$). The geometric average for dry-weather was 10 CFU per 100 mL and for wet-weather was 60 CFU per 100 mL. For Venice Beach, MRSA concentrations during wet-weather events were also significantly greater than during dry-weather events ($t = 3.09$, $p < 0.01$). The geometric average for dry-weather was 17 CFU per 100 mL and for wet-weather was 48 CFU per 100 mL. There were no significant differences between the dry-weather concentrations observed at Venice and El Porto ($t = 1.63$, $p = 0.11$), nor significant differences between the two beaches' wet-weather MRSA concentrations ($t = 0.44$, $p = 0.66$).

The maximum concentrations of MRSA from El Porto and Venice Beach correspond to first flush events during the early 2018 wet weather season. MRSA levels for El Porto were greater than 800 CFU per 100 mL after 1.02 inches of precipitation on November 29, 2018 and

630 CFU per 100 mL after 1.91 inches on December 6, 2018. The most elevated MRSA level for Venice Beach was 790 CFU per 100 mL after the same December 6 rain event. The most elevated levels for the second year of monitoring occurred much later in the season following a 0.5-inch rain event on March 13, 2020. El Porto had 660 CFU per 100 mL and Venice had 590 CFU per 100 mL.

For El Porto there were no significant differences in MRSA concentrations from each year's dry and wet weather (2018-2019 vs 2019-2020 wet weather MRSA concentrations $t = 1.62$, $p = 0.12$ and 2018-2019 vs 2019-2020 dry weather MRSA concentrations $t = 1.25$, $p = 0.22$). For Venice there were no significant differences in MRSA concentrations from each year's dry and wet weather (2018-2019 vs 2019-2020 wet weather MRSA concentrations $t = 0.25$, $p = 0.8$ and 2018-2019 vs 2019-2020 dry weather MRSA concentrations $t = 1.41$, $p = 0.17$).

A small fraction of marine MRSA isolates was resistant to erythromycin (7.3%, 4/55), trimethoprim / sulfamethoxazole (1.9% , 1/54) and tetracycline (1.8%, 1/55). None of the samples were resistant to ciprofloxacin. Multi-drug resistance (MDR) is defined as resistance to three or more classes of antibiotics (Magiorakos et al. 2012). None of the isolates expressed resistance to more than two of the tested antibiotics, so they are not considered MDR, although they certainly could be resistant to classes not tested in this study.

For 2019 to 2020 season, Venice MRSA concentrations were significantly correlated with total coliform ($\rho = 0.50$, $p = 0.03$), *E.coli* ($\rho = 0.54$, $p = 0.01$) and *Enterococcus* ($\rho = 0.53$, $p = 0.02$). El Porto MRSA concentrations and total coliform, *E. coli*, *Enterococcus* were associated but not significantly correlated ($\rho = 0.30$, $p = 0.18$, $\rho = 0.20$, $p = 0.39$, and $\rho = 0.40$, $p = 0.08$, respectively). There were no significant relationships discovered between FIB and MRSA in 2018 to 2019 due to limited data from the database.

For the 2019 to 2020 monitoring season, for Venice the turbidity was significantly associated with MRSA concentrations ($\rho = 0.74$, $p < 0.001$). Venice's water temperature and conductivity were weakly associated with MRSA concentrations ($\rho = 0.37$, $p = 0.14$; $\rho = 0.21$, $p = 0.41$). El Porto's water temperature and conductivity were not found to be associated with MRSA concentrations ($\rho = -0.16$, $p = 0.52$; $\rho = 0.17$ $p = 0.50$). The turbidity was very associated with MRSA concentrations but not significant ($\rho = 0.36$, $p = 0.13$). For both beaches, dissolved oxygen did not seem to be associated with MRSA concentrations (Venice $\rho = 0.01$, $p = 0.97$; El Porto $\rho = 0.20$, $p = 0.42$). Due to a machine sensor error, the pH data were not a realistic measure of ocean conditions and could not be analyzed.

MRSA detected in surfers and controls

There were 566 total swabs collected from 186 surfers and 53 control subjects over the study duration (Table 1). For the duration of the study, MRSA was detected in 21/446 surfer swabs (4.71% of surfers) and in 2/120 control swabs (1.67% of controls). With the exception of one surfer, all of the incidences of MRSA never more than once in each positive individual. For the one surfer that had MRSA twice, there were two months in between where the individual tested negative many times, so the MRSA colonization was considered as two separate events.

Table 1: Demographic characteristics, incidences of dry and wet-weather surf events, and incidences of MRSA colonization.

Characteristics	Venice	El Porto	Controls
Number of Participants	164	22	53
Mean Age [years]	34	54	32
Female sex [n(%)]	14 (12.6%)	2 (10%)	35 (69%)
Total swabs collected in 2018 - 2019 [n(%)]	144 (42%)	128 (38%)	68 (20%)
Total incidences of MRSA colonization [n(%)]	10 (6.9%)	7 (5.5%)	1 (1.5%)
Wet-weather surf event [n(%)]	10 (6.9%)	33 (26%)	
Dry-weather surf event [n(%)]	112 (78%)	87 (68%)	
Excluded or unknown [n(%)]	22 (15%)	8 (6.3%)	
Total swabs collected in 2019 - 2020 [n(%)]	109 (48%)	65 (29%)	52 (23%)
Total incidences of MRSA colonization [n(%)]	3 (2.8%)	1 (1.5%)	1 (1.9%)
Wet-weather surf event [n(%)]	9 (8.3%)	12 (19%)	
Dry-weather surf event [n(%)]	80 (73%)	50 (77%)	
Excluded or unknown [n(%)]	20 (18%)	3 (4.6%)	

Surfers that surfed during wet-weather events were over three times more likely to be colonized by MRSA compared to surfers that surfed during dry-weather (risk ratio = 3.27, 95% CI 1.32 to 8.12, p = 0.01). Notably, surfers that surfed during wet-weather events were over six times more likely to be colonized by MRSA compared to controls (risk ratio = 6.56, 95% CI 1.40 to 30.7, p = 0.02). Surfers that surfed during dry-weather were colonized by MRSA at twice the rate of controls, but it was not significant (risk ratio = 2.00, 95% CI 0.45 to 8.92, p = 0.36).

During the first winter (2018 to 2019) El Porto surfers that surfed during wet-weather were over ten times more likely than controls to experience MRSA colonization (risk ratio = 10.3, 95% CI 1.25 to 84.68, p = 0.03). Similarly, the wet-weather El Porto surfers were six times more at risk for MRSA colonization than their dry-weather El Porto surfing counterparts (risk ratio = 6.60, 95% CI 1.34 to 32.32, p = 0.02). During this same monitoring period (2018 to 2019) Venice surfers that surfed during wet-weather were also over six times more likely than controls to experience MRSA colonization (risk ratio = 6.8, 95% CI 0.46 to 100, p = 0.02). Lastly, the wet-weather Venice surfers were slightly more at risk for MRSA colonization than the Venice dry-weather surfers (risk ratio = 1.60, 95% CI 0.22 to 11.74, p = 0.64). During the second winter season, there were only five incidences of MRSA. Unfortunately, due to the risks presented by the COVID-19 pandemic, human and environmental sample collection ended two months earlier than planned.

Of the MRSA collected from surfers, 9.5% (2/21) were resistant to tetracyclines, 19% (4/21) were resistant to trimethoprim / sulfamethoxazole, and 47.6% (10/21) were resistant to erythromycin. None of isolates were resistant to ciprofloxacin, although 19% (4/21) expressed intermediate resistance. Of the two incidences of MRSA in controls, one was resistant to trimethoprim / sulfamethoxazole and the other one was resistant to tetracycline.

Discussion

This project was designed to address several research gaps. First, this study aimed to increase understanding of the persistence of MRSA in recreational seawater through dry-weather monitoring and adaptive sampling during storm events. Secondly, this study investigated the role of the environment in the transmission of MRSA, which has important public health implications. Even at a relatively small scale, this study gave statistically significant results tying

MRSA ocean water exposure to nasal colonization. This work addresses the critical knowledge gap concerning the role of the environment in the growing and alarming worldwide spread of antibiotic resistant illnesses in humans.

These findings indicate that MRSA is pervasive in the Santa Monica Bay and might be more widespread than previously measured. In a 2008 survey of Malibu, Dana Point, and Catalina Island, MRSA was only detected in 1.6% of dry-weather seawater samples (Goodwin et al., 2012). For this study, MRSA was detected in all dry and wet-weather seawater samples. MRSA was detected at a much greater frequency in Venice and El Porto than in Hawaii and Florida, but the wet-weather and dry-weather concentrations for all sites are comparable (Economy et al., 2019; Plano et al., 2013). Clearly, there are complex, year-round sources of MRSA into the Santa Monica Bay.

Precipitation events were associated with a significant increase in MRSA concentrations in both beaches. This effect was particularly noticeable in 2018, when there were several major rainstorms following a period of intense drought beginning in 2011. This result was also observed in Hawaii, when rainfall after a dry period caused especially high concentrations of estuarine *S. aureus* (Economy et al. 2019). It is estimated that rain events in Los Angeles causes 10 billion gallons of polluted runoff to enter Santa Monica Bay (DPW, n.d.). Because of this, it is recommended to avoid swimming for 72 hours following rainfall. In 2018 at El Porto, five days after a large rainstorm, MRSA was still elevated (143.5 CFU / 100 mL), suggesting that the water is best avoided for even longer after a first flush event.

Regulatory agencies evaluate water quality through FIB monitoring instead of directly measuring specific pathogens. It is not known if traditional indicators can accurately reflect the persistence of emerging contaminants like ARB and ARGs. In the case of Venice Beach, all FIB

were significantly associated with MRSA. Interestingly, at El Porto both total coliform and *Enterococcus* were moderately correlated with MRSA, whereas *E.coli* had a weak association. Studies in Greece, South Africa, and Hawaii have found *S. aureus to be correlated with total coliforms, fecal coliforms, and Enterococcus in seawater and freshwater streams* (Gemmell and Schmidt 2013; Efstratiou et al. 1998; Viau et al. 2011). The differences in FIB correlation observed at Venice and El Porto might be attributed to different input sources of MRSA. Venice Beach might be more at risk for fecal pollution from the particularly dense population of homeless people.

There were also higher than average concentrations of MRSA at both beaches on irregular dry-weather days, suggesting that dry-weather runoff is an important driver of ARB pollution. An estimated 100 million gallons of dry-weather runoff flows through Los Angeles' storm drains daily and empties directly into the Santa Monica Bay without any treatment (DWP, n.d.). Dry-weather sources of MRSA pollution could be pet waste, sewage, or even runoff from fertilizer-applied crops or landscaping (Cinquepalmi et al. 2013; Boopathy 2017; Casey et al. 2013). In 2019 to 2020 there were over 148,000 gallons of sewage that spilled within the Los Angeles County watershed which could contribute to MRSA loading. Future research should investigate potential sources of both dry and wet-weather MRSA into the Santa Monica Bay.

There was increased nasal carriage of MRSA amongst surfers exposed to impaired stormwater runoff, suggesting that the environment serves as an open reservoir available for ARB transfer to humans. Most research on the proliferation of antibiotic resistance has focused on the medical field. The relatively small amount of knowledge we currently have on the relationship between *environmental* exposure and antibiotic resistant illnesses in humans has come from work in agricultural settings (Bisdorff et al., 2012; Carrel, Schweizer, Sarrazin,

Smith, & Perencevich, 2014; Feingold et al., 2012). In fact, a recent systematic review paper on environmental transmission of antibiotic resistance (Huijbers et al. 2015) examined many articles showing circumstantial evidence of increased levels of illness associated with ARB hotspots. However, the paper found no publications, "There were no publications, however, providing direct evidence of AMR [antimicrobial resistant] bacteria to humans resulting from exposure to the environment,"(Huijbers et al. 2015) One reason for this is that designing controlled human studies on environmental exposure is difficult, to say the least.

Surfers that chose to surf during wet-weather events had significantly more risk of MRSA colonization. While the environmental transmission of ARB during wet-weather has not been documented, this result is similar to other epidemiological studies that evaluated swimmers or surfers in wet-weather. In an extensive longitudinal study that followed Southern California surfers over two winters, it was shown that individual surf sessions matched to elevated FIB from storm events were strongly associated with illness, but only during wet weather periods (Arnold et al., 2017). Another survey of Pacific Northwest surfers reported diarrhea and fever were occurred more frequently in surfers that often surfed in rain events compared to those who never or sometimes did (Harding et al., 2015).

The incidence of MRSA in surfers was more frequent than control subjects, suggesting that surfers might be at a greater risk of ARB transmission from the ocean. While surfing in dry weather was riskier than not surfing at all, this group did have similar colonization rates to controls. The rate of colonization in control subjects (1.67%) was very similar to the estimated colonization rate for adults living in the United States (1%) (CDC, 2013). The majority of the controls identified as female, and the majority of surfers were male. This gender discrepancy is

not considered to influence differences in MRSA colonization between the groups, as gender is not a risk factor for *S. aureus* nasal carriage (Sakr et al. 2018).

Interestingly, the phenotypic antibiotic susceptibility patterns of the marine MRSA isolates matched the surfer MRSA isolates. For both types, expression of erythromycin resistance was most common, followed by trimethoprim / sulfamethoxazole, and then tetracycline. None of the surfer or marine samples were resistant to ciprofloxacin. This is a positive finding, as ciprofloxacin is a fluoroquinolone – antibiotic of last resort. While it is tempting to see such similar patterns of antibiotic resistance in both seawater and surfers, resistance to trimethoprim / sulfamethoxazole and tetracycline also manifested in the controls' MRSA isolates. Further genomic analysis of all isolates is needed in order to make definitive connections.

The composition of Venice Beach surfers closely matches other research on surfers. The majority of Venice surfers were younger (between 25 and 34) and male, essentially identical to other surfer epidemiological studies (Harding et al. 2015; Leonard et al. 2018; Arnold et al. 2017; Stone et al. 2008). The El Porto surfer population was older than the Venice surfers, and it is important to consider that age has been shown to be a risk factor for MRSA carriage, with individuals under the age of 20 or over the age of 71 having significantly more risk (Lu et al. 2005).While the average age of El Porto surfers was less than 71, there were some surfers who were that age. However, El Porto surfers reported surfing in wet-weather at twice the frequency of Venice surfers, so age might not be as much of a factor as the exposure increase.

In addition to the differences in age, there were important behavioral differences at the two beaches. At Venice, a large number of surfers volunteered for the study, whereas El Porto had a small group of surfers. The Venice surfers were more likely to give a sample once or a few times, whereas the El Porto group consistently provided nasal specimens almost every week over

the course of two winter seasons. Although there were many repeat surfers at Venice Beach, the Venice surfer was more likely to provide a snapshot of MRSA levels whereas El Porto allowed for long term temporal monitoring.

Individuals with *S. aureus* carriage are described as persistent or intermittent carriers (Sakr et al. 2018). Persistent carriers can have *S. aureus* colonization for over 154 days, whereas intermittent carriers will have colonization for only 14 days (Van Belkum et al. 2009). In this study, the surfers that tested positive for MRSA tested negative when swabbed at a later date, hence persistent nasal carriage was not observed (although a subset of MRSA positive participants did not return for a follow-up sampling event and therefore have an unknown colonization status). This is good news, as persistent nasal carriers are more at risk for *S. aureus* infection (Sakr et al. 2018).

While it is possible that surfers did not contract MRSA from ocean water exposure, their frequency of nasal carriage was much higher than controls after surfing during wet periods. It is also unknown if any participants developed subsequent *S. aureus* infections. It is also possible, that if the human nasal sample collection was not terminated due to COVID-19, the incidence of MRSA in dry-weather would have been significantly greater compared to controls. This study has a relatively small population of surfers, and even smaller population of control subjects.

In summary, surfers should follow public health recommendations and avoid ocean recreation for 72 hours following a rain event. More research is needed to elucidate if dry-weather surfers are at a significantly greater risk than nonsurfers, but all surfers should visit their primary care provider if they notice any changes in health following a surf event.

Chapter 2: Prevalence of antibiotic resistance genes and co-selection of metal(loid)s in stormwater biofilters in Southern California

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 with antibiotics. 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, are also responsible for 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 environments. 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 examples, efflux pumps will remove both different heavy metals and antibiotics from the cell. Co-regulation is the third mechanism for co-selection whereby a share regulatory response for both types of exposure can occur in response to one, 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, Eckert, and Corno 2016; Gao, Munir, and Xagoraraki 2012; Mao et al. 2015; Jang et al. 2018), agricultural soils affected by land application of biosolids or manure waste (Chee-Sanford et al. 2009; Ji et al. 2012), feedlots (L. Y. He et al. 2016), water bodies associated with waste discharges (D. W. Graham et al. 2011), and metal-spiked experiments in microcosms (Stepanauskas et al. 2006; C. W. Knapp et al. 2011; Q. 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 (Sidhu et al. 2012; Garner et al. 2017; Dorsey et al. 2013). Fecal contaminants found in stormwater are frequently observed to carry elevated levels of ARB and antibiotics (Karkman, Pärnänen, and Larsson 2019). While degradation of antibiotics are dependent on physicochemical properties and molecular structure (Cycoń, Mrozik, and Piotrowska-Seget 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 (Najera et al. 2005). A detailed study in this area is needed considering the significant consequences of impaired urban stormwater quality.

The aims of this study consisted of several tasks: (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 strategery developments in urban biofilters.

Material and methods

Study area and sample collection

Urban biofilters/bioswales (hereafter, we use the term "biofilters") were sampled over three seasons (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. At the time of this study, Irvine, Santa Barbara, and San Diego had approximate populations of 282,572, 91,350, and 1,425,976, respectively (United States Census Bureau, 2018). Within each of the three campuses, two biofilter sites were sampled (**Error! R eference source not found.**). Each sampling site was represented by four subsamples of soil covering the length of the bioswales for Manzanita (MZ), Sierra Madre (SM), Culver (CUL), Verano (VER), Sanford (SAN), and each individual sub-basin for the infiltration basin at Altman Clinical and Translational Research Institute (ACT). A total of seventy-two soil samples across three time points were collected.

After aboveground vegetation, rocks, and mulch were removed, a composite soil sample of surface soils (0–10 cm) was collected from each of three soil cores at each location, using cylindrical stainless-steel coring cups (5.08 cm diameter x 10 cm length) alongside 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.

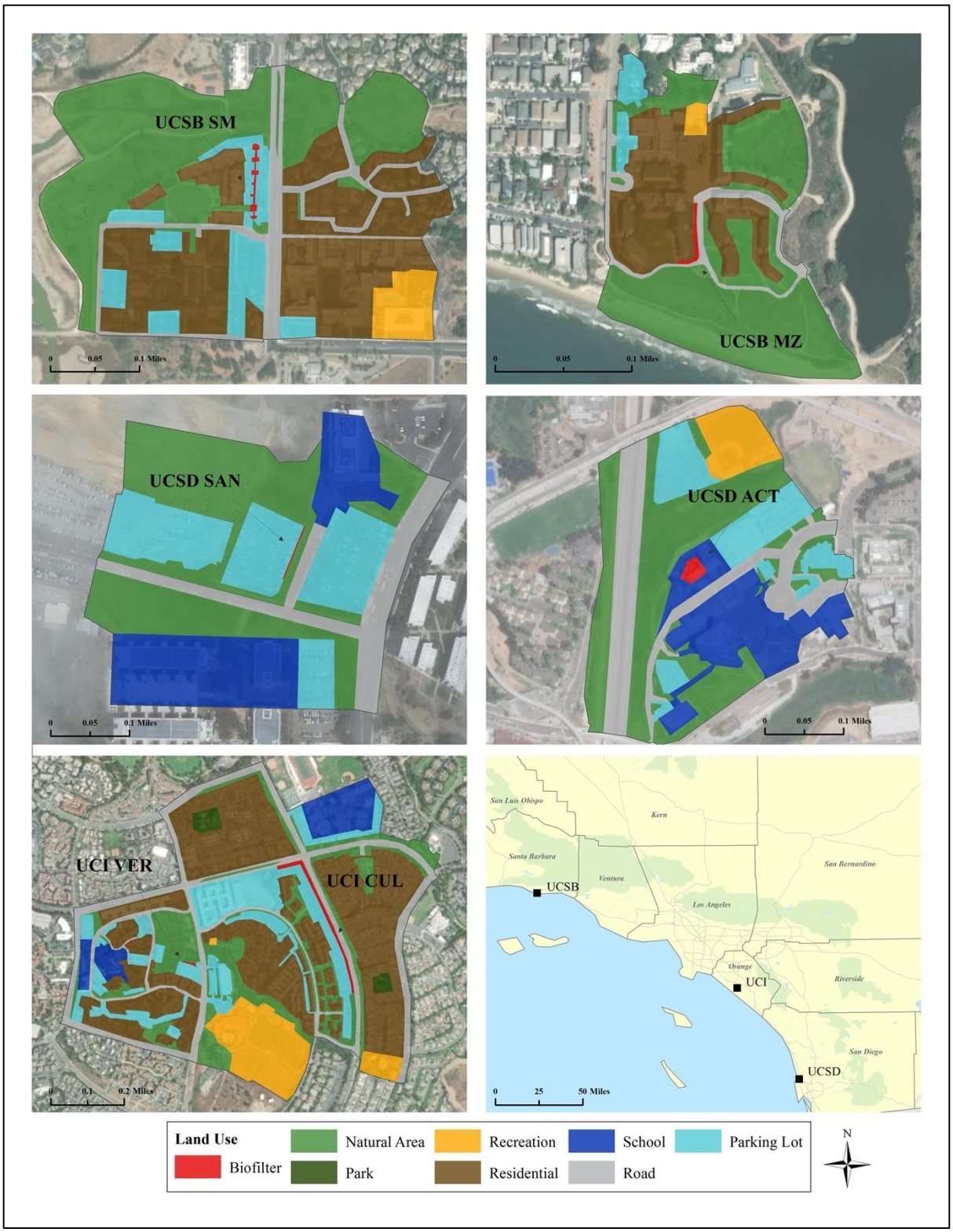


Figure 3: Land-use types near six biofilter sites located at UCI, UCSD, and UCSB. Exact drainage areas of biofilters were not shown on this map. Abbreviation: UCSB: University of California, Santa Barbara; UCI: University of California, Irvine; UCSD: University of California, San Diego; MZ: Manzanita; SM: Sierra Madre; CUL: Culver; VER: Verano; ACT: Altman Clinical and Translational Research Institute; SAN: Sanford.

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 (Sheldrick and Wang 1993; Rible and Quick 1960; Association of Official Analytical Chemists (AOAC) 1997). 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 (Nelson and Sommers 1996; Gardner 1986). 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 □m 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).

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, Campbell, and Bisson 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 nano pure water containing 6.8%-7% (v/v) HNO_3, and 0.9% (v/v) H_2O_2. All chemicals were analytical grade or better, 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 UCR 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.

DNA extraction and real-time quantitative Polymerase Chain Reaction (qPCR) 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 (*int*I1), 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 (**Error! Reference source not found.**) and thermocycling conditions (**Error! Reference source not found.**) 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).

Table 2: Primer sequences and concentrations of qPCR reactions.

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	(Y. 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, Garrigues-Jeanjean, and Mackie 2001)
	*tet*W-R		GGGCGTATCCACAATGTTAAC		
*erm*F	*ermF*-F	500	TCGTTTTACGGGTCAGCACTT	246	(C. Knapp et al. 2010)
	*erm*F-R		CAACCAAAGCTGTGTCGTTT		
*int*I1	*int*I1-F	200	GGCTTCGTGATGCCTGCTT	424	(Y. Luo et al. 2010)
	*int*I1-R		CATTCCTGGCCGTGGTTCT		
16S rRNA	16S rRNA-F	500	ATGGCTGTCGTCAGCT	351	(Pan and Chu 2018a)
	16S rRNA-R		ACGGGCGGTGTGTAC		

Abbreviations: F: Forward; R: Reverse.

Table 3: 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
*int*I1	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

Statistical analysis and geographic information systems (GIS)

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 other 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. Spearman's correlation was undertaken to identify correlations among ARGs, metal(loid) concentrations, and soil properties. Both are nonparametric methods that do not assume normal distribution. A p-value of < 0.1 was set to be significant considering the high variability of environmental samples. 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 for further analysis.

Results and discussion

Prevalence of ARGs in biofilters in Southern California

Biofilter soil samples were collected at six biofilter sites from three different UC campuses in Southern California. Soil DNA was extracted and analyzed for selected ARGs, MGE (*int*I1), and 16S rRNA (surrogate of total bacteria) in **Error! Reference source not found.**. Relative gene abundances (normalized to 16S rRNA genes) were often used to account for efficiencies during DNA extraction and size of the microbial community. 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 *int*I1 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 *int*I1 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 *int*I1 gene abundances from UC biofilter soil samples were compared in soils to those found in biosolids from waste water treatment plant (W. Hung 2020), San Diego park soils (Echeverria-Palencia et al. 2017), commercially available manure fertilizers (Cira et al., 2021), pristine soils from nearby hiking trails (Cira et al., 2021) as shown in Figure . From this analysis, both relative *sul*1 and *int*I1 levels in biofilters in this study may be considered from 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 *int*I1 except for San Diego park soils as that information was not available. These gene disparities between soil environments should receive more attention. In particular in soils containing relatively low concentrations of metal(loid)s, biofilters are likely to be new hot spots and present risks to the environment in the long-term.

Management of biofilters also played a critical role in the ARG levels as shown in **Error! Reference source not found.**. Garden amendments and reclaimed water that are f requently used for fertilizing and irrigating could contain ARGs and thus be capable of increasing soil ARGs (Fahrenfeld et al. 2013; F. H. Wang et al. 2014; Echeverria-Palencia

2018). In the present study, MZ, SM, CUL, and SAN were irrigated with reclaimed water irrigation while VER and ACT were irrigated with portable water. Among all biofilters, MZ was the only biofilter reported not to receive garden amendments and had relatively lower relative ARG abundances than those in the rest of biofilters. Except for fertilizers, operations and practices of biofilters do not appear to affect levels of ARGs in biofilters (Figure 5). Levels of relative gene abundances of *sul*1, *sul*2, *tet*W, and *int*I1 were the highest at ACT compared to those in other biofilters, possibly due to condensation drainage from air conditioners. Other factors including history, areas, and impervious drainage areas were not observed to have affected the ARG levels within the biofilters.

Table 4. 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.

	16S rRNA	*int*I1	*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

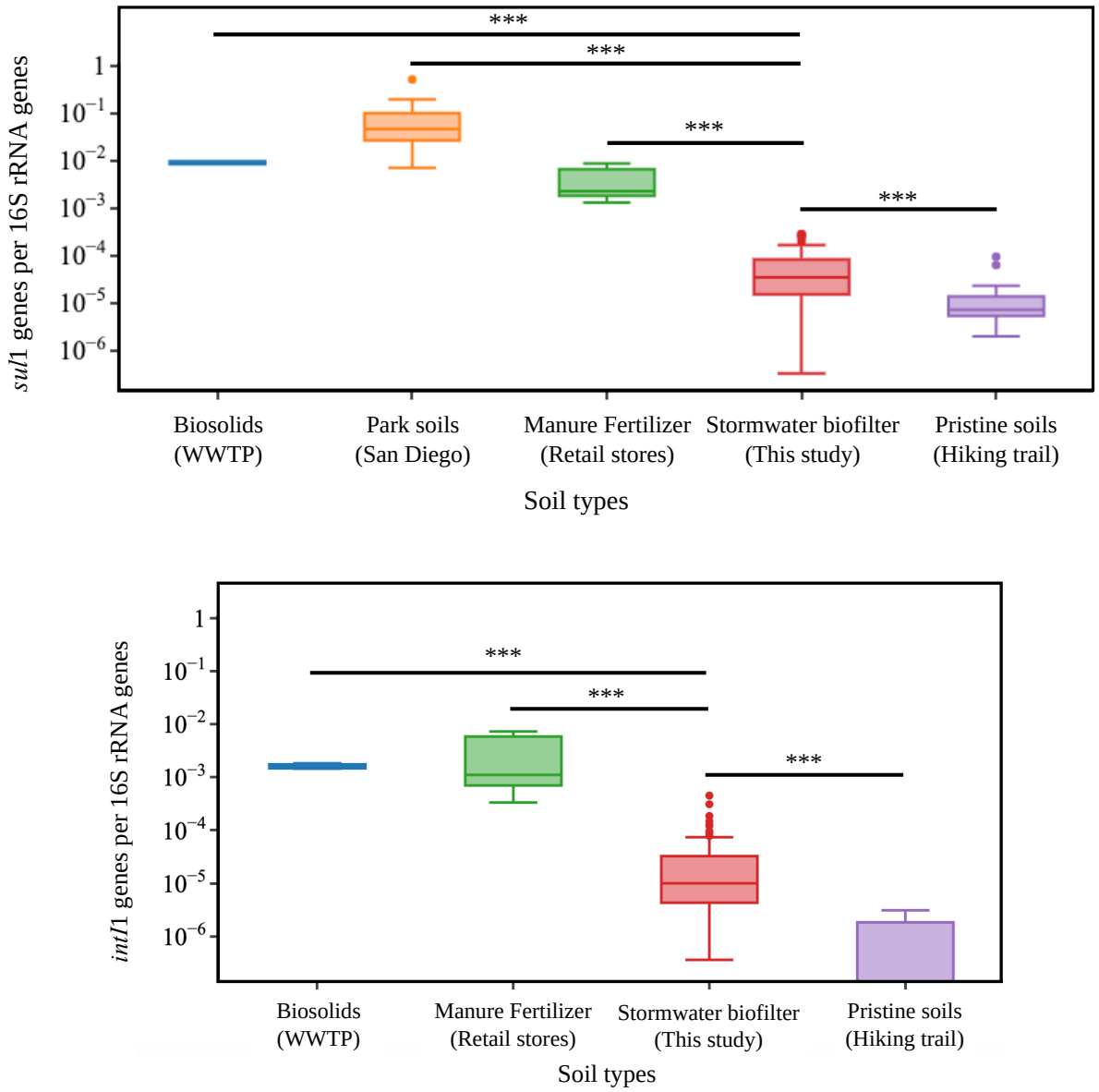


Figure 4: Relative *sul*1 (top) and *intI*1 (bottom) gene abundances among different soil types. Statistical differences between soil types significant at $p < 0.01$, $p < 0.05$, and $p < 0.1$ level are marked with ***, **, and *, respectively.

Table 5: 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 roads	1,997	2001	RW	RW, runoff
SM	UCSB	126	Bioswale	Parking lot	1,571	2016	RW	RW, runoff, fertilizer
CUL	UCI	1,330	Bioswale	Parking lot and natural area	21,400	2007	RW	RW, fertilizer, runoff
VER	UCI	146	Bioswale	Parking lot and natural area	1,878	2016	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: Portable Water.

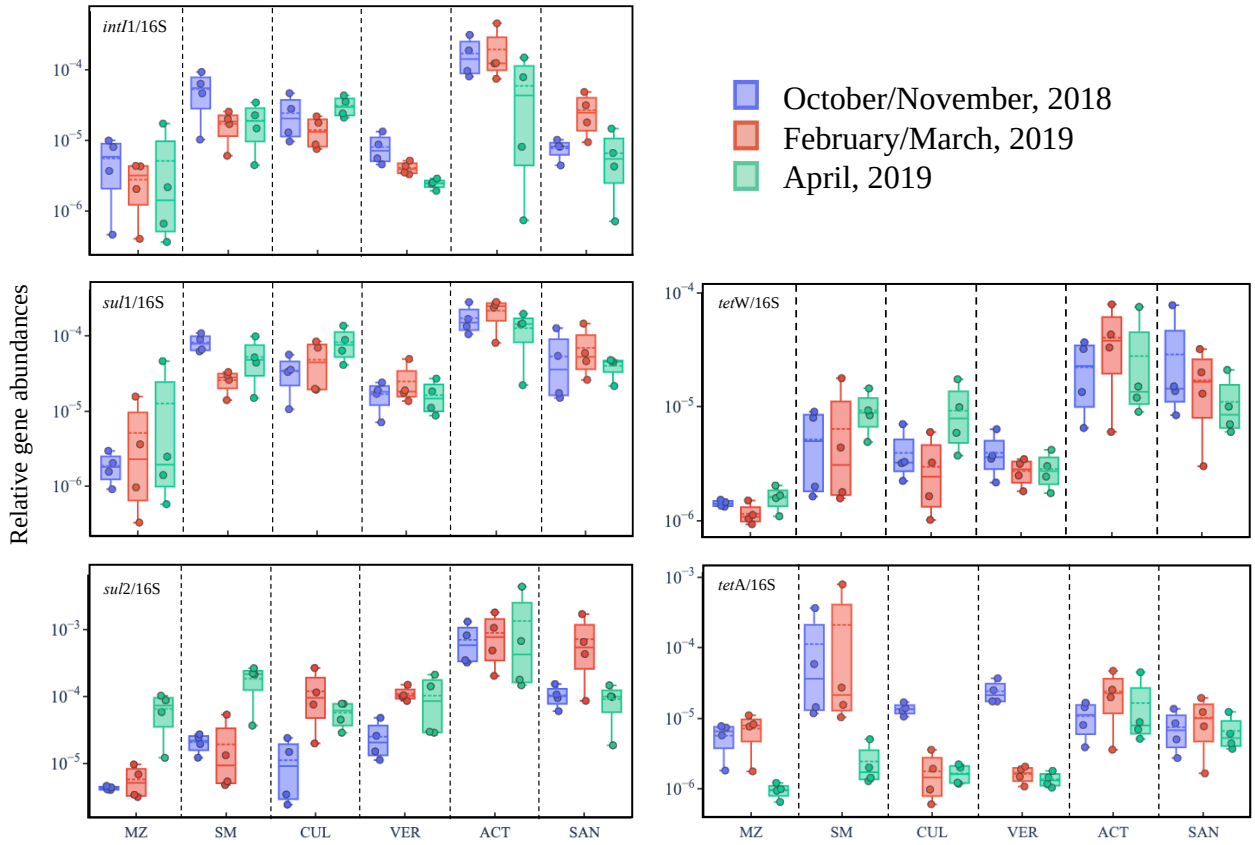


Figure 5: Temporal and spatial variation of the relative gene abundances in six biofilter sites on October/November 2018 (blue), February/March 2019 (orange), and April, 2019 (gray). Abbreviations: MZ: Manzanita; SM: Sierra Madre; CUL: Culver; VER: Verano; ACT: Altman Clinical and Translational Research Institute; SAN: Sanford.

Soil characteristics of campus biofilters in Southern California

The descriptive statistics of soil properties in biofilters are summarized in **Error! R eference source not found.**. Based on the soil textural triangle from the United States Department of Agriculture (USDA), biofilters were characterized as sandy loam with a few identified as loamy sand and sandy clay loam (Figure). 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.

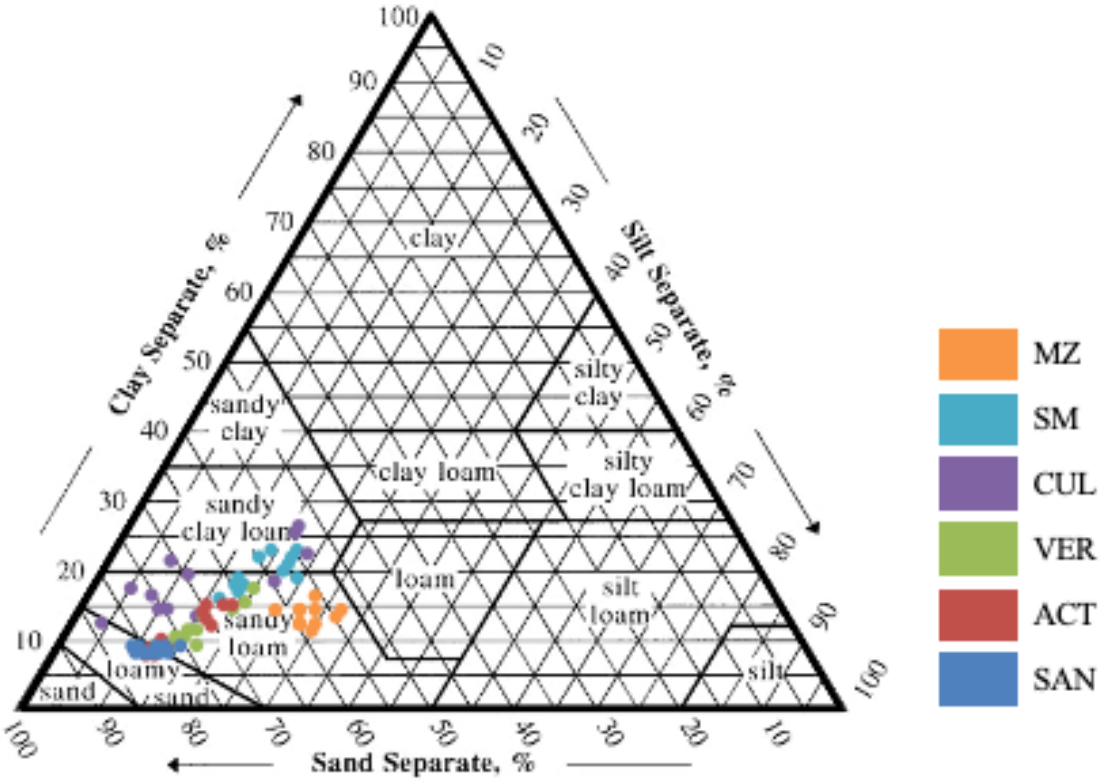


Figure 6: Soil texture of 72 biofilter samples collected from 6 biofilter sites in three UC campuses (MZ = orange, SM = blue green, CUL = purple, VER = green, ACT = red, SAN = blue). Abbreviation: MZ: Manzanita; SM: Sierra Madre; CUL: Culver; VER: Verano; ACT: Altman Clinical and Translational Research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

Spearman's correlations between gene abundances and soil properties were examined (**Error! Reference source not found.**). The percentage of sand and silt, SOM, density, total C , and total N within biofilters were significantly correlated with relative gene abundances of *sul*1, *sul2*, and *tet*W ($\rho = -0.649$ to 0.614, $p < 0.05$). 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; T. 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.

Table 6: Spearman's correlation between gene abundances and soil properties within biofilters. Significance levels at $p < 0.01$ and $p < 0.05$, < 0.1 are marked as ***, **, and *, respectively.

	Sand	Silt	Clay	Moisture	SOM	Density	pH	CEC	Total C	Total N	PO_4	NO_3	NH_4
	(%)	(%)	(%)	(%)	(%)	(g/cm^3)	(-)	(meq/100 g)	(%)	(%)	(ppm)	(ppm)	(ppm)
*intI*1/16S	0.152	–0.331***	0.209	0.262**	–0.16	0.291	0.366**	0.194	–0.036	–0.108	0.377**	–0.177	–0.214
*sul*1/16S	0.398**	–0.550***	0.01	–0.035	–0.363***	0.423**	0.325**	–0.185	–0.349**	–0.425***	0.174	–0.331***	–0.111
*sul*2/16S	0.614**	–0.519***	–0.452***	–0.211	–0.547***	0.519**	–0.051	–0.510***	–0.556***	–0.630***	–0.141	–0.332***	–0.074
*tet*A/16S	–0.02	–0.048	0.051	0.258**	–0.125	0.103	0.074	–0.079	–0.273	–0.252	0.156	–0.128	–0.377***
*tet*W/16S	0.490**	–0.456***	–0.292**	–0.033	–.572***	0.393*	0.062	–0.487***	–0.553***	–0.649***	–0.124	–0.376***	–0.240**

Abbreviations: BDL: Below Detection Limit; SOM: Soil Organic Matter; CEC: Cation Exchange Capacity; C: Carbon; N: Nitrogen

Table 7: 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.

	Sand (%)	Silt (%)	Clay (%)	Moisture (%)	SOM (%)	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

Abbreviations: BDL: Below Detection Limit; SOM: Soil Organic Matter; CEC: Cation Exchange Capacity; C: Carbon; N: Nitrogen.

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 . 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 (D. B. Smith et al. 2013) with several exceptions. High total arsenic (40.9 mg/kg), total chromium (96.8 mg/kg), and total nickel 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), 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. 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 from parking lots and building roofs, 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 (**Error! Reference s ource not found.**). In this study, the levels of heavy metals in biofilters varied over the course of time (**Error! Reference source not found.**). 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. 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. 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 should not be ignored in the case of heavy metal driven co-selection.

Table 8: 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.

	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 (D. B. Smith et al. 2013)										25.8	0.3	0.3	6.4	66	17.9	17.7	36	60

Abbreviations: BDL: Below Detection Limit; N.A.: Not Available.

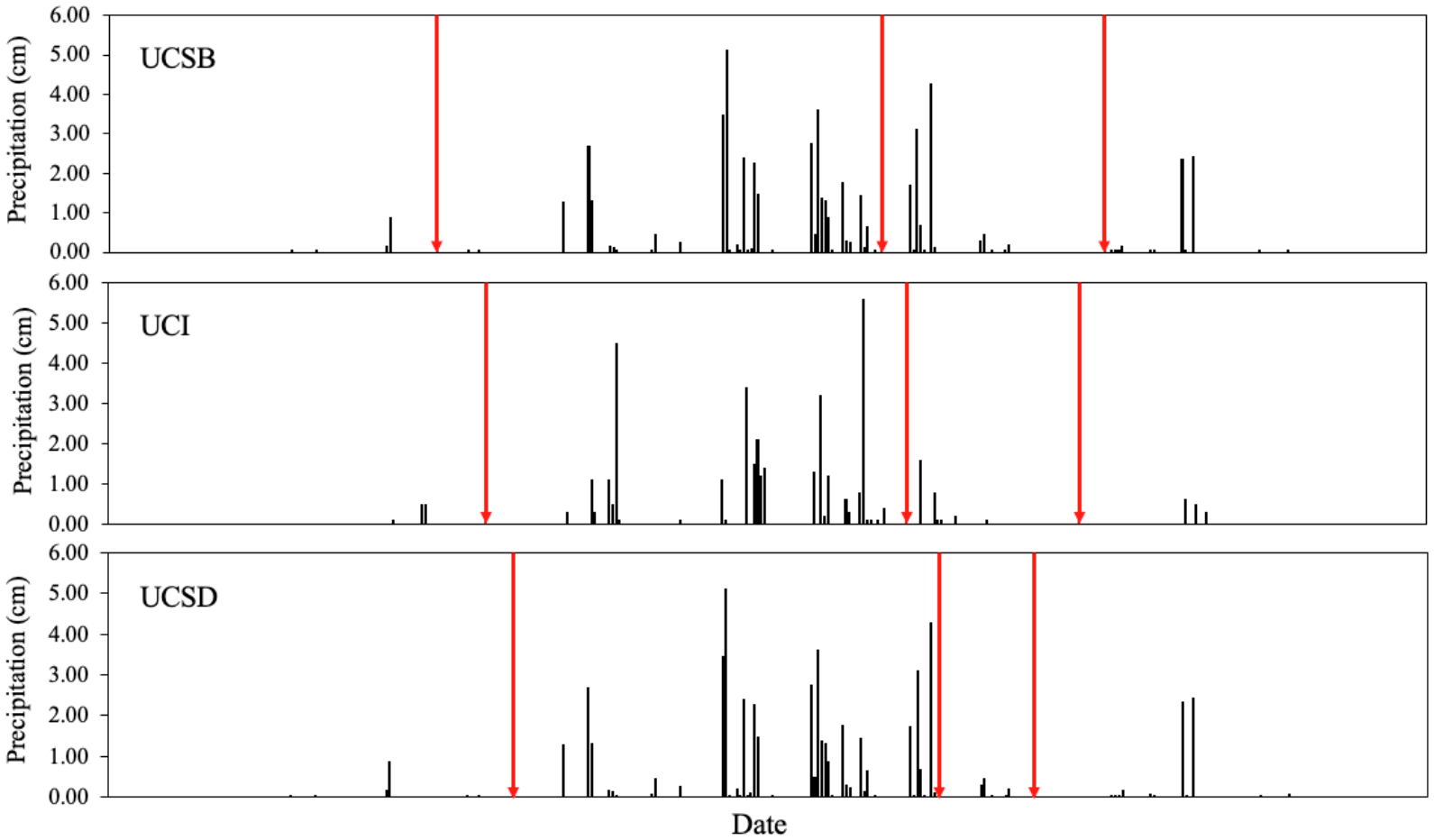


Figure 7: Daily rainfall data reported in areas near each campus. Red lines indicated the dates of sampling.

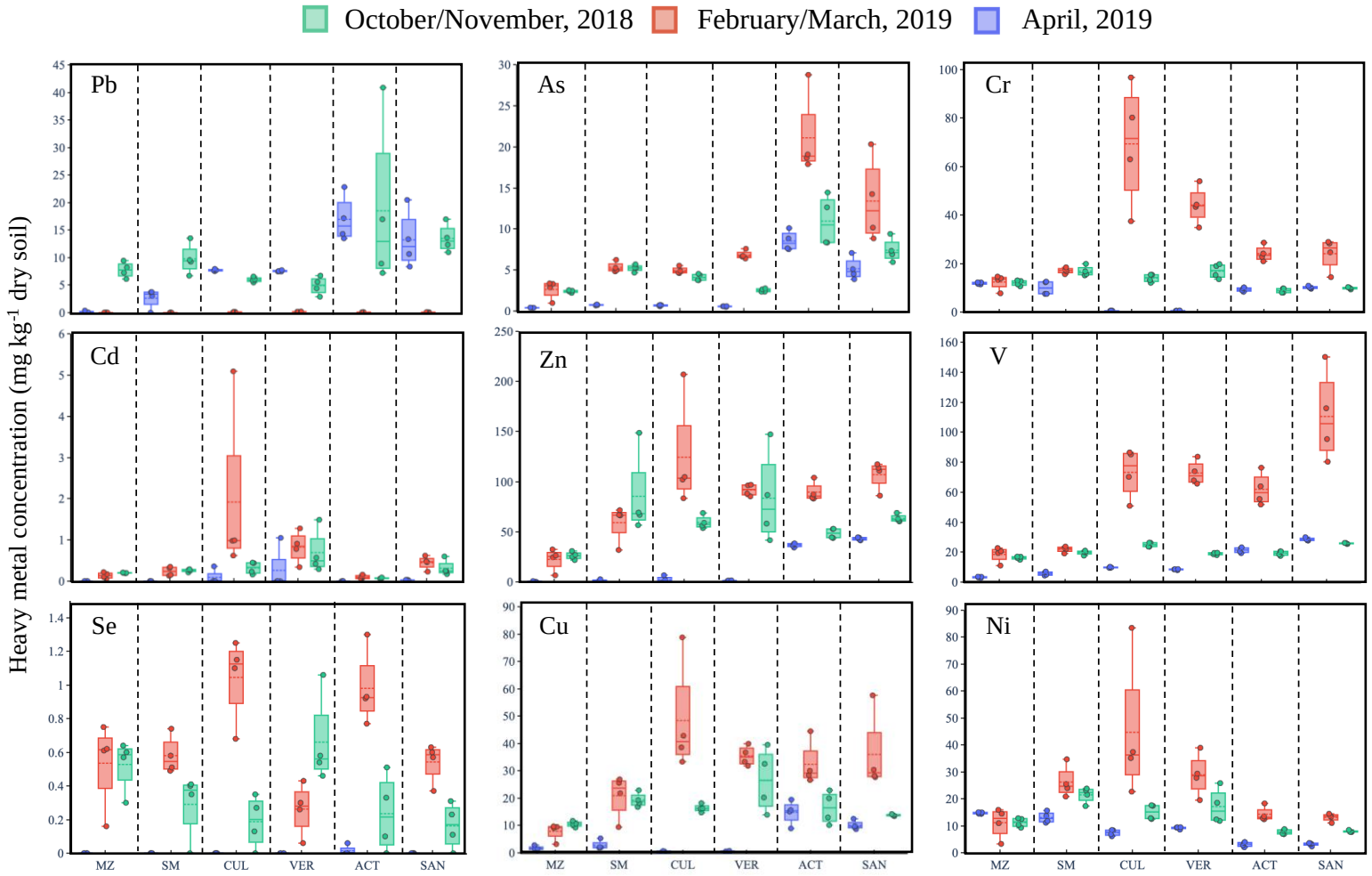


Figure 8: Temporal variation of nine metal(loid) concentrations, including arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu), nickel (Ni), lead (Pb), selenium (Se), vanadium (V), and zinc (Zn) in six biofilter sites on October/November 2018 (blue), February/March 2019 (orange), and April, 2019 (grey). Abbreviations: MZ: Manzanita; SM: Sierra Madre; CUL: Culver; VER: Verano; ACT: Altman Clinical and Translational Research Institute; SAN: Sanford.

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 9. Many significant correlations were found between ARGs and total heavy metal levels in biofilters. Total metal(loid)s of As, Cu, Pb, V, and Zn exhibited significant correlations individually with selected ARGs: *sul*1/16S (As: $\rho = 0.57$, $p < 0.01$; Cu: $\rho = 0.31$, $p < 0.05$; Pb: $\rho = 0.26$, $p < 0.01$; V: $\rho = 0.3$, $p < 0.01$; Zn: $\rho = 0.29$, $p < 0.05$), *sul*2/16S (As: $\rho = 0.71$, $p < 0.01$; Cr: $\rho = 0.32$, $p < 0.1$; Cu: $\rho = 0.52$, $p < 0.01$; Pb: $\rho = 0.36$, $p < 0.01$; Se: $\rho = 0.25$, $p < 0.05$; V: $\rho = 0.55$, $p < 0.01$; Zn: $\rho = 0.54$, $p < 0.01$), and *tet*W/16S (As: $\rho = 0.70$, $p < 0.001$; Cu: $\rho = 0.35$, $p < 0.01$; Pb: $\rho = 0.30$, $p < 0.05$; V: $\rho = 0.50$, $p < 0.001$; Zn: $\rho = 0.40$, $p < 0.001$). Additional correlations were also detected between *int*I1/16S and total As concentrations ($\rho = 0.37$, $p < 0.01$). Yet, there was a negative correlation between total Cd and *int*I1/16S ($\rho = -0.27$, $p < 0.05$). Notably, total heavy metals of As, Cu, Pb, V, and Zn also showed significantly negative correlations with *tet*A/16S (Cd: $\rho = -0.46$, $p < 0.01$; Cr: $\rho = -0.47$, $p < 0.01$; Cu: $\rho = -0.43$, $p < 0.01$; Ni: $\rho = -0.47$, $p < 0.01$; Se: $\rho = -0.29$, $p < 0.05$; Zn: $\rho = -0.39$, $p < 0.01$). 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 (Zhang et al. 2018; C. W. Knapp et al. 2011, 2017). Moreover, the mobile genetic element *int*I1 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., (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. Total heavy metals have been clustered into four major groups as shown in HCA in Figure 10: (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, for example, 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 As was significantly correlated with relative abundance of *sul*1 genes ($\rho = 0.30$, $p < 0.05$), *sul*2 genes ($\rho = 0.54$, $p < 0.01$), and *tet*W genes ($\rho = 0.33$, $p < 0.01$). Levels of bioavailable Zn were also found to be significantly associated with *int*I1/16S ($\rho = 0.39$, $p < 0.01$), *sul*1/16S ($\rho = 0.33$, $p < 0.01$), and *tet*A/16S ($\rho = 0.39$, $p < 0.01$). Bioavailable Cu concentrations, however, were negatively correlated with *intl*1/16S ($\rho = –0.21$, $p < 0.1$), *sul*1/16S ($\rho = –0.23$, $p < 0.05$), *sul*2/16S ($\rho = –0.25$, $p < 0.1$), and tetW/16S ($\rho = –0.21$, $p < 0.1$). Overall, most correlations 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 10). 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, Balgobind, and Pillay 2013).

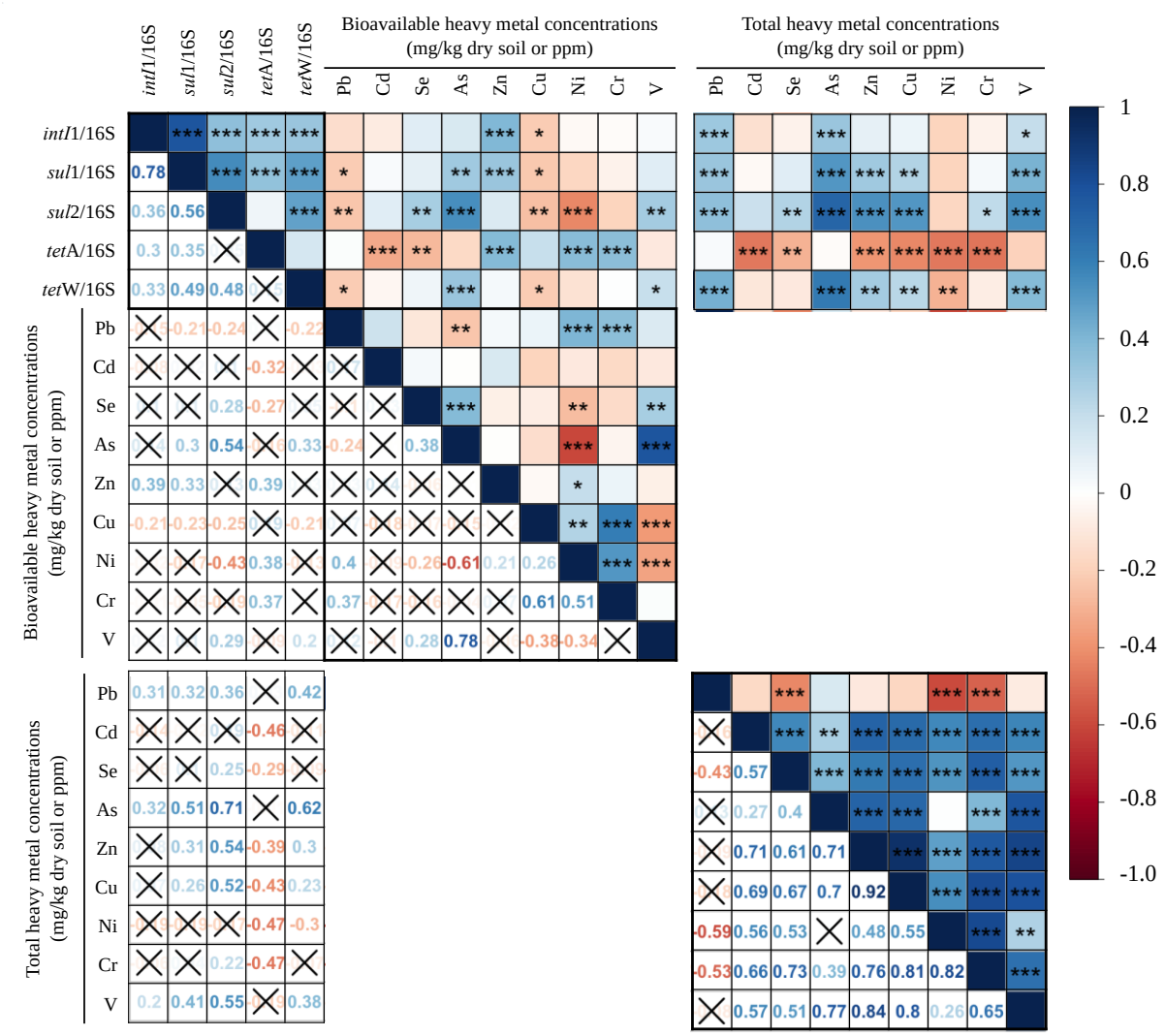
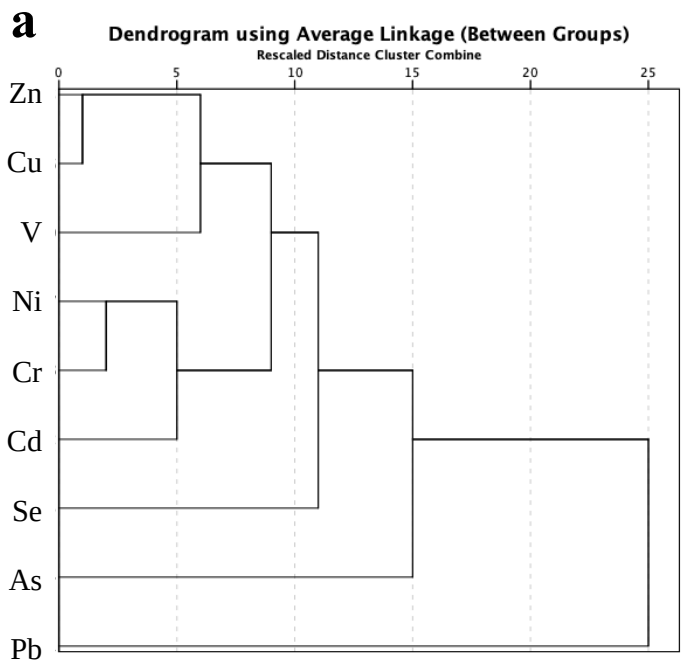
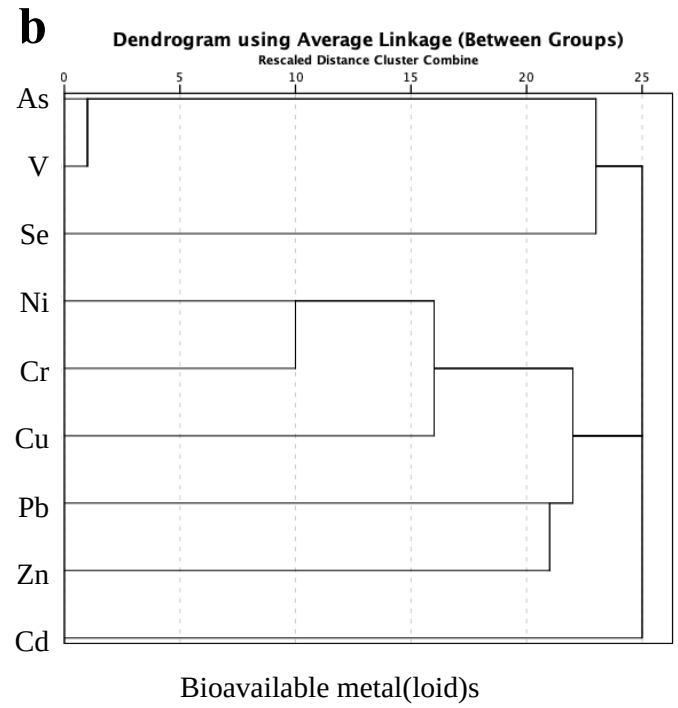


Figure 9: Spearman's correlation coefficients for combination of antibiotic resistance genes (ARGs), mobile genetic elements (MGEs), bioavailable metal(loid)s, and total metal(loid)s (Positive = blue, negative = red, X = not significant). Correlations significant at $p < 0.01$, $p < 0.05$, and $p < 0.1$ level are marked with ***, **, and *, respectively.



Total metal(loid)s



Bioavailable metal(loid)s

Figure 10: Dendrogram of HCA for (a) total metal(loid)s and (b) bioavailable metal(loid)s.

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 (Y. Zhou et al. 2017). Recent studies that showed significant correlations between ARGs and heavy metals in soils are summarized in Table 9. 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 (C. W. 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 9: Correlations between ARGs and heavy metals in soils reported by previous studies (Cui et al. 2016; L. Y. He et al. 2014; J. Zhang et al. 2018; C. W. Knapp et al. 2011, 2017; Ji et al. 2012) Bordered genes indicate correlations are overlapped with present study.

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, *sul*2, *sul*3	1	*bla*$_{TEM}$
arsenic (As)	1	*bla*$_{SHV}$	11	*bla*$_{SHV}$, *tet*BP, *fex*A, *fex*B, *cfr*, *sul*1, *int*I1, *tet*H, *tet*O, *tetQ*, *tet*W
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, *sul*1, *sul*2, *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, *sul*2	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 (-), *tet*W
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, *tet*W	11	*sul*A, *sul*3, *tet*L, *tet*W, *tet*Q, *sul*1, *sul*2, *fex*A, *fex*B, *cfr*, *int*I1
Bioavailable heavy metals				
arsenic (As)	0		8	*tet*A, *tet*L, *tet*M, *tet*W, *tet*Q, *sul*1, *cfr*, *fex*A
copper (Cu)	0		2	*tet*A, *flo*R
iron (Fe)	0		1	*tet*M
zinc (Zn)	0		10	*int*I1, *tet*B, *tet*M, *tet*Q, *tet*X, *sul*1, *sul*2, *cfr, cml*A, *flo*R

Abbreviation: Ct: Count.

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 (C. W. 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 10). Only variables shown 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 11): (1) *int*I1/16S ($R^2_{adj} = 0.329$, $p < 0.01$), (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 *int*I1 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 *int*I1 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 10: 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}

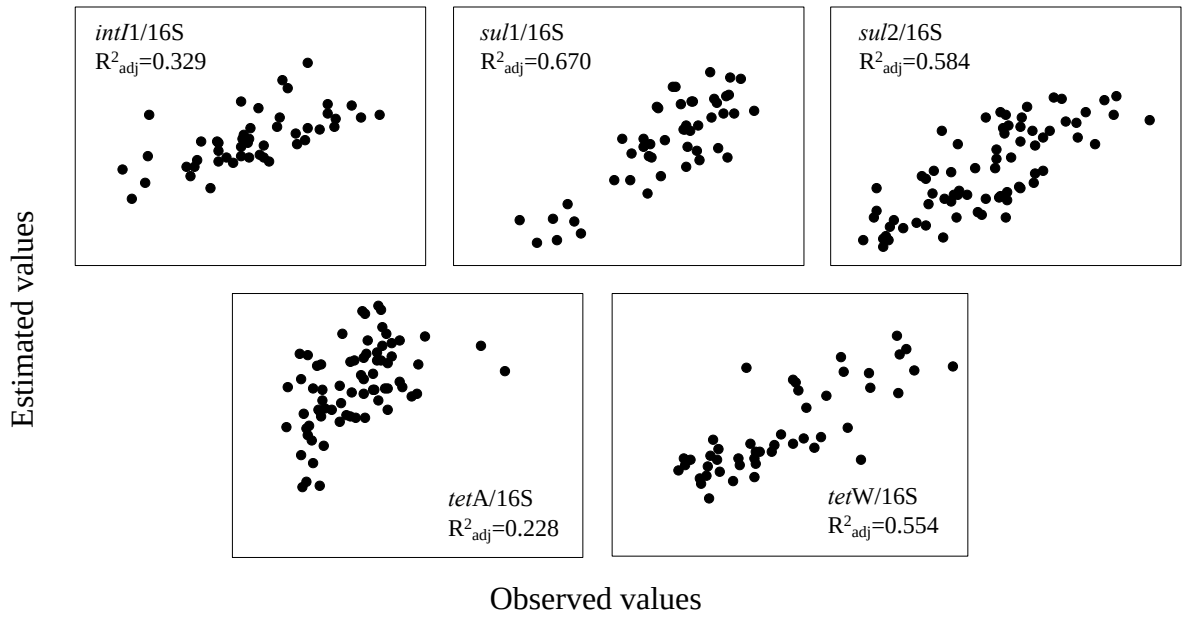


Figure 11: Multiple linear regression between observed and estimated values of relative abundances of *sul*1, *sul*2, *tet*A, *tet*W, and *int*I1 genes.

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.

Chapter 3: Do stormwater biofilters remove pathogens and antibiotic resistance genes to a similar extent as traditional fecal indicators and novel fecal source markers?

Introduction

Microbial pollution in stormwater is a major cause of surface water contamination. In California, microbial pollutants drive large spending in planned low impact development (LID) best management practices (BMPs). Biofilters are included in LID BMPs for meeting compliance goals aimed at public health protection. Stormwater biofilters (also called bioretention systems, rain gardens, or natural treatment systems) can capture and treat stormwater through natural processes of vertical soil filtration, sedimentation, extended detention, and biological uptake (Allison, Francey, CSIRO (Australia), & Melbourne Water Corporation., 2005; Hatt et al., 2009; Dietz and Clauson, 2005; other Bratieres 2008). In addition to stormwater volume reduction and other benefits, improving stormwater quality is a major motivation for managers to implement such systems. While biofilters have been shown to remove total suspended solids, heavy metals and nutrients (Bratieres et al. 2008; Davis et al. 2001), research into their efficacy at removing microbial pollutants is not consistent. It is not clear whether biofilters sufficiently remove important pathogens or emerging contaminants like antibiotic resistant genes (ARGs). To effectively use biofilters for water quality improvement, understanding of efficiency, mechanism, and predictability of stormwater pollutant removal by such systems is required.

Our understanding of microbial contaminant removal is limited due to the complexities of studying biofilter infrastructure. While some microbial removal studies have been done on field-scale biofilter systems, most studies have been conducted using laboratory-scale columns. In addition, they have focused on FIB (Chandrasena et al., 2014; Afrooz et al. 2018; Mohanty and

Boehm 2014; Mohanty et al., 2014), rather than pathogens that drive the public health risk or human fecal markers that are more closely related to relevant fecal sources of risk than are FIB. In fact, to our knowledge there has not yet been any study examining removal of human fecal markers through biofilter systems. It is unknown if and how results from laboratory studies can be extrapolated to field performance, and if and how removal of FIB can be related to removal of fecal source markers and pathogens. Additionally, there is a disconnect between current compliance regulation, which is based on fecal indicator bacteria (FIB), and public health risk management, which is based on pathogens. Although FIB are used as indicators of human fecal waste to infer public health risk, FIB themselves are generally not pathogens and can also originate from non-human or even non-fecal sources (Converse et al., 2011; Field and Samadpour 2007). Therefore, reduction in FIB through biofilters may not reflect reduction in public health risk.

Recognizing the disconnect between compliance regulation and public health protection, the water quality management community is also considering another category of fecal indicators called fecal source markers as potential compliance tools. For example, human fecal source markers are genetic markers that are associated with human fecal sources, which present the highest public health risk compared to non-human fecal source (Harwood et al. 2014; Colford et al. 2007; Soller et al. 2010). As direct pathogen measurement may be hindered due to low pathogen concentrations (but also low infectious dose) in human fecal material and the environment, measuring the higher abundance human fecal source markers has become an attractive option for compliance monitoring more directly related to public health risk than FIB (Rodrigues 2017; Savichtcheva and Okabe 2006). However, FIB and fecal source markers are different with respect to both the organism themselves but also in measurement methods (culture

vs. genetic methods), it is also unclear if and what relationship exist between removal of FIB and human fecal markers.

A holistic study of fate and transport of microbial pollutants through field-scale biofilter systems was conducted to better understand the extent, pattern, and mechanisms of microbial pollutant removal through such systems. A suite of microbial parameters was studied for removal in a full-scale biofilter using natural stormwater spiked with primary influent as source of fecal contamination. In addition to traditional fecal indicators, one bacterial (HF183) and two viral fecal source markers were evaluated. The plant pathogen pepper mild mottle virus (PMMoV) and crAssphage are viral markers that have high specificity to human feces and are expected to be better environmental indicators of viral pathogens. PMMoV is the most common RNA virus in human feces (Kitajima, Sassi, and Torrey 2018). CrAssphage is a bacteriophage predicted to infect *Bacteroides,* the target of human-associated fecal markers such as HF183 (Stachler et al. 2017). Also included are two ARGs, *sul*1 and *erm*B, as well as *intI*1, a class 1 integron considered to play an important role in the proliferation of environmental antibiotic resistance (Stalder et al. 2012). CrAssphage has also shown to be associated with ARGs (including *sul*1) and *intI*1 in a sewage-impacted urban stream (Stachler, Crank, and Bibby 2019). *Salmonella, campylobacter,* and adenovirus were also evaluated for removal as they are common pathogens in surface water.

This was a large-scale, synergistic study that served as a unique opportunity to evaluate removal using natural stormwater, since pilot-scale studies are rare in the literature. This study was also novel in the detailed investigation of biofilter hydrology and utilization of bromide to normalize the microbial parameter of interest. The general experimental approach was to conduct challenge experiments where the biofilters were dosed with stormwater mixed with

sewage. Microbial targets (FIB, human fecal markers, pathogens, ARGs that occur in sewage, and microbial communities), along with other relevant abiotic and biotic parameters, were monitored in the influent, effluent, and media of biofilters, during and after the dosing experiments. The purpose of the challenge tests was to evaluate 1) the removal efficiencies of fecal source markers, pathogens, and emerging contaminants such as ARGs and 2) if fecal source markers or FIB accurately reflect pathogen fate and transport behavior in these systems.

Methods

Systematic Review

A systematic literature review was conducted to evaluate 1) pathogen, fecal source marker, and ARG removal by stormwater biofilters 2) if traditional FIB such as *E.coli* are representative of pathogen, fecal source marker, or ARG removal within these systems. A modified version of the meta-analysis described in Rippy, 2015 was utilized to perform this analysis in December 2020. The analysis was conducted on Web of Science by searching: 1) biofilter, biofiltration, bioinfiltration, bioretention, or rain garden 2) AND Stormwater or runoff 3) AND bacteria or *Escherichia coli* or *Enterococcus* or total coliform or fecal coliform 4) AND pathogen or fecal source marker or *Campylobacter* or *Salmonella* or Entero1A or coliphage or adenovirus or virus or MRSA or *Staphylococcus aureus* or HF183 or GenBac3 or crAssphage or PMMoV or *sul*1 or antibiotic resistance gene or antibiotic resistant bacteria or 16s rRNA or host-associated genetic marker or *intI*1 or *erm*B.

Studies were excluded if 1) No microbial contaminants were included or 2) only FIB removal were evaluated or 3) duplicate data was reported or 4) the experiment was not representative of

realistic biofilter conditions 5) pathogen removal was mentioned anecdotally. Average log reduction values for the parameter of interest were extracted or calculated from the included studies. In instances where there were multiple experimental configurations tested for one microbial parameter, the log reductions were averaged. In this case, the standard deviation reflects the difference in experimental configurations.

Biofilter construction

Experiments were conducted with four constructed stormwater biofilter test cells located on the Orange County Public Works (OCPW) campus in Orange, CA (33.825633, -117.851344). Orange, CA has a Mediterranean climate characterized by warm, dry summers and mild winters. The annual precipitation is 36.58 cm and storm events typically only occur during winter months. For the purpose of this paper, the only results included are those of a challenge test conducted on cell four (C4) since this was the only cell that received both Bromide as a conservative tracer and a sequence of five storms. The experimental setup and hydrological characteristics are described fully in Parker et al 2020. Briefly, test cells were 1.5 m x 2.4 m x 1. 8 m and constructed out of concrete sealed by Polycoat-Aquaseal 5000 waterproofing (**Error! Reference source not found.**2**)**. The bottom of each cell contained a lateral 10 cm diameter PVC pipe underdrain connected to a cleanout outlet for effluent sampling. The biofilters were packed with a 15 cm course aggregate layer immediately over the underdrain collection pipe, followed by a 7.6 cm #8 Choke stone layer, 7.6 cm sand layer, and 1.1 m of soil media which left 0.5 m of freeboard at the top of the biofilter. Per the biofilter design specification, the soil (approximately 1.1 m deep) is a mixture of fine sand and compost, measured on a volume basis

(65% Sand, 20% Sandy Loam, 15% compost) to achieve the following gradation: 85-88% Sand, 8-12% Fines (from Sandy Loam Topsoil), 3-5% Organic Matter.

Figure 12: The cells were vegetated with *Carex spissa* and contained six spray heads for irrigation. In addition to infrequent rains received since completion of construction and planting, the biofilter cells were mainly irrigated with tap water to support plant growth. Biweekly weeding was implemented to maintain a monoculture plant species in these four biofilter cells.

Stormwater collection and spiking with primary influent

C4 went through three phases of storm events described in full in Parker et al., 2021. Briefly, phase IB focused on hydrological characterization and involves running stormwater (from on-site underground stormwater cistern) through all four biofilter cells (referred as biofilter dosing herein). The main purpose was to characterize important hydrological parameters (e.g. infiltration rate, water balance), to acclimate the biofilter cells (which have been irrigated mainly by tap water), to confirm logistics in running the challenging experiments, and to measure background pollutant concentration, in preparation for Phases II work. Each biofilter cell will be dosed separately, under a transient flow setup. Phase II focuses on characterizing pollutant breakthrough curves and vertical distribution of pollution removal within the biofilter cells. In this phase, C4 was challenged with sewage-spiked stormwater to investigate removal efficiency and vertical distribution of microbial targets and other pollutants (via post-challenge experiment coring). The other two biofilter cells (referred as control cells) will be treated as replicate cells to the test cells for examining vertical distribution of various analytes prior to introducing sewage-spiked stormwater into the test cell via the same coring procedure as that would be used for the test cell. These soil media cores will be sectioned at different depth to provide a pre-sewage dosing depth profile of various analytes. C4 will proceed to phase III, where it is flushed with unspiked storm water for three days (four individual transient flow experiments) to assess retention and release of captured water and constituents from previous experiments.

In summary, water samples were collected in Phases I, II, and III. While Phase II water samples were subjected to the most complete suite of analysis, measurements were only performed on selected samples or analytes in Phases I and III. Soil core samples were collected

on one control cell (C2) following Phase I, and on C4 upon completion of Phase III "flushing" experiments.

For Phase I, stormwater was collected on the OCPW site in an 80,000-gallon underground storage cistern (part of the Glassell LID campus project) over the winter and spring of 2019, for use in experiments in May and June 2019. The collected stormwater was mainly street runoff from parking lots but also included effluent from a modular treatment wetland. Prior to dosing C4, stormwater was first pumped into a 500-gallon plastic tank that was placed on a pallet scale (Figure 13). A sump pump (Zoeller 98-100) was inserted into the underground stormwater cistern and submerged a few feet, with care taken to avoid getting near the bottom of the cistern to prevent clogging from deposited sludge. A trash pump was inserted into the plastic tank to ensure continuous mixing and homogenization of the stormwater influent. The influent was applied to the test cell via a hose with a control valve. Due to the pressure changes induced by the trash pump mixing the influent, the pressure transducer needed for automatic control valve operation was inoperable, so manual control valve operation was used . All dosing experiments were conducted under simulated transient flow condition following a realistic hydrograph from past Orange County storms.

For Phase II, approximately 1500 liters of primary influent sewage (passing the bar screen) was collected from Orange County Sanitation Department (OCSD) using eight 55-gallon barrels. For phase II, 1400 pounds of sewage and 1400 pounds of stormwater were mixed together with the trash pump in the plastic 500-gallon tank. OCSD's influent monitoring data (2014-2018) indicate that 50% spike would yield appropriate concentration range for metal-related research work. Test cell C4 received sewage-spiked dose as the first storm event on day one. After the one phase II storm event, C4 received phase III treatment which consisted of four

stormwater-only storms (3600 pounds of stormwater were applied for each subsequent storm event).

The effluent was collected from the biofilter cleanout outlet in a large, acid-washed glass jar. Ten samples were collected in five-liter portions throughout the storm's duration.
The remaining effluent was transported into the shared basin and removed by a separate sump pump into a different 500-gallon plastic tank on a pallet scale. The effluent was weighed to quantify volume loss.

Figure 12: The experimental setup consisted of a 500-gallon tank with a trash pump to ensure homogenous concentration of the influent. The control valve is pictured on the white stool.

Vertical distribution of microbial targets

Six cores were taken for test cell C4 and a paired control cell. Each core was sectioned by sawing the polycarbonate sleeve (or by extruding the soil) to generate subsamples for each relevant depth interval (0 – 10 cm, 10 – 20 cm, 30 to 40 cm, 50 – 60 cm). For each unit, each subsample at each depth was combined into one composite per unit at each depth. The mixing was done quickly at ambient temperature in a large bag to ensure homogenization. Soil core samples were processed to allow for storage in -20°C to allow later DNA extraction and subsequent molecular analysis and to obtain eluent (i.e. soil wash off) for immediate culture-based analysis and filtration for subsequent molecular analysis. Standard washing procedure was performing following the methods in Boehm et al., 2009.

Sample Processing for molecular and culture-based analysis

Samples were immediately processed after their collection in OCPW's onsite laboratory. Samples underwent membrane filtration following standard methods using polycarbonate filters for bacteria and nixed mixed nitrocellulose for viruses. For bacteria, a maximum of 100 mL was vacuum filtered through sterile 0.22 µm Supor filters (PALL), with the exact volume of water filtered recorded (30-50 mL for raw sewage, 50-100 mL for samples). For antibiotic resistant genes, integron, 16s rRNA gene and Salmonella analysis, the influent and effluent water samples underwent membrane filtration on 0.4 µm polycarbonate filters. The bacteria filters were rolled and placed into 5 mL tubes using sterile forceps and stored on dry ice until transport to the labs where they were stored at -20°C until extraction. For the virus filters, up to 300 mL was pre-treated with 3 mL of 5M MgCl2 for 5 minutes, then filtered through 0.45 µm HA filters (Whatman), the vacuum was then released, and the filters were treated with 0.5 mL RNAlater for

3-5 minutes, followed by removal by vacuum. As with the DNA filters, the actual volume filtered was recorded (30-50 mL for raw sewage, 100 to 300 mL for samples). The virus filters were similarly placed into 5 mL tubes, stored at 4°C overnight, then placed on dry ice until transport to the labs where they were stored at -80C until extraction. For MRSA analysis filtration processing was conducted (as described in Goodwin & Pobuda, 2009) with gridded 0.45 µm filter paper (EMD Millipore, Burlington, MA). A 20 µm net filter (EMD Millipore, Burlington, Massachusetts) was stacked directly on the gridded filter to minimize difficulty in counting colonies on turbid filters. The volume of stormwater and stormwater/sewage mixture filtered varied according to appropriate volume necessary to yield 20 - 60 colonies. Concentrations of *S. aureus* were calculated in colony forming units (CFU) per 100 mL of water. Each volume of seawater filtered was preceded by a 20 mL phosphate buffer solution (PBS) prime and followed by a 20 mL PBS rinse. The filters were incubated on CHROMagar™MRSA. In addition to a CHROMagar™MRSA filter blank, a method blank filter was incubated on tryptic soy agar (TSA), which was made from Tryptic Soy Broth (BD Bacto, Fisher Scientific, Waltham, MA) and agar (Fisher Science, Hampton, NH).

For the soil eluents, for each depth, 4 replicates of ~25 g of soil was added to 250 mL of sterile PBS in 500 mL bottles and shaken by hand for 2 minutes. The samples were allowed to settle for ~50 minutes. The supernatant from the 4 replicates was removed using serological pipettes and combined prior to filtration (30 to 50 mL each for both DNA and virus filters, actual volumes recorded, processed as described above). Filter blanks (nanopure water for DNA & virus filters, sterile PBS for soil eluents) were processed for each date, following the same procedure as with the samples.

Membranes were shipped on dry ice to their respective university lab from the OCPW lab. Upon arrival, the virus filters were stored at -80°C until further processing. Extractions for viruses were performed using the Qiagen PowerViral DNA/RNA extraction kit using betamercaptoethanol; extractions were performed using the manufacturer's instructions, except bead beating was extended to 10 minutes and DNA/RNA was eluted from spin columns using two rounds of 50 µl of DNA/RNA-free water (for a total of 100 µl of template). The bacteria filters were stored at -20°C until subsequent DNA extraction with PowerWater Isolation Kit (Qiagen, Hilden, Germany).

Fecal indicator bacteria were quantified by two laboratories, Orange County Health Care Agency (HCA) and Enthalpy. HCA used EPA Method 1600 (2002) for *Enterococcus*, EPA Method 1603 (2014) for *E. coli*, and APHA 9222 D for fecal coliform. Enthalpy lab used Standard Methods 9230-D for *Enterococcus* and Standard Methods 9223-B-b for *E. coli* and fecal coliform. Coliphage was quantified by Double-Layer Agar plaque assay (without enrichment) (EPA Method 1601, 2001).

Quantitative Real-Time PCR (qPCR) and Droplet Digital PCR (ddPCR)

*Sul*1, *erm*B, and *intI*1

Amplification for all genes was performed in a StepOnePlus (Applied Biosystems, Foster City, CA) in 20 µL reaction mixtures on 96-well plates containing PowerUp SYBR® Green Master Mix (Applied Biosystems, Foster City, CA), 4µL aliquots of template DNA, and forward and reverse primers at 200nM final reaction concentration (**Error! Reference source not found.**). For each plate a seven-point standard curve was used along with negative control. Due to inhibition, all DNA templates were diluted with RNA/DNA-free water to a concentration of

1.0 ng/µL. The reaction conditions for *sul*1 are as follows: an initial denaturation at 95°C for 15 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 65°C for 30 seconds, and extension 72°C for 30 seconds. The reaction conditions for *erm*B are as follows: an initial denaturation at 95°C for 15 minutes, followed by 45 cycles of denaturation at 94°C for 20 seconds and annealing at 60°C for 1 minute. The reaction conditions for *intI*1 are as follows: an initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, and extension 72°C for 30 seconds.

16s rRNA

The 16s rRNA assay was performed in a StepOnePlus (Applied Biosystems, Foster City, CA) in 25 µL reaction mixture on 96-well plates containing PowerUp SYBR® Green Master Mix, 2 µL aliquots of template DNA, and forward and reverse primers at 600nM final reaction concentration. For each plate a five-point standard curve was used along with negative control. Each sample was performed in triplicate wells, from which the standard error was calculated. Due to inhibition, all DNA templates were diluted with RNA/DNA-free water to a concentration of 1.0 ng/uL.

invA

For Salmonella, the *invA* Taqman assay was used following the primers described in González-Escalona et al., 2009. Amplification was performed in a StepOnePlus (Applied Biosystems, Foster City, CA) in 20 µL reaction mixtures on 96-well plates containing TaqMan™ Environmental Master Mix 2.0 (Applied Biosystems, Foster City, CA), 2µL aliquots of template DNA, forward and reverse primers at 10uM final reaction concentration, and probe at 7.5uM

final concentration. For each plate a seven-point standard curve was used along with negative control. The reaction conditions are as follows: an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension 72°C for 15 seconds.

HF183 and GenBac3

HF183 were quantified using the SIPP HF183 method (Layton et al. 2013; Cao, Griffith, and Weisberg 2016; Griffith et al. 2013) via the automatic SMART platform and GenBac3 were quantified following Epa Method B (2010).

Campylobacter

For Campylobacter, the Taqman assay was used following the primers, probes, and reaction conditions described in (Cao, Griffith, and Weisberg 2016).

crAssphage

crAssphage was detected using the methods of Stachler & Bibby, 2014. Each 25 μL reaction included: 1X Environmental Master Mix 2.0, 1 μM of forward and reverse primers, 0.08 μM probe, 5 μg BSA, 2 μL of template, and the balance water. Standard curves were constructed using DNA oligos; the range of the curve ranged from 15.6 gc/rxn to 1.56 x 10^6 gc/rxn. All samples were run in duplicates and undiluted in qPCR. Thermocycling conditions were as follows: 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds then 60°C for 60 seconds.

AdV

Adenovirus was detected using the methods of Sassoubre et al., 2012. Each 20 μL reaction included: 1X Fast Virus Master Mix, 0.25 μM of forward and reverse primers, 0.125 μM probe, 2 μL of template, and the balance DNA/RNA-free water. Standard curves were constructed using DNA oligos; the range of the curve was from 12 gc/rxn to 1.2 x 10^6 gc/rxn. Due to inhibition, all extracts were run at 1:10 dilutions in RNA/DNA-free water. All samples were run in duplicate. Thermocycling conditions were as follows: 50°C for 5 minutes, 95°C for 20 seconds, followed by 45 cycles of 95°C for 3 seconds then 60°C for 30 seconds.

PMMoV

A two-step RT-qPCR assay was performed to quantify PMMoV in samples using the methods of Symonds et al., 2014. Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit with RNase inhibitor following the manufacturer's instructions. qPCR was performed in a 25 μl reaction, with the following reagents: 1x Environmental Master Mix 2.0, 0.4 μM of forward and reverse primers, 0.125 μM of probe, 2 μL of template cDNA, and the balance DNA/RNA-free water. Standard curves were constructed using DNA oligos; the range of the curve ranged from 12 gc/rxn to 1.2 x 10^6 gc/rxn. All samples were run in duplicates and undiluted in qPCR. Thermocycling conditions were as follows: 95°C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds then 60°C for 60 seconds.

Inhibition testing for AdV, PMMoV, and crAssphage

A subset of five of the samples were randomly chosen to test for inhibition using a spike and dilute approach. Each of those five samples, after reverse transcription, was spiked with a

standard for each respective qPCR target. Then, those spiked samples were diluted 1:10 in RNA/DNA-free water. The spiked and spiked then diluted samples were quantified for each target using qPCR, and the change in C_q was calculated. If the change between the diluted and spiked sample was greater than 2.3 cycles, the sample was considered uninhibited. If the change in Cq was less than 2.3 cycles, the sample was considered inhibited and all samples were run at a 1:10 dilution in RNA/DNA-free water. Using this approach, we determined that the qPCR assay for crAssphage and PMMoV could be run undiluted, while the assay for AdV was inhibited in our samples and needed to be run at a 1:10 dilution.

Table 11: The primers and probes for all qPCR and ddPCR assays.

Gene	Forward primer/reverse primer	Probe	Reference
*sul*1	F:CGCACCGGAAACATCGCTGCAC R:TGAAGTTCCGCCGCAAGGCTCG	NA	Pei et al., 2006
intI1	F:GGCTTCGTGATGCCTGCTT R:CATTCCTGGCCGTGGTTCT	NA	Luo et al., 2010
ermB	F:AAAACTTACCCGCCATACCA R:TTTGGCGTGTTTCATTGCTT	NA	Knapp et al., 2010
16s rRNA	F: ATGGCTGTCGTCAGCT R: ACGGGCGGTGTGTAC	NA	Pan & Chu, 2018
Campylobacter	F: CACGTGCTACAATGGCATAT R: GGCTTCATGCTCTCGAGTT	CAGAGAACAATCCGAACTGGGACA	Griffith et al., 2016
invA	F: CAACGTTTCCTGCGGTACTGT R: CCCGAACGTGGCGATAATT	CTCTTTCGTCTGGCATTATCGATCAGTACCA	González-Escalona et al., 2009
GenBac3	F: GGGGTTCTGAGAGGAAGGT R:CCGTCATCCTTCACGCTACT	CAATATTCCTCACTGCTGCCTCCCGTA	EPA Method B, 2010
HF183	F: ATCATGAGTTCACATGTCCG R: CTTCCTCTCAGAACCCCTATCC	[6-FAM]-5'-CTAATGGAACGCATCCC –MGB [VIC]-5'-	Griffith et al., 2013

		AACACGCCGTTGCTACA –MGB	
Adenovirus A-F	F:GGACGCCTCGGAGTACCTGAG R:ACNGTGGGGTTTCTGAACTTGTT	CTGGTGCAGTTCGCCCGTGCCA	Sassoubre et al., 2012 Jothikumar et al., 2005
crAssphage	F:CAGAAGTACAAACTCCTAAAAAACGTAGAG; R:GATGACCAATAAACAAGCCATTAGC	AATAACGATTTACGTGATGTAAC	Stachler & Bibby, 2014
PMMoV	F:GAGTGGTTTGACCTTAACGTTTGA R: TTGTCGGTTGCAATGCAAGT	CCTACCGAAGCAAATG	Haramoto et al., 2013; Rosario et al., 2009 Symonds et al., 2014

Data Analysis of relative breakthrough and log reduction

Contaminant removal was evaluated by calculating the total mass in and out of the cell for each microbial parameter. To account for volume and mass loss due to leakage issues in the biofilter construction, microbial parameter removal was calculated using a modified protocol of Ryan et al., 1999. Relative breakthrough (RB) is the ratio of cumulative fraction of initial microbial mass recovered at the outlet over time and the cumulative fraction of initial bromide mass recovered at the outlet over time.

$$RB = \frac{\left(\frac{M}{M_0}\right)}{\left(\frac{B}{B_0}\right)}$$

where M is the cumulative microbial mass recovered, M_0 is the total microbial mass in the influent, B is the cumulative bromide mass recovered, and B_0 is the total bromide mass in the influent. If RB > 1, then the biofilter is a source of microbial pollutants. If RB < 1, then the biofilter is a sink of microbial pollutants.

$$Attenuation\ (\%) = 1 - RB$$

$$Log\ reduction = -\left(log_{10}\left(\frac{Attenuation}{100} + 1\right)\right)$$

Attenuation was calculated as 1 – RB, and then logarithmic transformation to provide microbial removal as a log reduction.

Results

Systematic Review

The literature search generated 36 total articles. Eight articles met the inclusion criteria. One of the studies was a laboratory-scale column experiment. Four of the studies were field mesocosms: three were column experiments conducted in greenhouses and one consisted of rectangular plastic basins in a tent. Only three papers detail experiments conducted in full-scale stormwater biofilter cells. All eight papers evaluated *E. coli* removal and three also evaluated *Enterococcus* removal. Removal of these two indicator bacteria was compared to one or more other microbial parameters (**Error! Reference source not found.**). Most strikingly, no studies have evaluated the removal of fecal source markers, antibiotic resistance genes or genetic elements such as integrons. Integrons are capable of disseminating antibiotic resistance within environmental compartments, and the soil of stormwater biofilters may serve as an important

reservoir for resistance genes. Therefore, it is imperative to evaluate the removal of both integrons and antibiotic resistance genes. Within the three articles that consisted of full-scale biofilter studies, only *E.coli, Enterococcus,* and coliphage were included as microbial parameters.

Excluding one coliphage measurement, all of the microbial parameters observed in the studies did not experience any leaching events and had a net positive reduction. *E.coli* average log reduction ranged from 0.23 to 3.16 and *Enterococcus* average log reduction ranged from 0.28 to 1.03. F+RNA coliphage, a type of virus infecting *E. coli* was evaluated in seven of the studies, highlighting its common use as an indicator of human fecal pollution. In one field mesocosm, coliphage was not removed (average log reduction -0.02) but otherwise the removal ranged from 0.29 to 4.23. The fecal pollution indicator, *C. perfringens*, and pathogen *Campylobacter* each appeared in three studies. *Staphylococcus* and *Salmonella* removal were only included in the laboratory microcosm experiment. Similarly, Adenovirus, the only human viral pathogen, was included in one field mesocosm (Chandrasena et al. 2017) experiment (Afrooz et al. 2018) (cite). These three pathogens have never been studied for removal within a large, field-scale biofilter.

It is important to note that six of the studies contained media amendments that are not commonly used in full-scale biofilters. For example, Jung et al., 2019 utilized an antimicrobial media composed of a Copper-coated zeolite in a laboratory mesocosm. Novel media amendments are easily studied within column experiments, just not practically incorporated into the construction of actual biofilters. There is clearly a need to further understanding in how full-scale biofilters without media amendments remove pathogens in addition to traditional indicator bacteria.

Table 12: Average log removal of microbial contaminants in biofilters reported by literature.

	AUTHOR	MEDIA AMENDMENTS	EC	ENT	FRNA	CAM	CP	SA	SAL	ADV	CO
LABORATORY MICROCOSM	Afrooz et al. 2018	Biochar	1.9 ± 0.1		1.76 ± 0.32			3.9 ± 0.2	3.7± 0.2		
		Sand	0.23 ± 0.03		0.39 ± 0.1			0.33 ± 0.02	0.37 ± 0.03		
FIELD MESOCOSM	Chandrasena et al. 2017	Sugarcane mulch, pinewood chips	1.2		-0.02	0.7	2.1			1	1.7
	Jung et al. 2019	Woodchips	2.54 ± 0.00	2.4†	2.2†	2.45†	3.5†				
		Copper-coated zeolite	3.16 ± 0.00	2.5†	3.1†	3.2†	3.5†				
	Li et al. 2012	Vermiculite, perlite, redgum wood chips and pea straw	1.6 ± 1.0		4.32		3.11				
	Kranner et al. 2019	Biochar	0.58 ± 0.06	0.51 ± 0.04	1.28 ± 0.01						
		Sand	0.35 ± 0.08	0.28 ± 0.07	0.83 ± 0.02						
BIOFILTER	Chandrasena et al. 2016	Sand	1.27 ± 0.31			0.84 ± 0.26					
	Davies et al. 2008	Sand	1.6 ± 0.00		1.51 ± 0.01						
	Youngblood et al. 2017	Fly-Ash	0.35 ± 0.14	1.03 ± 0.04	0.29 ± 0.12						

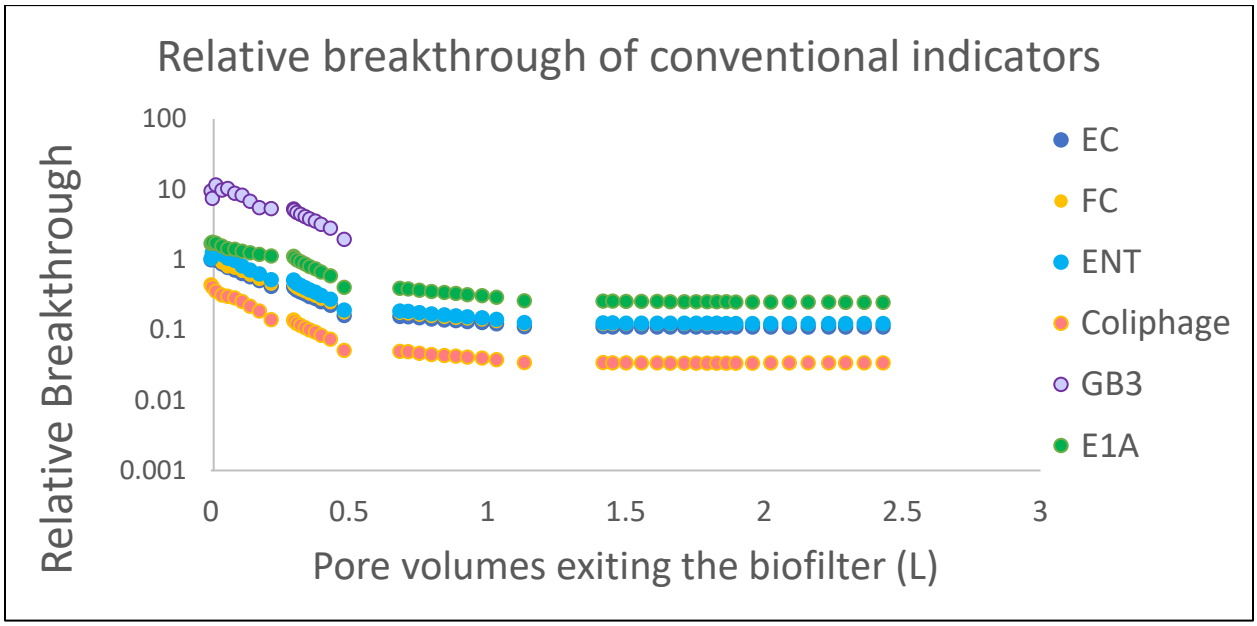
Abbreviations: EC, *E.coli*; ENT, *Enterococcus*; FRNA, male-specific coliphage; CAM, *campylobacter*; CP., *C. perfringens;* SA, *S. aureus*; SAL, *Salmonella*; ADV, adenovirus; CO, Cryptosporidium oocysts

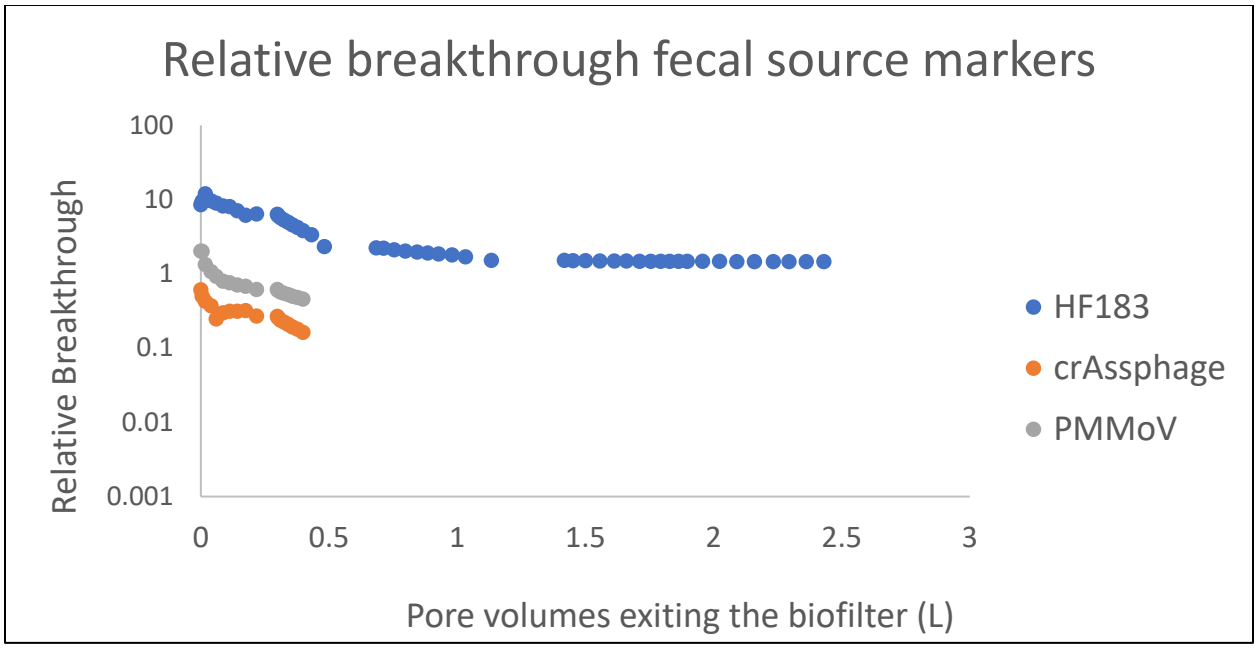
†Indicates that the author only provided median log reduction value

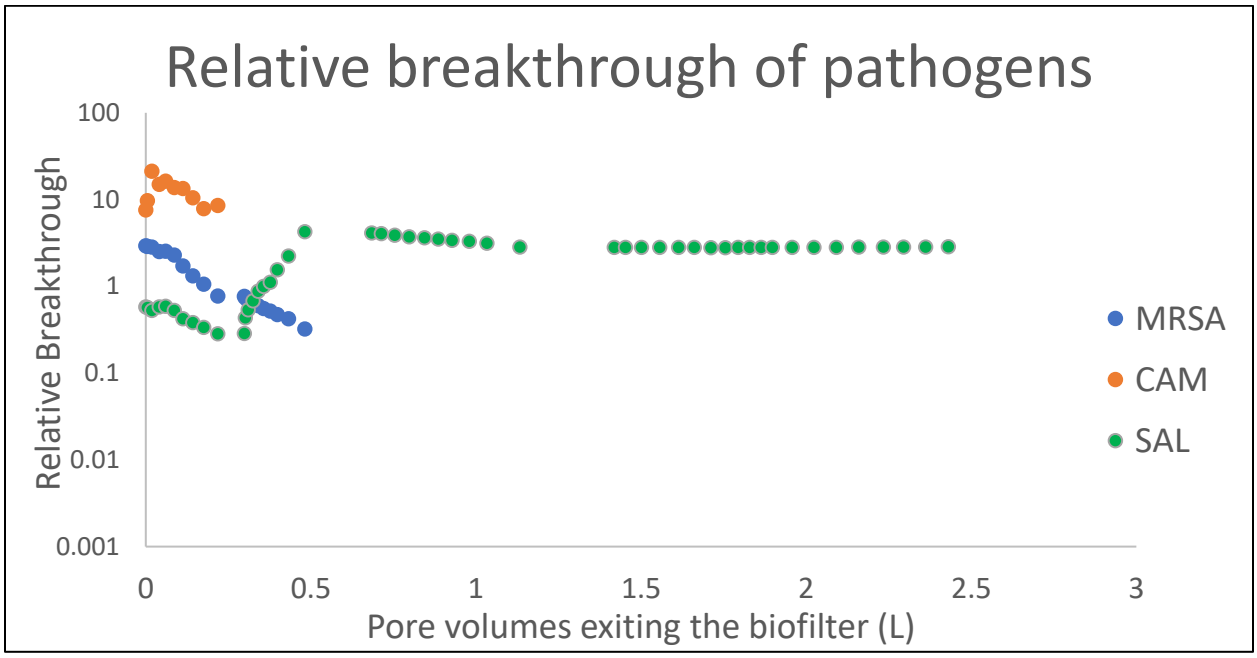
Relative breakthrough of FIB, fecal sources markers, pathogens, and ARGs

Initially FIB mass appears to go straight through the biofilter without any log reduction, either from short circuiting or perhaps from natural FIB growth within soils. However, by the last effluent sampling of the first storm, which was composed of both stormwater and raw influent, FIB is reduced 0.38, 0.35, and 0.29 for *E.coli*, fecal coliform, and *Enterococcus* respectively. The cell is flushed four more times with just stormwater. Between the tail end of the second stormwater-only flush and the last effluent collected from the final storm, the log reduction in FIB mass stays constant (Figure 14).

Salmonella initially is removed by the biofilter, but then increases after the first stormwater flush and remains with a relative breakthrough greater than one—indicating the biofilter is a source. Both *Bacteroides* markers—GenBac3 and HF183—remain above one the entire time, suggesting that they pass straight through the system.




Relative breakthrough fecal source markers
Relative Breakthrough
100
10
1
0.1
0.01
0.001
0
0.5
1
1.5
2
2.5
3
Pore volumes exiting the biofilter (L)
HF183
crAssphage
PMMoV


Relative breakthrough of pathogens
Relative Breakthrough
100
10
1
0.1
0.01
0.001
0
0.5
1
1.5
2
2.5
3
Pore volumes exiting the biofilter (L)
MRSA
CAM
SAL

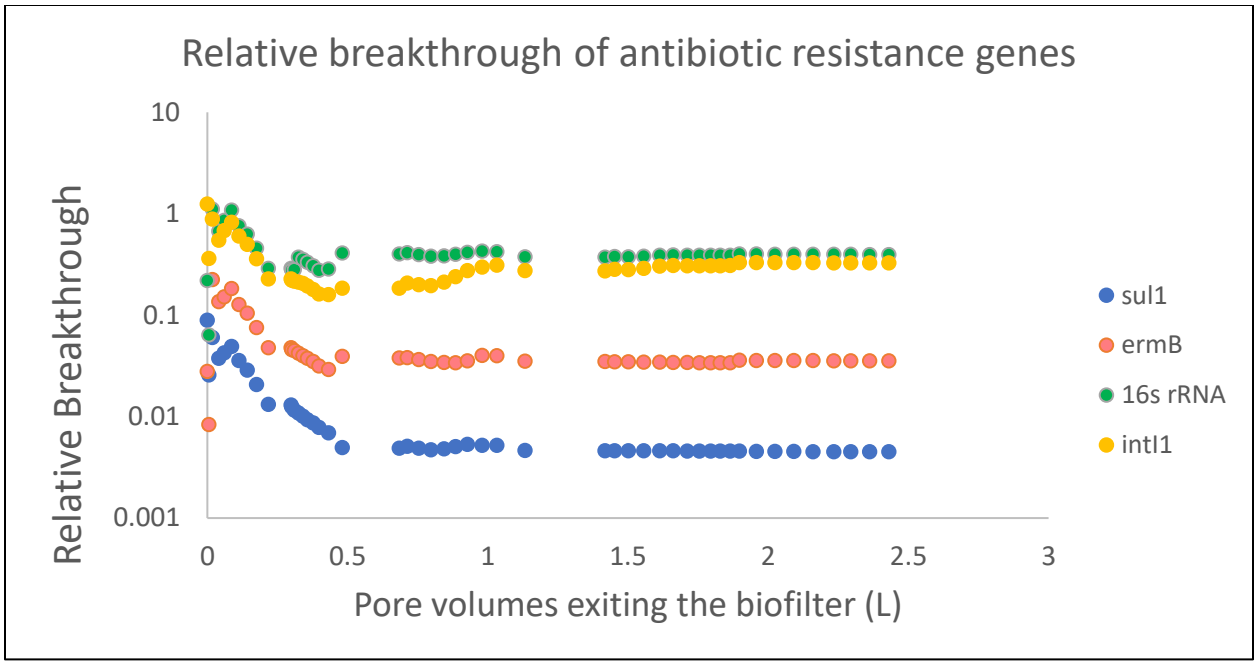


Figure 13: Relative breakthrough of microbial parameters grouped by: (from top to bottom) conventional viral and bacterial indicators, fecal source markers, pathogens, and lastly antibiotic resistance genes.

Log reduction of FIB, fecal source markers, pathogens, and ARGs

Monitoring of biofilter effluent after addition of stormwater showed that C4 was effective at removing culture-based FIB with log reductions ranging from 0.91 and 0.96 (**Error! Reference source not found.**). Genetic markers for conventional bacterial indicators GenBac3 and Entero1A were also reduced, but not to the extent of their culture-based counterparts. Coliphage was the only culture-based viral fecal indicator and achieved excellent removal with a log reduction of 1.47.

There were differences in the viral fecal source markers and the bacterial fecal source marker. PMMoV and crAssphage experienced satisfactory removal while HF183 experienced a net leaching from the system. MRSA was the only culture-based pathogen measured and achieved almost a half log reduction. The two other bacterial pathogens, *Salmonella* and *Campylobacter*, which were quantified using genetic methods, both experienced significant

leaching out of the biofilter. Adenovirus was the only viral pathogen and achieved log reduction of 1.3.

The proxy for total cells, 16s rRNA, experienced a log reduction of 0.40, showing that the biofilter was responsible for decreasing the total bacterial load. *Sul*1 experienced the largest log reduction of 2.35. Additionally, the relative abundance of *sul*1 (not normalized with Bromide) was reduced tenfold by the biofilter. The influent from the combined sewage and stormwater storm was $7.8x10^{-1}$ *sul*1 gene copies per 16s rRNA gene copies and the geometric mean of the effluent was $7.5x10^{-2}$. *Erm*B was also well removed with a log reduction of 1.47. The relative abundance of *erm*B (not normalized with Bromide) was dramatically reduced by the biofilter by almost two orders of magnitude. The influent from the combined sewage and stormwater storm was 9.0 $x10^{-2}$ *erm*B gene copies per 16s rRNA gene copies and the geometric mean of the effluent was 1.1 $x10^{-3}$. The absolute abundance of the *IntI*1 gene decreased but to a lesser extent than the two ARGs. The relative abundance of *IntI*1 (not normalized with Bromide) was actually slightly increased by the biofilter. The influent from the combined sewage and stormwater storm was 1.4 $x10^{-2}$ *intI*1 gene copies per 16s rRNA gene copies and the geometric mean of the effluent was 1.8 $x10^{-2}$.

Table 13: The final log reduction in mass of all microbial parameters.

	E.coli	Fecal Coliform	*Enterococcus*	Coliphage	Entero1A
Log Reduction	0.96	0.93	0.91	1.47	0.61
	Campylobacter	AdV	*Salmonella*	MRSA	
Log Reduction	-0.93	1.30	-0.63	0.49	
	HF183	GenBac3	crAssphage	PMMoV	
Log Reduction	-0.16	-0.29	0.79	0.34	
	16s	*sul*1	*intI*1	*erm*B	
Log Reduction	0.40	2.35	0.49	1.47	

Pathogens, FIB, fecal source markers in soil cores

E. coli and fecal coliform were below the limit of detection in the control cell but ranged between 3400 to 15400 MPN /100 mL in C4 (Table 14). *Enterococcus* and MRSA had similar concentration in both C2 and C4 were very similar, indicating that either the soil media was a bacterial source itself, or that there was growth after previous stormwater dosing. Coliphage was below the limit of detection in C2 and C4. HF183 was detected but not quantifiable, or not detected in both C2 and C4. Entero1A was not detected in either cell excluding the top ten cm of C4.

Table 14: The soil cores of test cell C4 and control cell C2.

	Depth	MRSA (CFU / 100 mL)	*E.coli* (MPN / 100 mL)	Fecal Coliform (MPN / 100 mL)	*Enterococcus* (MPN / 100 mL)	Coliphage (MPN / 100 mL)	Entero1A (copies / 100 mL)	HF183 (copies / 100 mL)
C4	0 - 10 cm	4500	6600	7300	380	< 100	5000.00	DNQ
	10 - 20 cm	2900	2300	3500	150	< 100	DNQ	ND
	30 - 40 cm	3400	14500	15400	720	< 100	DNQ	ND
	50 - 60 cm	3400	3400	3100	350	< 100	DNQ	ND
Control Cell (C2)	0 - 10 cm	5300	< 9	< 9	750	< 100	DNQ	DNQ
	10 - 20 cm	12800	< 9	< 9	430	< 100	DNQ	ND
	30 - 40 cm	9600	< 9	< 9	230	< 100	ND	ND
	50 - 60 cm	2600	< 9	< 9	160	< 100	ND	ND

Discussion

Novel fecal source markers may provide a better approach for monitoring surface water quality, but their fate and transport in stormwater biofilters has never been investigated. While FIB removal in biofilters has been fairly well studied, it is not known if stormwater biofilters are effective at removing disease causing pathogens. It is also not known whether FIB or fecal source markers offer a better indication of pathogen removal or lack thereof within these infrastructure systems. Identification of stormwater biofilters' capacity to remove microbial contaminants is important for helping direct future design requirements and best practices.

In this study, a full-scale stormwater biofilter test cell was evaluated for FIB, fecal source marker, pathogen, and ARG removal during five storm events. The OCPW test cell C4 received mixed sewage and stormwater influent followed by four stormwater-only flushing events. C4 effectively removed most conventional fecal indicators, viral fecal source markers and viral pathogen Adenovirus, and two ARGs. The test cell was also a reservoir for bacterial pathogens

Campylobacter and *Salmonella* which proliferated in the system. Both *Bacteroides* markers, HF183 and GenBac3, seemed to pass through the test cell with no observed log reduction. Microbial pollutants have unique characteristics that complicate their removal by biofilters. They are biological organisms that can grow and interact not only with each other but also with their environment (Rippy 2015). The extent and speed of growth differ depending on the type of microbes and the environmental biotic and abiotic conditions (Peng et al., 2016; Maurya et al., 2020). Many natural treatment systems have reported increases in FIB levels likely due to growth of these microbes within the treatment systems. Biofilters can also leach FIB from their filter media due to the intermittent nature of stormwater infiltration, where the wetting fronts or air-water interfaces can eject previously attached bacteria, causing effluent concentration to be greater than influent concentration (Mohanty et al., 2013).

With the exception of GenBac3, all bacterial fecal indicators were successfully reduced by C4. FIB appeared to resist mobilization throughout the following four-flushing events that followed the sewage influent. C4 showed greater FIB removal compared to the average FIB removal for biofilters without saturated zones reported in Rippy et al.'s meta-analysis (2015). Rippy found the average log reduction for *E.coli, Enterococcus,* and fecal coliform to be 0.86, 0.57, and 0.85, respectively. While C4's removal of *E.coli* and fecal coliform are comparable, the *Enterococcus* log reduction is much greater. It should also be considered that the Rippy analysis incorporates laboratory and field scale column experiments, which may not accurately reflect real life biofilter *Enterococcus* removal conditions. The only full-scale biofilter experiment to examine *Enterococcus* removal, retrieved by the systematic review discussed previously, found a similar removal in an Oklahoma biofilter amended with fly-ash. Although

the OCPW achieved almost one log reduction, it is important to consider that the effluent concentrations still greatly exceed safe levels recommended for recreational water quality.

Entero1A is a relatively new molecular method for quantifying FIB. C4 appeared to better remove culture-based *Enterococcus* compared to Entero1A. Some studies have found Entero1A to be an overestimation of *Enterococcus* quantity, which could explain the removal differences (Raith et al., 2013). This can be explained by the differences in detection methods. Culture-based methods depend on cell viability and would not account for damaged cells. *Enterococcus* cells could die off or be compromised during the filtration process and molecular methods would capture these bacteria.

C4 reduced MRSA while the other two bacterial pathogens, *Salmonella* and *Campylobacter*, experienced a net leaching effect. This finding does not agree with other biofilter experiments that observed positive reductions in these two pathogens. The increase in *Campylobacter* and *Salmonella* in effluent can possibly be attributed to regrowth and proliferation within the biofilter soil. *Campylobacter* can also form biofilms for survival and for persistence (Bronowski et al., 2014). *Salmonella* can also persist in soil (Jechalke et al., 2019). *Staphylococcus aureus* had previously been examined for stormwater biofilter in one laboratory experiment. In a sand column *Staphylococcus aureus* achieved 0.33 reduction, less than our observed reduction for methicillin-resistant *Staphylococcus aureus.* The difference in removal between MRSA, *Salmonella*, and *Campylobacter* could be attributed to the contrasting culture-based and molecular methods with the latter method accounting for nonviable cells.

There was no reduction in either general fecal indicator GenBac3 or human-associated fecal source marker HF183 observed in the biofilter test cell. In fact, these two bacterial parameters were observed to have a leaching effect. However, this may just be a result of

normalizing the results with Bromide as opposed to an indication that there is some type of bacterial regrowth within the soil. Most likely these two markers were not retained at all by the test cell and passed straight through in the effluent. The other two viral fecal source markers, PMMoV and crAssphage, were actually greatly reduced by the biofilter.

In fact, all viral parameters were removed by the OCPW test cell. In addition to the two viral source markers, the viral indicator (coliphage) and viral pathogen (adenovirus) were effectively removed. In water quality monitoring, coliphage is considered to be a better indicator of viral pathogens that FIB due to the different physical and chemical properties of viruses and bacteria(EPA, 2015). Coliphage has been relatively well-studied within the context of stormwater biofilters. Appearing in seven out eight articles collected during the systemic review, it has been used as a proxy for understanding virus removal within treatment systems. In C4, the coliphage is adequately removed; the log reduction is greater than FIB. The reduction in coliphage is less but similar to the log reduction reported in a similar study conducted with a full-scale sand biofilter (Davies et al. 2008). Adenovirus has only been studied in a laboratory-scale column experiment where it achieved one log removal (Chandrasena et al. 2017), which was very comparable to the log reduction removed by C4. In the C4 Challenge experiment, coliphage was found to be an appropriate indicator for adenovirus removal.

In surface water or stormwater, the fate of bacteria and viruses deviates due to differences in chemical and physical properties. Complex abiotic and biotic factors impact their survival and life cycle. Additionally, general indicators, fecal source markers, and pathogens vary greatly in size, shape, surface property and physiology. In this biofilter, it appears that FIB is not an appropriate reference for all pathogen removal. For *Campylobacter* and *Salmonella*, HF183 and GenBac3 are a better approximation for the fate and transport within the system. Accordingly,

the viral fecal source markers and indicators persist differently in surface water compared to bacterial fecal source markers such as HF183. Similarly, these fecal source markers could be removed differently by treatment systems such as stormwater biofilters.

The results of the systematic review indicate that ARG fate and transport has not been previously studied in stormwater biofilters. It has not been clear whether biofilters can sufficiently remove emerging contaminants like ARB and ARGs. Studies have shown that stormwater carries ARGs into freshwater water bodies (S. Zhang et al. 2016; Garner et al. 2017). Biofilters may be a potential hotspot for ARGs proliferation due to the existing of biofilms (fostering horizontal gene transfer) and selective and co-selective factors such as heavy metals and antibiotics (Baker-Austin et al. 2006). Biofilters can accumulate both commensal and pathogenic bacteria from stormwater. Additionally, many biofilters are constructed with geomedia such as compost which may serve as a source of ARGs (Riber et al. 2014; Sharma and Reynnells 2016). It is not understood if the detection of FIB or fecal source markers or pathogens in an environmental corresponds to the presence of ARGs.

C4 effectively reduced both the absolute and relative abundances of both ARGs, *erm*B and *sul*1. These findings indicate that stormwater biofilters may reduce transmission of antibiotic resistance in the environment. For extracellular ARGs, DNA molecules could be removed from influent due to adsorption on sediment (Cai et al., 2006; Hua et al., 2013; Xue & Feng, 2018). The observed reduction in *sul*1 and *erm*B is consistent with some studies of both natural and constructed wetlands that examined ARG removal (Chen & Zhang, 2013; He et al., 2018; Hsu, et al., 2017). Constructed wetlands are manmade systems designed to treat stormwater or wastewater through natural wetland processes like filtration, UV degradation, and adsorption (Lamori et al. 2019). Wetlands have also been shown to be a sink for ARB (Kawecki et al.,

2017). It is useful to compare microbial removal in wetlands to microbial removal in stormwater biofilters due to their similarity. Both biofilters and wetlands rely on natural processes for stormwater remediation, but wetlands typically have longer residence times. Three constructed wetlands treating wastewater greatly reduced *sul*1 and *erm*B (Chen and Zhang 2013; Yujie He et al. 2018). However, another constructed wetland treating wastewater found that *sul*1 proliferated within the treatment system and had a -262% removal while another urban stormwater wetland found "no overall reduction" in *sul*1 (He et al., 2018; Hsu et al., 2017).

The results of this study indicate that stormwater biofilters can effectively reduce ARGs, but this study is limited by only examining two genes. There are hundreds of known ARGs, all of which may exhibit gene-specific behavior. Also, the ARG removal might seem more pronounced when raw sewage is the microbial source and may seem less dramatic when stormwater is the less concentrated microbial source. C4 also reduced in *intI*1 and 16s rRNA, but not the same degree as the two ARGs. *IntI*1 is removed up to 90% in the majority of constructed wetland studies. There was one instance of leaching with -310% *intI*1 detected in the effluent. In all of the instances where 16s rRNA was quantified in constructed wetlands, it only increased in the effluent with percent removals ranging from -210% to -620%. In these complex ecosystems, it is understandable how the total cells could increase. The relative abundance of both ARGs was decreased, indicating that this biofilter is not a reservoir for the dissemination of antibiotic resistance. The relative abundance of *intI*1 slightly increased in the effluent, indicating that horizontal gene transfer was occurring within the biofilter.

It is possible that raw sewage might not always be representative of typical microbial contamination in stormwater. More research is needed with full-scale biofilters and real stormwater. Additionally, more research is needed with replicate experiments. So far, the

existing literature (including this study) seem to generate very site-specific results. Consistent results are needed to make appropriate recommendations for stormwater infrastructure construction.

In summary, these results indicate that viral fecal source markers and viral fecal indicators are appropriate for assessing viral pathogen fate and transport within these systems. However, the relationship between bacterial indicators and fecal markers with bacterial pathogens is more complicated. Relying purely on traditional indicator bacteria, it would appear that the sewage is reduced by the OCPW test cell. However, the two Bacteroides markers suggest that the biofilter causes no reduction in fecal pollution. Future research warrants characterization of microbial surface properties (such as hydrophobicity or surface charge) to properly understand mechanisms of removal or lack thereof within particular biofilters.

Chapter 4: Conclusion

Antibiotic resistant infection rates have been increasing in the last few decades. The ocean may be an understudied reservoir of antibiotic resistant bacteria and play an important role in pathogen transmission. Surfers are particularly vulnerable to these environmental exposure events, particularly during wet-weather events when levels of microbial contamination can increase. This epidemiological surfer research was one of the few studies to document an association of environmental ARB exposure and subsequent colonization in a non-agricultural setting. More research is needed to determine the connection between environmental ARB and human disease.

In California, new legislation requires all new development projects to treat the first 0.75 inches of a rain on-site. In order to implement new policies such as this, it is critical to understand how stormwater biofilters can remove microbial pollution, particularly ARGs and ARB. The test cell located on the OCPW campus demonstrated that more research is needed on full-scale biofilters to fully evaluate pathogen removal. In this biofilter, certain bacteria species were removed while other species passed straight through the cell.

Additionally, the fate and transport of ARGs within topsoil was studied to diagnose biofilters as a potential reservoir of ARGs. 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.

Major health organizations including WHO and the CDC are beginning to promote a *One Health* perspective for combatting antibiotic resistance. The idea of One Health is that antibiotic resistance can be decreased or effectively managed by maintaining the synergistic health of humans, animals, and the environment. Environmental solutions to AR are increasingly needed, as ARB are no longer a concern unique to the healthcare industry. Stormwater biofilters are a solution to minimize AR proliferation in the environment, and their installation may decrease downstream ARB colonization events in recreational waters. Research should continue to be performed to further understanding of the fate and transport of these important new contaminants, and their effects on human health. This research can be applied to appropriately regulate both ARB and ARG in surface water, wastewater, and drinking water.

Chapter 5: References

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