MICROBIAL ASSEMBLY AND RESPONSES TO ROAD RUNOFF CONTAMINANTS IN THE EUGENE, OREGON URBAN STORMWATER SYSTEM

by

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THESIS ABSTRACT

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Contaminants like trace metals and polyaromatic hydrocarbons can be carried by stormwater road runoff from into receiving waters, potentially compromising ecosystem health. The microbial communities dispersed by stormwater include populations that are carried into green infrastructure designed to mitigate the effects of stormwater. Microbial communities may influence the contaminant filtering functions of engineered bioswales, but this depends on the ability of microbes to survive dispersal and tolerate contaminants.

Evidence from microbial communities in stormwater and associated bioswales collected during a heavy precipitation event suggests that fungi and bacteria are assembled by different mechanisms, and that interactions with metals can produce specific responses in growth motility-related behaviors for some bacteria isolated from contaminant-amended stormwater. This research is intended to develop methods to identify contaminant-sensitive indicator taxa for stormwater road-runoff monitoring, and the network of bioswales in Eugene, Oregon is a promising system to study for this purpose.

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I: INTRODUCTION

Increasing urbanization alters the spatial and chemical environments that microbial communities must respond to, but there are gaps in our understanding of how urban infrastructure might affect microbial assembly processes and specific adaptive responses. Microbial communities can be assembled by stochastic processes like dispersal, and by deterministic processes like species sorting, depending on complex interactions between factors like environmental heterogeneity, the number of cells moving between locations, and microbial traits like dispersal ability and resource specialization (Adams, Crump & King, 2014). Notably, the ability to disperse alive has been identified as an important trait for microbial community assembly in water-associated habitats (Langenheder and Lindström, 2019), suggesting that the same may be true of microbes dispersed via stormwater runoff.

Most urban centers are required by law to manage stormwater, and green infrastructure projects have been initiated to mitigate the environmental effects of urban stormwater runoff by reducing stormwater flows to surface waters. Green infrastructure systems channel stormwater into bioswale soils and permeable pavements, substrates and surfaces to infiltrate and evotranspirate stormwater. Stormwater contaminants can persist after being sorbed into soils, and the ability of green infrastructure microbial communities to degrade, sequester, or transform contaminants can be a significant component of stormwater contaminant mitigation strategies (Gill, Lee & McGuire, 2017).

Urban stormwater transports a mixture of contaminants along roadways that are a significant source of inputs for urban water and soil systems (Liguori et al., 2021), and these contaminants can act alongside hydrodynamic forces as an additive stressor affecting the ability of microbes to survive dispersal through stormwater or colonize and persist in new environments (Langenheder and Lindström, 2019). Specific microbial responses to contaminants like metals are among the traits that could affect microbial community assembly in stormwater runoff (Liguori et al., 2021), for example by reducing the abundance of specialists to generalists (Qian et al., 2022). On the other hand, exposure to contaminants in stormwater could increase overall community tolerance by selecting for contaminant tolerant taxa with a variety of phenotypic adaptations (Cyriaque et al., 2020).

Heavy metals and PAHs detected together in urban stormwater samples during monitoring often co-occur as depositions from tire abrasion, vehicle brake emission, lubricants and other petroleum products build on impervious road surfaces (Liguori et al., 2021). Metals are naturally present in the environment, but trace metals that are essential micronutrients can be toxic at high concentrations and the concentrations in urban stormwater catchments can be several times higher than normal environmental background levels (Ancion et al., 2014). Metals have high electrostatic attraction potential and ligand binding affinities, which can allow them to interrupt the binding of other essential metals to their normal sites, disrupting cellular regulation. Metal-tolerant microorganisms can sequester metals intracellularly or extracellularly, expel them via permeability barriers or active transport, and transform or detoxify them (Prabhakaran et al., 2016). Efficient aggregation, biofilm formation and dissimilatory metal-reduction are all traits that can enhance the metal tolerance of some microbial taxa.

Contaminants in stormwater could induce priming responses, where a milder preceding environmental stress would theoretically prepare microbes for a more efficient stress response when the intensity of the stressor increases (Wesener & Tietjen, 2019). There is some evidence for prior acclimation history and the duration of contaminant exposure enhancing tolerance to contaminants (Xiao et al., 2019). This suggests that adaptive changes in traits important for successful dispersal and colonization, particularly growth and motility, could be induced incrementally by gradual increases in contaminant concentrations. Motility is a metabolically costly trait, and there are typically tradeoffs between migration and growth rate, with fast-moving, slow-growing groups and slow-moving fast-growing groups in mixed microbial populations (Gude, 2020). Stress may increase microbial investment in chemotactic motility (Ni et al., 2019), but chemical stressors may also inhibit motility depending on the class and concentration of chemical. There are still gaps in our knowledge of how phenotypic adaptations to specific contaminants might influence growth-motility tradeoffs and dispersal patterns in microbial assemblies.

The effect of contaminants on microbial assemblies can be assessed at the community level using molecular techniques to quantify taxonomic changes relative to contaminant concentrations (Ventorino et al. 2018, Birrer, Dafforn and Johnston, 2017). However, studies that assess the effect of contaminants on community assembly as well as adaptive traits in contaminant-resistant isolates enriched from these communities are rarer. The goal of this study

is to identify microbial assembly processes for bacteria and fungi in the Eugene urban stormwater system, and ultimately, to determine if differences in the enrichment or reduction of specific taxa might indicate the presence of specific contaminants. Individual bacterial isolates from this system may also have specific traits related to growth or motility that change in response to contaminants in consistent ways. This can help explain assembly processes at a finer scale, and potentially develop contaminant-sensitive microbial indicator taxa and strains relevant for this system.

This thesis addresses the following research questions:

1. Are differences in the microbial assemblages of stormwater road runoff and the microbial assemblages of associated stormwater management infrastructure explained primarily by stochastic or by deterministic processes?

2. Can variation in the growth and motility responses of individual bacterial isolates under metal stress explain dispersal and persistence patterns in the urban stormwater system?

To answer these questions, I conducted a community survey of fungal and bacterial taxa in stormwater road runoff and bioswale soil samples that were collected during a heavy rainfall event. I also investigated the growth and motility parameters of selectively cultured isolates to determine what changes might be consistently inducible by exposure to metals.

II. MATERIALS AND METHODS

Stormwater and Bioswale Sampling

Stormwater and bioswale soil samples were collected from 10 stormwater facility sites within the Eugene/Springfield urban growth boundary during first-flush rainfall between October 8-10, 2020. These sites were selected as a representative range of locations near the Willamette River and Amazon Creek monitoring basins (Figure 1).

Five 10 cm cores of bioswale soil adjacent to stormwater inlets at each of the 10 sites (n=50) were collected with a stainless steel soil probe (Grainger, Portland OR) and stored in individual Whirlpak bags. All implements were cleaned with 70% ethanol between core samples. At least 100 ml of stormwater runoff from 5 of the 10 sites was collected in sterile 50 ml falcon tubes, facing into the stormwater flow slightly upstream of the bioswale site. All stormwater samples were taken within 24 hours of the beginning of heavy rains, and all bioswale samples were collected within 48 hours of the beginning of heavy rains. Samples were transported from field sites in coolers and stored at -18 ℃ in the lab no later than 4 hours after collection.

Figure 1. Location of 10 selected bioswale sites within the Eugene/Springfield urban growth boundary.

Microbial Community DNA Extraction

All contamination-sensitive procedures were conducted in a biosafety cabinet equipped with a uv lamp. Bioswale soil cores were hand homogenized and 0.25 g of soil from every core (n=50) was weighed in autoclave- and uv-sterilized 2 ml eppendorf tubes. DNA was extracted using a Qiagen DNeasy PowerSoil single tube extraction kit (Qiagen, Germantown, MD) following the manufacturer's protocol.

At least 100 ml of the stormwater runoff from each site where first flush stormwater could be collected (n=5) was filtered through 0.2 um Sterivex syringe filters (MilliporeSigma, Burlington, MA). The remaining stormwater filtrate was autoclave-sterilized, filtered again with a 0.2 um filter (MilliporeSigma, Burlington, MA), and stored at 4°C for use as undefined stormwater (SW) growth media. Filters were removed from their casings and cut into sections using uv and ethanol-sterilized implements. Two sections from each filter were stored in a uv-sterilized 2 ml eppendorf tube. The method used to extract DNA from bioswale soil samples was also used to extract DNA from the filter.

DNA Sequencing and Sequence Processing

For each sample, the 16S rRNA V4-5 region was amplified with the universal primers 505f and 806r and the ITS1 rRNA region was amplified with the ITS1F and ITS2 primers. PCR reactions were performed according to a two-step protocol. PCR1 was performed in 20µl reactions according to the following recipe per sample: 1µl DNA, 0.5µl each of 10µM stock primer pools, 10µl GoTaq Green Master Mix (Promega, Madison, WI), 0.1µl BSA, 7.9µl nuclease free water. PCR conditions for 16S and ITS were: denaturation at 94℃ for 3 mins, 30 45 second amplification cycles at 94℃, 1 min at 54℃, 90 sec at 72℃, and a final 10 min extension at 72℃. PCR2 was performed in 25 µl reactions according to the following recipe per sample: 3µl PCR1 Product, 2µl of 5µM working plate primers, 10 µlGoTaq Green Master Mix (Promega, Madison, WI), and 10µl nuclease free water. PCR conditions for 16S and ITS were: denaturation at 94°C for 3 mins, 12 amplification cycles of 45 sec at 94°C, 1 min at 52°C and 90 sec at 72°C, with a final 10 min extension at 72°C.

1 µl of PCR2 products were quantified in flat-bottom 96 well plates using a PicoGreen dsDNA fluorophore (Invitrogen, Waltham, MA). RFUs were measured in a SpectraMax plate reader (Molecular Devices, San Jose, CA), and the values were used to calculate the volume of PCR2 product from each sample to be added to ensure a final concentration of 1ng per sample in the the final DNA pool for 16S and ITS amplicons. Pooled PCR2 products were purified using Qiagen QIAquick PCR purification kits (Qiagen, Germantown, MD). All sequences were processed using the Illumina NovaSeq platform. Sequences for all reads were de-multiplexed, quality filtered and processed using QIIME (Caporaso et al., 2010). Amplicon sequence variant (ASV) tables were assembled using the DADA2 pipeline (Callahan et al., 2016). For ITS sequences, taxonomy was assigned using the UNITE reference database. For 16S sequences, taxonomy was assigned using the Greengenes reference database. Statistical analyses were performed with the “phyloseq” and “dplyr” packages in R (v.4.1.2).

Experimental Contamination Enrichments

Inoculum from stormwater samples was obtained by suspending a separate section of the same sterivex filter used to filter stormwater samples for DNA extraction (n=5) in a sterile 15 ml falcon tube with 10 ml of SW media. A composite bioswale inoculum suspension for each site (n=10) was made by adding 0.5 g of bioswale soil from the 5 soil cores taken at each site to a sterile 15 ml falcon tube and suspending in 10 ml of SW media.

An experimental design with contaminant incubation treatments arranged in 96-well plates, similar to the experimental metacommunity described in Low-Décarie et al. (2015), was developed to investigate the specific effect of contaminants in the absence of dispersal on microbial community assembly. A review of stormwater contaminant concentrations reported from monitoring agencies in Oregon and Washington was conducted to identify experimental concentration gradients that might be ecologically relevant for the microbial communities in the Eugene urban stormwater system (Table 1).

Table 1. Concentration of contaminants detected in stormwater runoff during stormwater monitoring in the Pacific Northwest.

Contaminant	Source	Concentration (μM)
Zinc (Zn) Copper (Cu)	City of Eugene (2018)	0.0184-0.8810 0.0084-0.0931
Zinc (Zn) Copper (Cu) Naphthalene Pyrene	City of Seattle (2016)	1.08-6.42 0.16-1.01 3.90×10^{-4}-0.01638 2.47×10^{-4}-0.003659
Zinc (Zn) Copper (Cu)	City of Salem (2017)	1.372 0.199
Zinc (Zn) Copper (Cu)	City of Portland (2006)	0.576 0.128
Zinc (Zn) Copper (Cu) Naphthalene Pyrene	Washington State Department of Ecology (2015)	0.0214-19.73 0.005978-3.4296 3.12 x 10^{-4}-0.0172 3.46 x 10^{-4}-0.1285

Three different concentrations of copper, zinc, naphthalene and pyrene (Table 2) were selected to simulate a low, medium and high contaminant concentration gradient. Metals and PAHs were mixed to their final concentrations in SW media as one metal treatment and one PAH treatment at three different concentrations each. This strategy is similar to the method described in Ancion et al. (2010), and is intended to simulate the effects of co-contamination within a single contaminant class. Stock single contaminant solutions were prepared from autoclave- and filter-sterilized milliQ water and stored in the dark at room temperature.

Table 2. Final concentration of contaminants added to filtered stormwater media.

Contaminant	Treatment	Concentration (µM)
Zinc (ZnSO4)	Control Low Moderate High	n/a 1.0 5.0 10.0
Copper (CuSO4)	Control Low Moderate High	n/a 0.5 1.0 5.0
Naphthalene	Control Low Moderate High	n/a 0.0001 0.01 0.1
Pyrene	Control Low Moderate High	n/a 0.0001 0.01 0.1

Contaminant enrichments contained 1500 µl of culture (1200 µl amended SW media + 300 µl of inoculum) at the final concentrations in Table 2. For each composite stormwater and bioswale sample (n=15), there were six experimental treatments, representing a three-concentration gradient for metals and PAHs, with one replicate control treatment per sample in unamended SW media. All contaminant treatment cultures were incubated in the inner 60 wells of 2 ml 96-well deep well plates (MilliporeSigma, Burlington, MA). Samples were arranged as replicate columns with the sample order randomized for each plate, and treatments were arranged

across the inner six rows. Deep-well plate cultures were incubated in the dark at 30°C with gentle daily manual shaking. Airpore tape sheets (Qiagen, Germantown, MD) were used to seal the plates to allow gas exchange while preventing contamination. Inoculum from each culture was sampled at 7 and 14 days for sequencing (data pending) and culturing.

Bacterial Cultures and Strains

100 µl of inoculum from high and medium concentration treatments was suspended in 5 ml of stormwater growth media prepared in sterile 15 ml falcon tubes. Based on results from preliminary research with test bioswale soil suspensions conducted in Winter 2019, serially diluted 10^1 - 10^3 to obtain single colonies. 100 µl of each dilution was plated on 1% nutrient broth (NB) agar. Plates were incubated in the dark at 30°C and monitored daily for the growth of colonies for up to a month. Distinct colonies were subcultured 3 consecutive times on 1% NB agar plates to ensure that the cultures were axenic. Liquid growth media was inoculated with single colonies from axenic cultures using a sterile inoculating loop, incubated overnight, and preserved as 25% glycerol stocks stored at -80°C. Isolated cultures were tested for optimum growth in liquid 1/100 NB, 1X NB, 1X Luria broth (LB) or R2A broth, which, in addition to the use of relatively low-nutrient solid media, is consistent with a cultivation strategy to maximize the likelihood of obtaining oligotrophic isolates described in Pulschen et al. (2017).

To identify bacterial isolates, the 16S rRNA V4-V5 region was amplified from newly isolated colonies using a single-colony PCR kit (Cytiva, Marlborough, MA) with the universal primers 505f and 806r. PCR products were Sanger sequenced and the trimmed paired-end consensus sequences were searched using the NCBI database's Basic Local Alignment Search Tool (BLAST).

Minimal Inhibitory Concentration Tests

Stock solutions of 10 mM ZnSO4 and CuSO4 (Fisher Scientific, Waltham, MA) were prepared in autoclave- and filter-sterilized LB (10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl)

or modified Caulobacter growth media (2 g/L peptone, 0.2 g/L MgSO4). A 5 ml culture of the appropriate liquid media was prepared from glycerol stocks approximately 20 hours (for *Bacillus thuringiensis* ASWHM) or 70 hours (for *Brevundimonas* sp. CDWHM) prior to MIC and growth experiments.

MIC tests were carried out in replicate clear flat-bottom 96-well plates, with each isolate tested separately to avoid cross-contamination. Stock metal solutions were serially diluted in sterile growth media two-fold to a final volume of 100 µl across the rows of each plate, with at least one positive control column of 8 wells and one negative control column of 8 wells per replicate plate. 5 µl of overnight culture from the isolate being tested was inoculated across rows from wells 11-1 (positive control to highest concentration of inhibitor) in that order. Plates were sealed with Airpore tape and incubated in the dark at 30℃. After initial MIC results for each isolate were obtained, "challenge" incubation treatments were prepared by inoculating 5 ml of liquid growth media, either unamended or with Zn or Cu concentrations close to the initial MIC (2mM for *Bacillus thuringiensis*, and 100 µm for *Brevundimonas*) with a loopful of glycerol stock.

Microplate Growth Experiments

Prior to the start of experiments, pre-cultures for each isolate strain were diluted to a starting OD 600 of 0.1 nm in the inner 60 wells of clear 96-well flat-bottom polystyrene microplates, to a final volume of 150 µl per well. All plate set-ups included a blank row with uninoculated growth media. OD at 600 nm was measured every ten minutes at 30℃ in a microplate reader (Agilent, Santa Barbara, CA) with shaking for either 20 hours or 48 hours. When possible, growth experiments were repeated on different days to ensure consistent results.

Live Imaging

For motile isolates, overnight cultures were prepared in appropriate liquid growth media, and 1 ml of the overnight culture was spun down, resuspended in fresh media, diluted 10^2, and

stained with 1µl of the SYTO BC fluorescent stain described in the section below. Stained samples were prepared for live imaging by mixing 10 µl of the sample with 30 µl of melted 0.3% agarose gel and suspending the mixture in a glass capillary. The capillary was mounted for imaging on a custom light-sheet microscope, as described in Wiles et al. (2020). Images were recorded and processed using imageJ.

Laboratory Optimization of In-situ Chemotaxis Assay

A preliminary test of the in-situ chemotaxis assay and protocol as described in Clerc et al. (2020) was performed to determine optimal concentrations of test media with zinc to elicit specific tactic responses from motile bacterial isolates. A synthetic freshwater control media containing no organic substances was prepared as a control media suitable for isolates from stormwater (Environmental Protection Agency, 2002). Each row (n=4) of five replicate wells of the chip shown in Figure 1 were filled with a different 2 µm filter-sterilized solutions: a control row with synthetic freshwater, a row with 1% LB, a row with 1% LB amended with 1mM Zn, and a row with SW media amended with a 10 µM Zn.

An overnight culture of a test *Pseudomonas* isolate was spun down at 15,000 g for 10 minutes and re-suspended in the control freshwater media. The ISCA was deployed in an autoclave- and uv-sterilized lid from a 1000 µl tip box, and submerged in the bacterial suspension, and incubated for 1 hour in a biosafety cabinet. After incubation, the contents of the five technical replicate wells for each test substance were removed using a sterile 1 ml syringe with a 27G needle and pooled in an autoclave- and uv-sterilized 2 ml eppendorf tube.
A bacteria counting kit (ThermoFisher, Eugene, OR) containing SYTO BC fluorescent dye in DMSO and a 6 µm microsphere bead standard was used to stain bacterial cells retrieved from the ISCA wells. The stained cells were analyzed at a standard collection rate of 25 µl/min with an Attune Acoustic Focusing Cytometer.

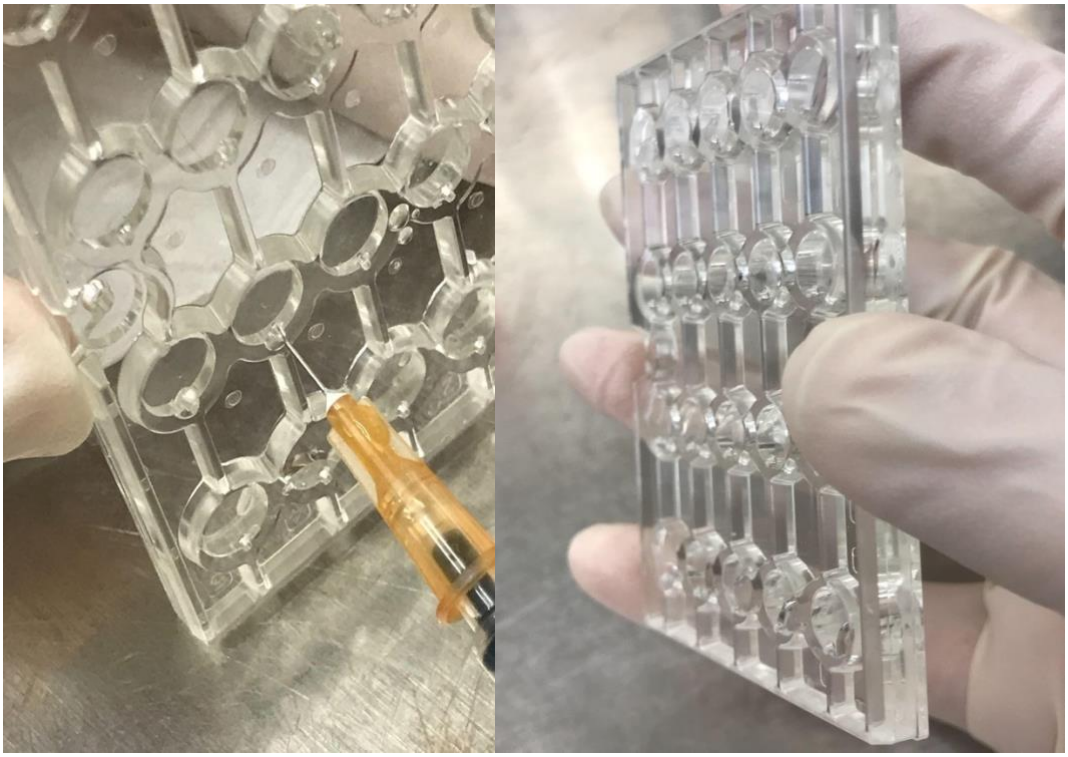

Figure 2. View of In-Situ Chemotaxis Assay (ISCA) showing arrangement of technical replicate wells and removal of well contents.

III: RESULTS

Microbial Community Survey

Permanova for bray-curtis distances between fungal samples was significant for all grouping variables, including location and sample type ($p<0.001$). Saprotrophs and animal pathogens were present at all sites and in all sample types.

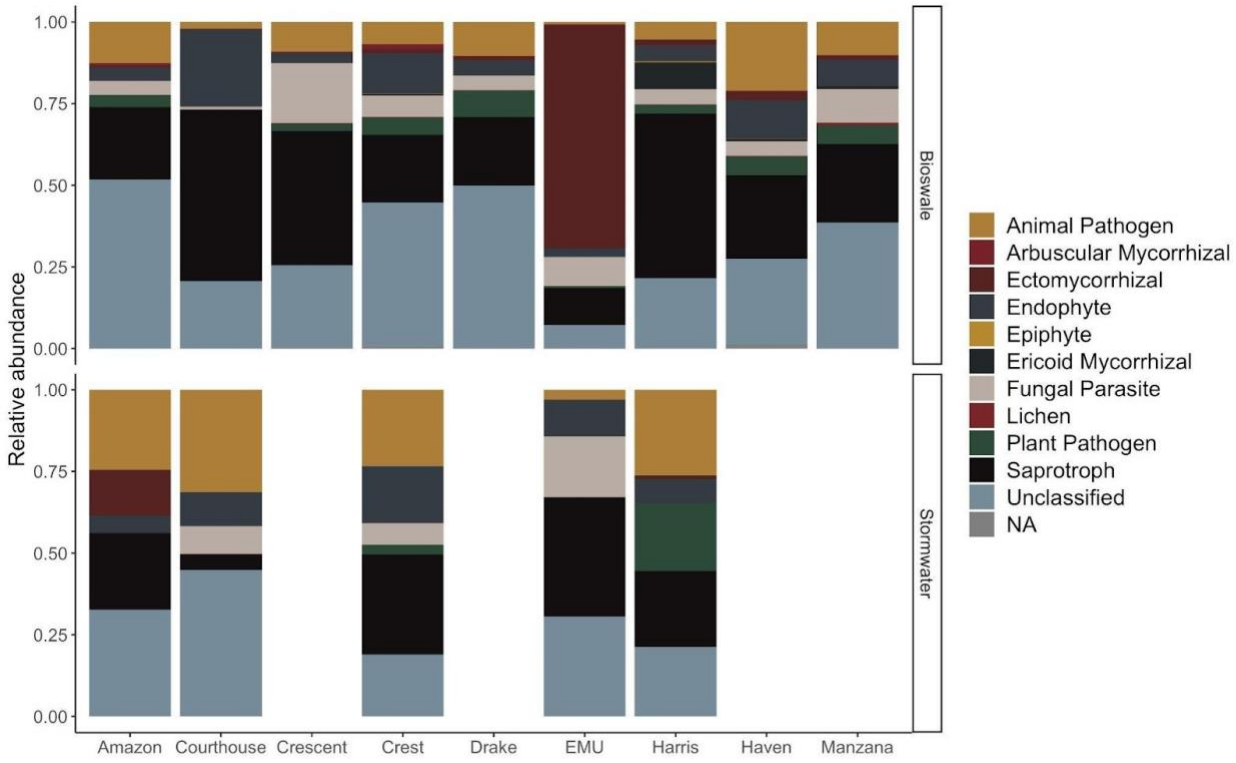

Figure 3. Relative abundances of fungal functional guilds in stormwater and bioswale samples from different sites ($p<0.001$).

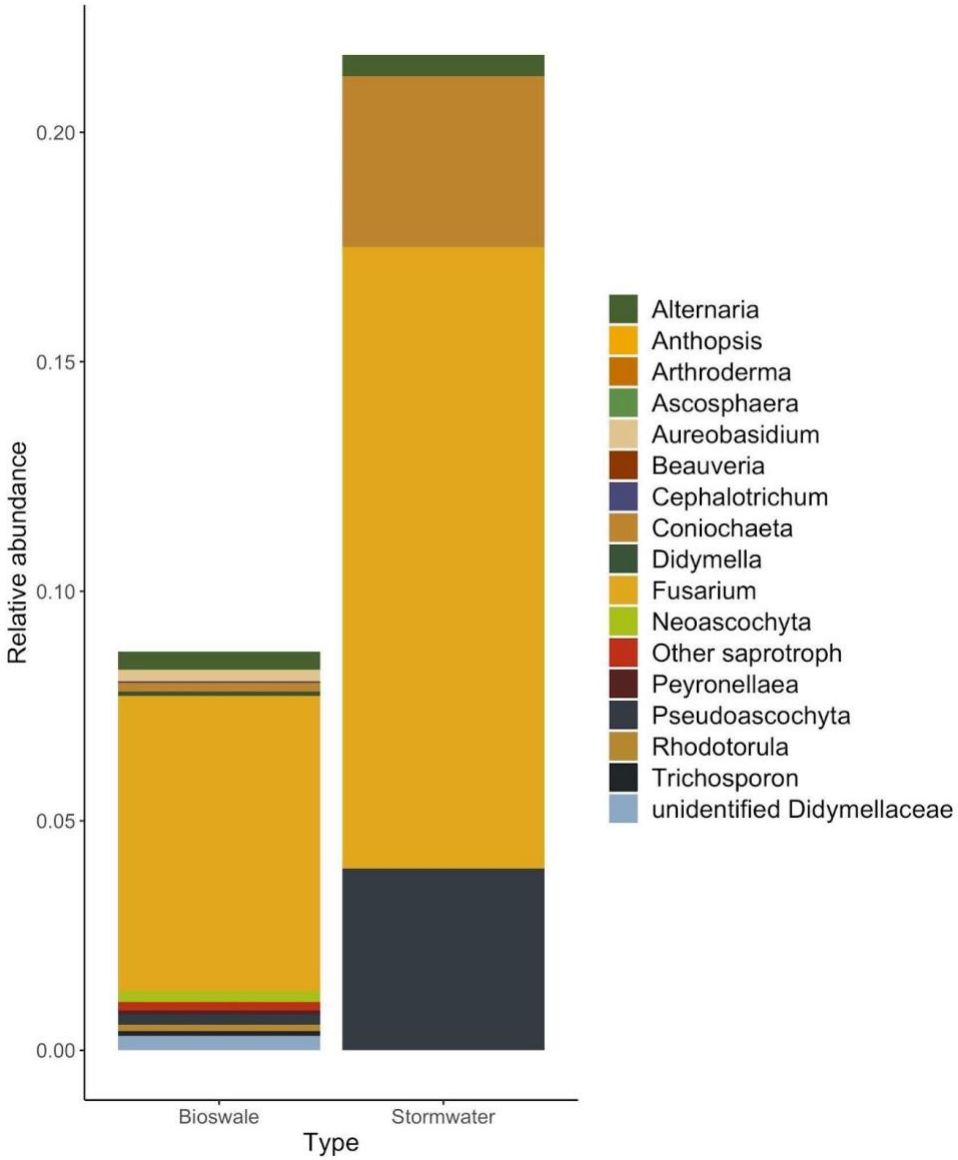

Figure 4. Relative abundances of top genera in the animal pathogen functional guild between bioswale and stormwater samples. Only four genera of animal pathogens (*Pseudoascochyta*, *Fusarium*, *Coniochaeta*, and *Alternatria*) were present in stormwater samples. *Fusarium* was the most abundant genus in both stormwater and bioswale samples.

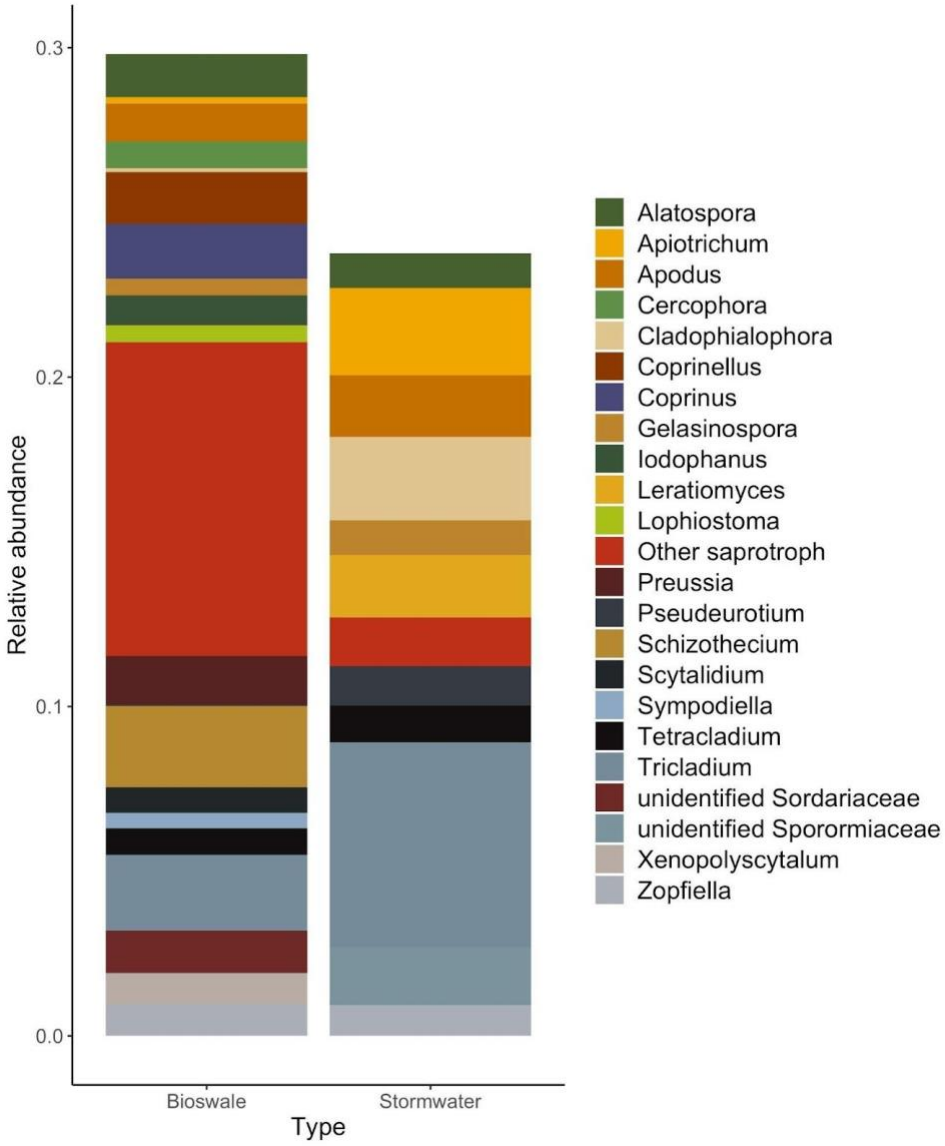

Figure 5. Relative abundances of top genera in the saprotroph functional guild between bioswale and stormwater samples.

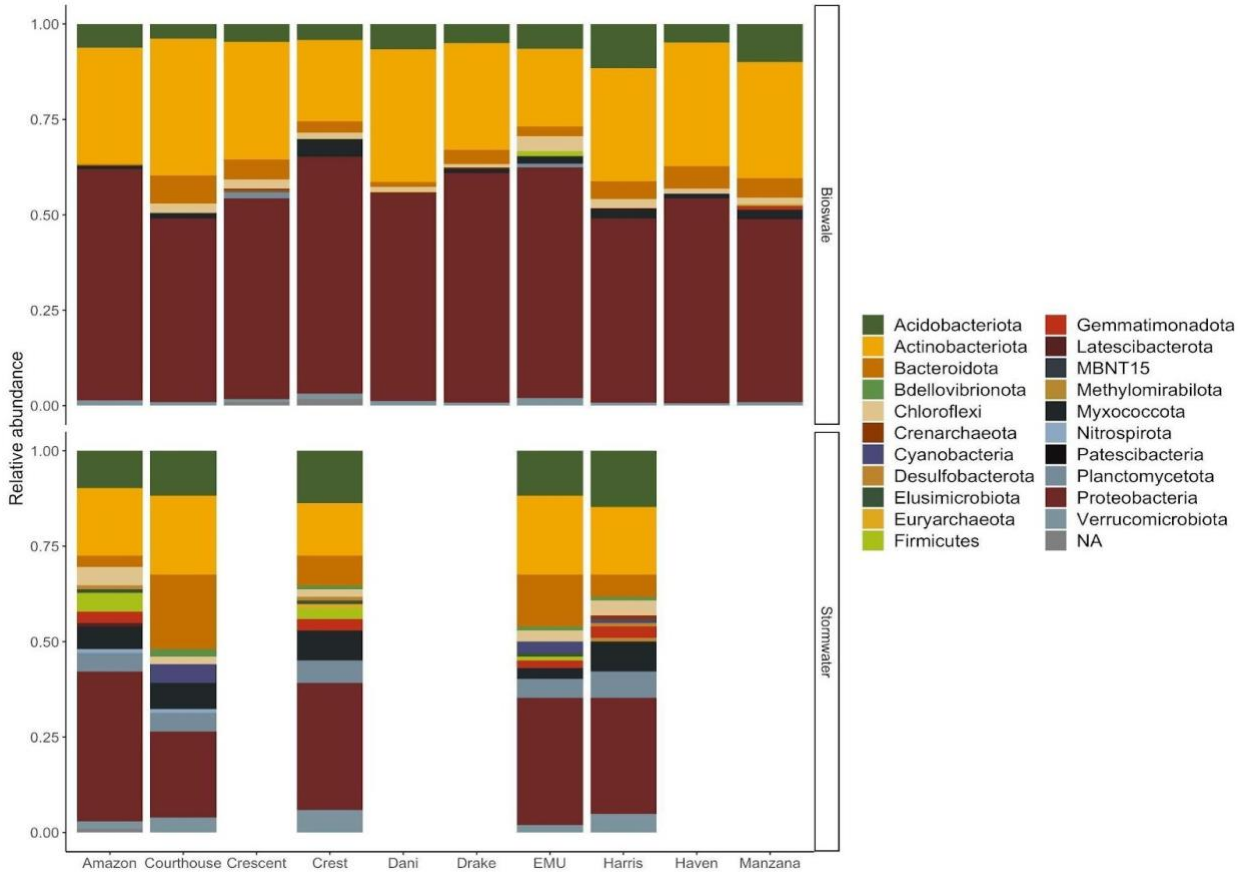

Figure 6. Relative abundances of bacterial phyla in stormwater and bioswale samples (p=0.019) from different sites.

Permanova for bray-curtis distances between bacterial samples was not significant for any grouping variable except sample type (p=0.019). *Proteobacteria* and *Actinobacteriota* were the most abundant phyla present at all sites in both sample types.

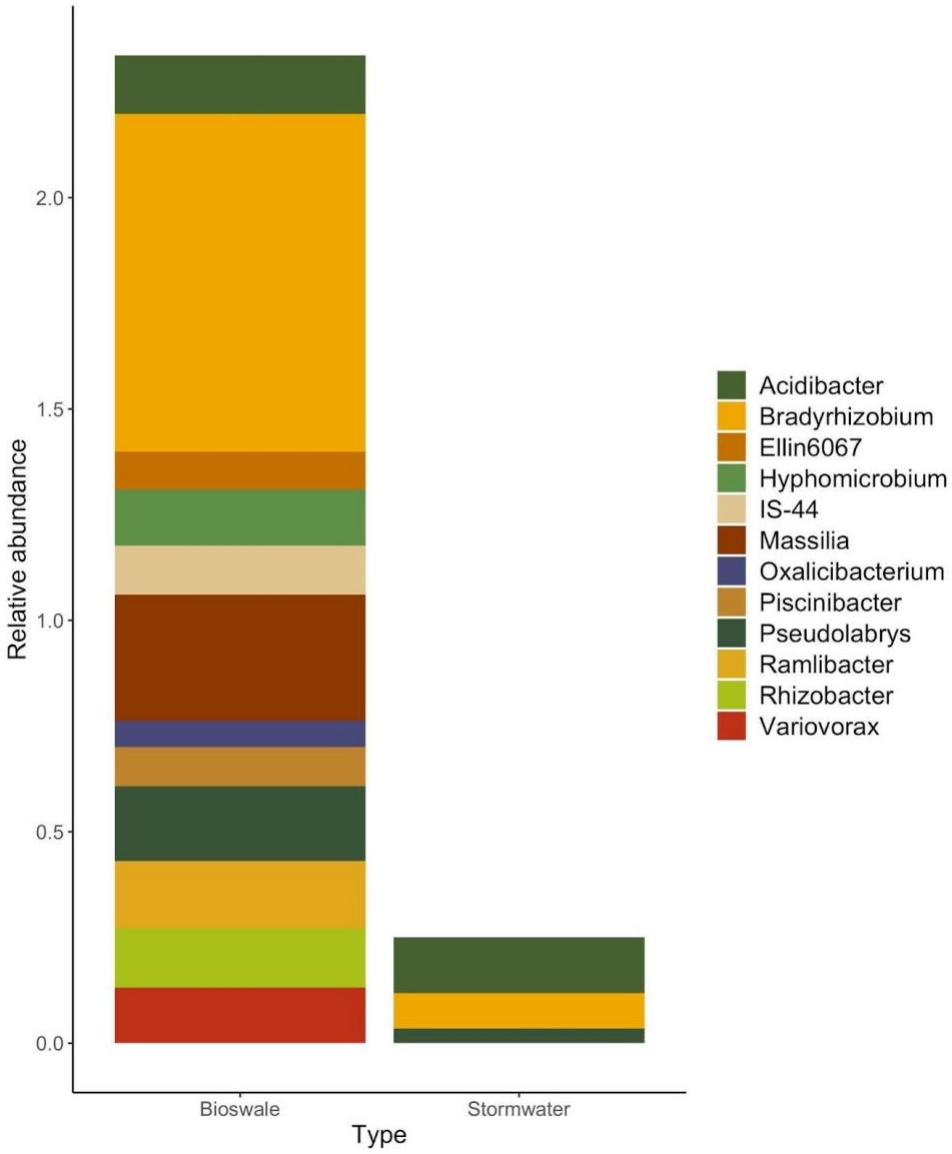

Figure 7. Relative abundances of top genera in phylum *Proteobacteria* between bioswale and stormwater samples. Only three of the top genera of *Proteobacteria* were present in stormwater samples (*Psedolabrys*, *Bradyrhizobium*, and *Acidibacter*).

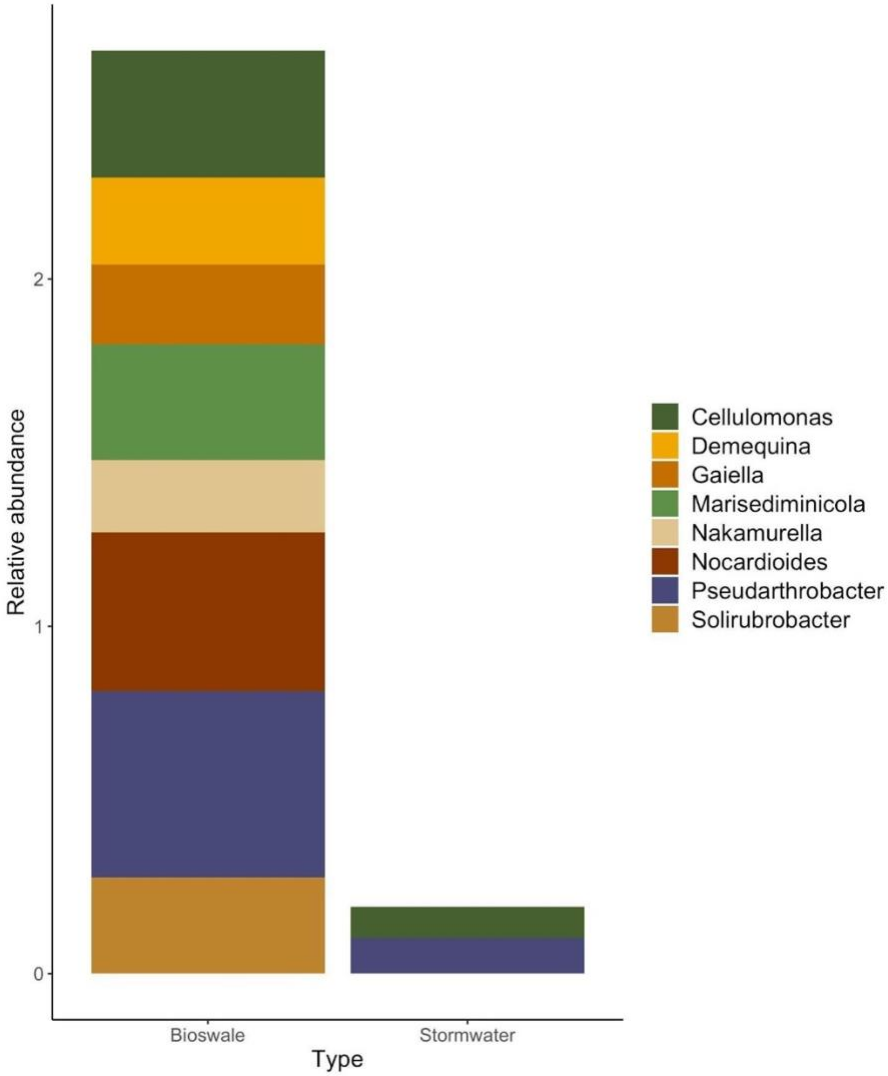

Figure 8. Relative abundances of top genera in phylum *Actinobacteriota* between bioswale and stormwater samples. Only two genera among the top ten *Actinobacteriota* were present in stormwater (*Pseudarthrobacter*, and *Cellulomonas*).

Bacteria Isolated from Contaminant Enrichments

Of the 55 isolates that could be cultured more than once, 18 with distinct colony morphologies were selected for Sanger sequencing. Nine isolates with >97% BLAST grade scores were considered identifiable to at least the genus level (Table 3).

Table 3. Isolates identified to the genus level from 16S rRNA V4 amplicons.

Isolate ID	Highest 16S rRNA sequence similarity	BLAST grade (%)	Sample Type
ASWHM	*Bacillus thuringiensis*	99.6	stormwater
CDBMM	*Sphingobacterium* sp.	100	bioswale
CDWHM	*Brevundimonas* sp.	100	stormwater
CTBMP	*Flavobacterium branchiarum*	97.1	bioswale
CTWHM	*Bradyrhizobium* sp.	99.8	stormwater
CTWHP	*Arsenicicoccus bolidensis*	97.8	stormwater
CVBHM	*Pseudomona*s sp.	100	bioswale
CVBHP	*Brevibacillus* sp.	99.8	bioswale
HAWMM	*Rhodococcus* sp.	98.8	stormwater

A pair of isolates with different growth and motility traits were selected for further study. Isolate ASWHM is a gram-positive fast-growing (visible colonies form as early as 2 hours after streaking on LB agar plates), strain of *Bacillus thuringiensis* that is motile via peritrichous flagella. Isolate CDWHM is a light orange-pigmented gram-negative, slow growing (3 days to form colonies on low-nutrient media, up to 3 months on rich media) strain identified as a member of the Genus *Brevundimonas*, motile by a polar flagellum. After this isolate was identified, I tested a modified caulobacter media (2 g/L peptone, 0.2 g/L MgSO4) and found it to be the optimal growth media for this strain.

Isolate MICs

Table 4. Initial MIC for isolates grown in unamended growth media.

Isolate ID	Metal	Concentration (mM)
Bacillus thuringiensis	ZnSO4	2.5
Brevundimonas	ZnSO4	0.125
Bacillus thuringiensis	CuSO4	2.5
Brevundimonas	CuSO4	0.08

MICs for *Bacillus thuringiensis* and *Brevundimonas* ranged between 0.08mM and 2.5mM. *Bacillus thuringiensis* MICs were the same for both metals, but *Brevundimonas* was more sensitive to Cu.

Table 5. MIC for isolates after priming in amended growth media.

Strain and treatment	Metal	Concentration (mM)
Bacillus control	ZnSO4	2.5
Bacillus control	CuSO4	2.5
Bacillus Zn	ZnSO4 2mM	2.5
Bacillus Zn	CuSO4 2mM	2.5
Bacillus Cu	ZnSO4 2mM	2.5
Bacillus Cu	CuSO4 2mM	2.5
Brevundimonas control	ZnSO4	0.125
Brevundimonas control	CuSO4	0.08
Brevundimonas Zn	ZnSO4	0.5
Brevundimonas Zn	CuSO4	0.25
Brevundimonas Zn	ZnSO4	0.5
Brevundimonas Cu	CuSO4	0.25

MICs for *Bacillus thuringiensis* remained unchanged after treatment incubations, suggesting a hard limit for both Zn and Cu at 2.5mM. *Brevundimonas* MICs increased 2x for Zn and over 3x for Cu for the strains in both metal incubation treatments. The control strain MICs remained unchanged from the initial MIC.

Growth Experiments

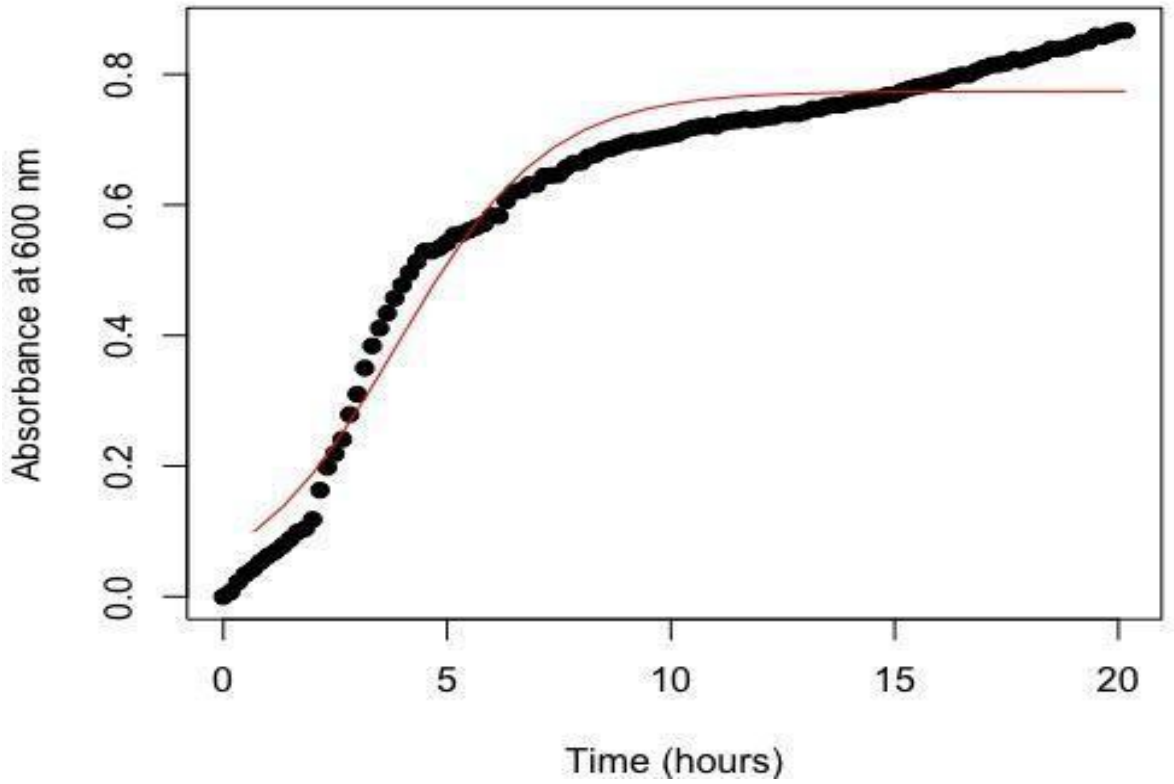

Figure 9. Optical density of *Bacillus thuringiensis* strain ASWHM grown in LB media over 20 hours. K (carrying capacity) = 0.774, r (intrinsic growth rate) = 0.597.

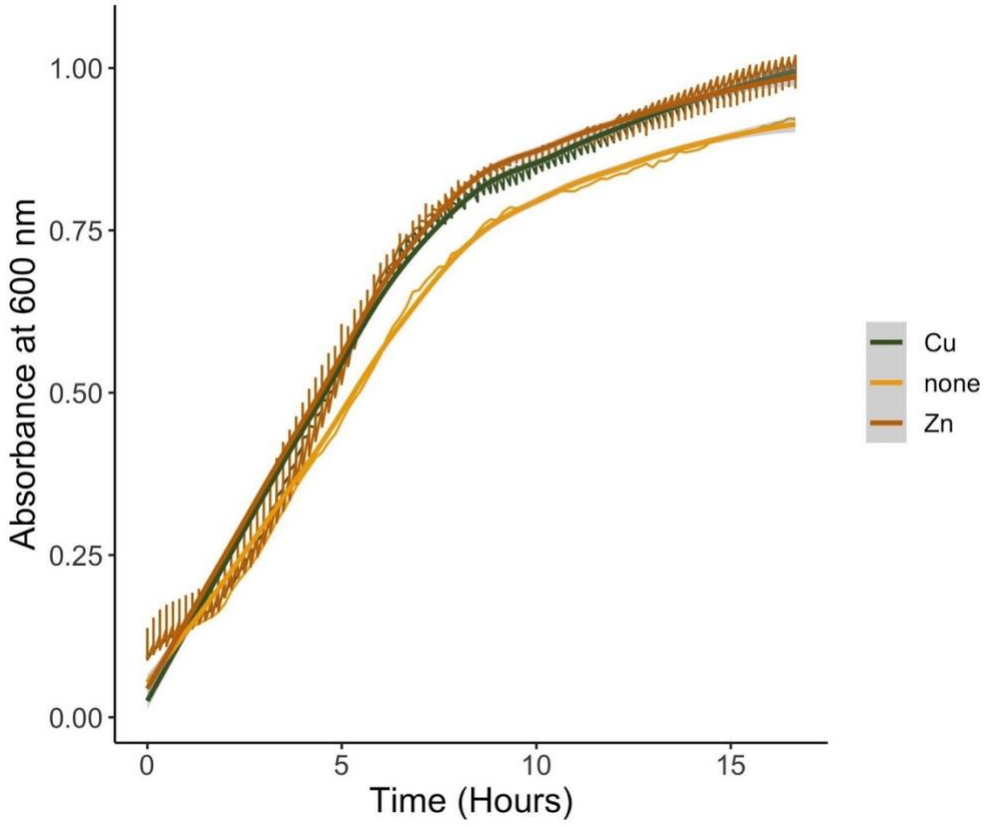

Figure 10. Optical densities of *Bacillus thuringiensis* strain ASWHM after incubation in unamended growth media, media amended with 2mM Zn, and media amended with 2mM Cu ($p=0.11$). Optical density was measured in a microplate reader at 30℃ at 10 minute intervals over a 20 hour period.

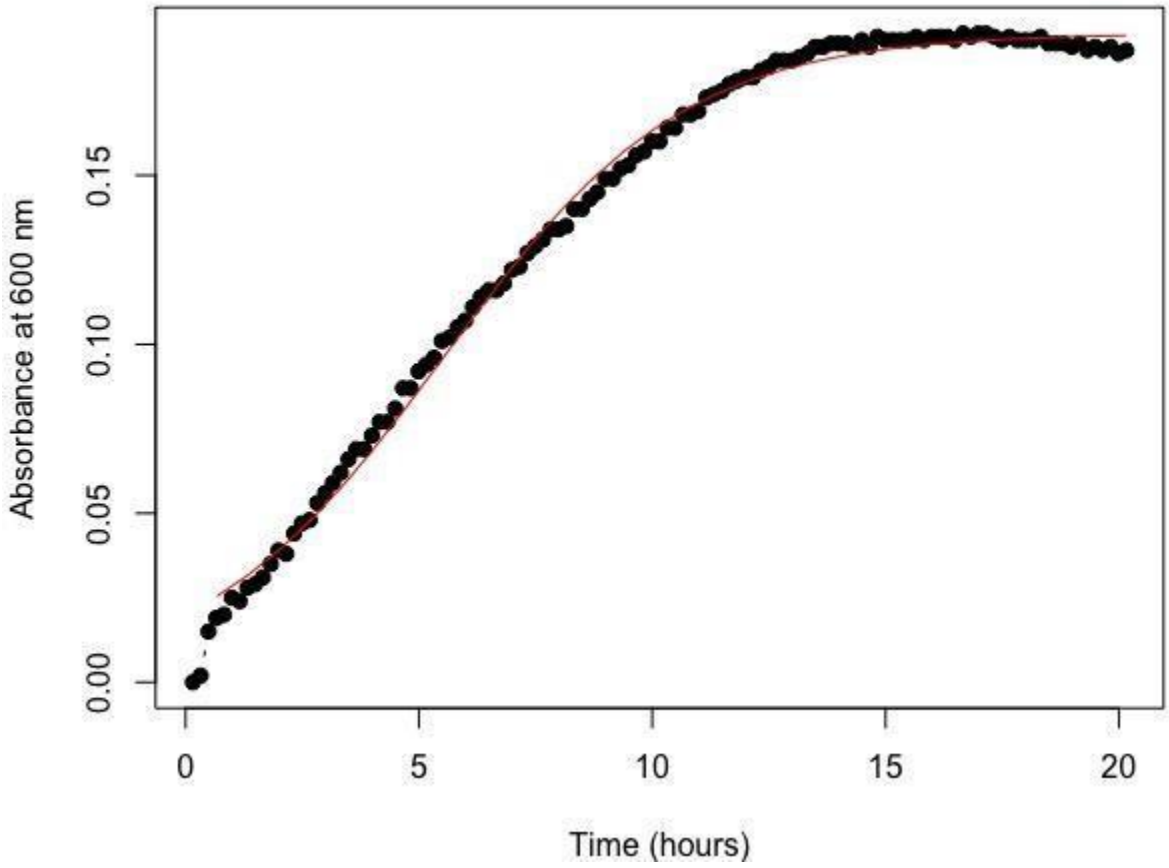

Figure 11. Optical density of *Brevundimonas* sp. strain CDWHM grown in caulobacter media over 20 hours. K (carrying capacity) = 0.192, r (intrinsic growth rate) = 0.388.

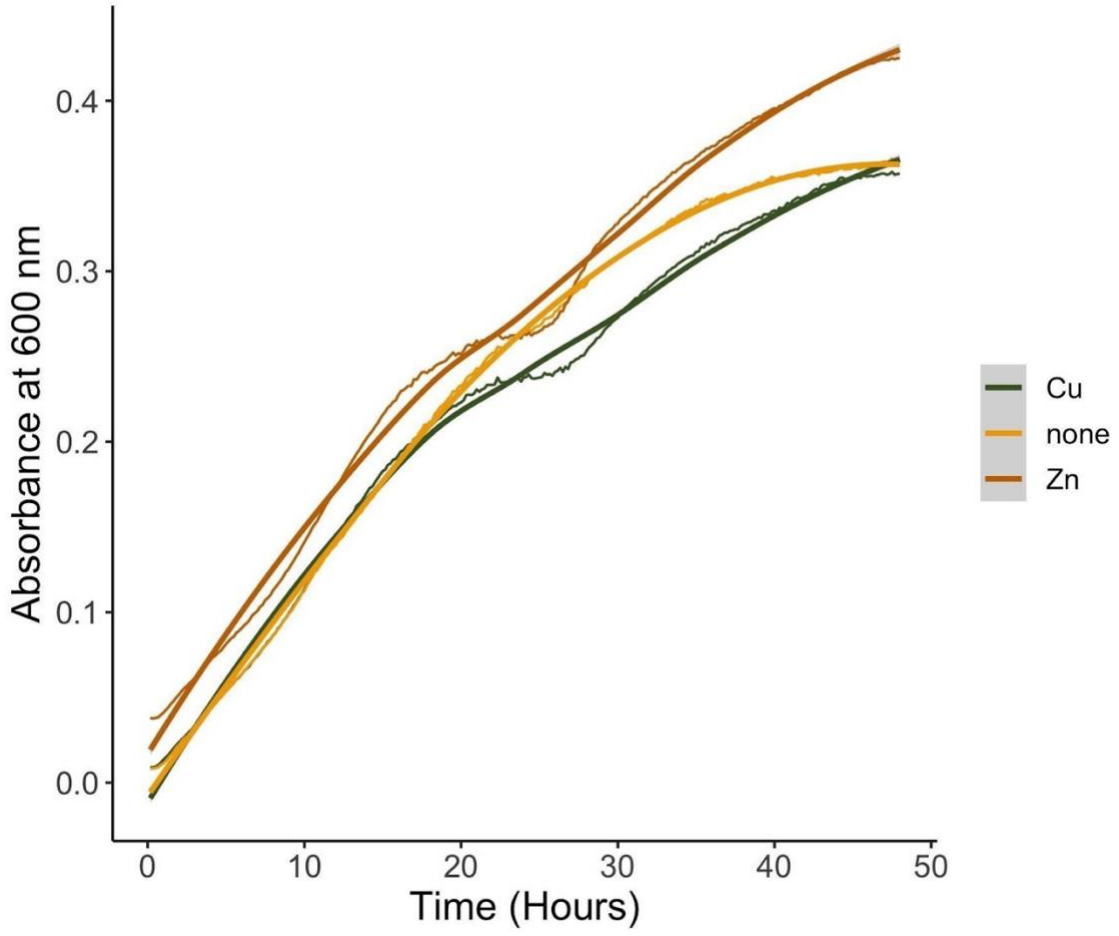

Figure 12. Optical densities of *Brevundimonas* sp. strain CDWHM after incubation in unamended growth media or in media supplemented with 100 µM Zn or 100 µM Cu ($p<0.001$). Optical density was measured in a microplate reader at 30℃ at 10 minute intervals over a 48 hour period.

In-situ Chemotaxis Assay

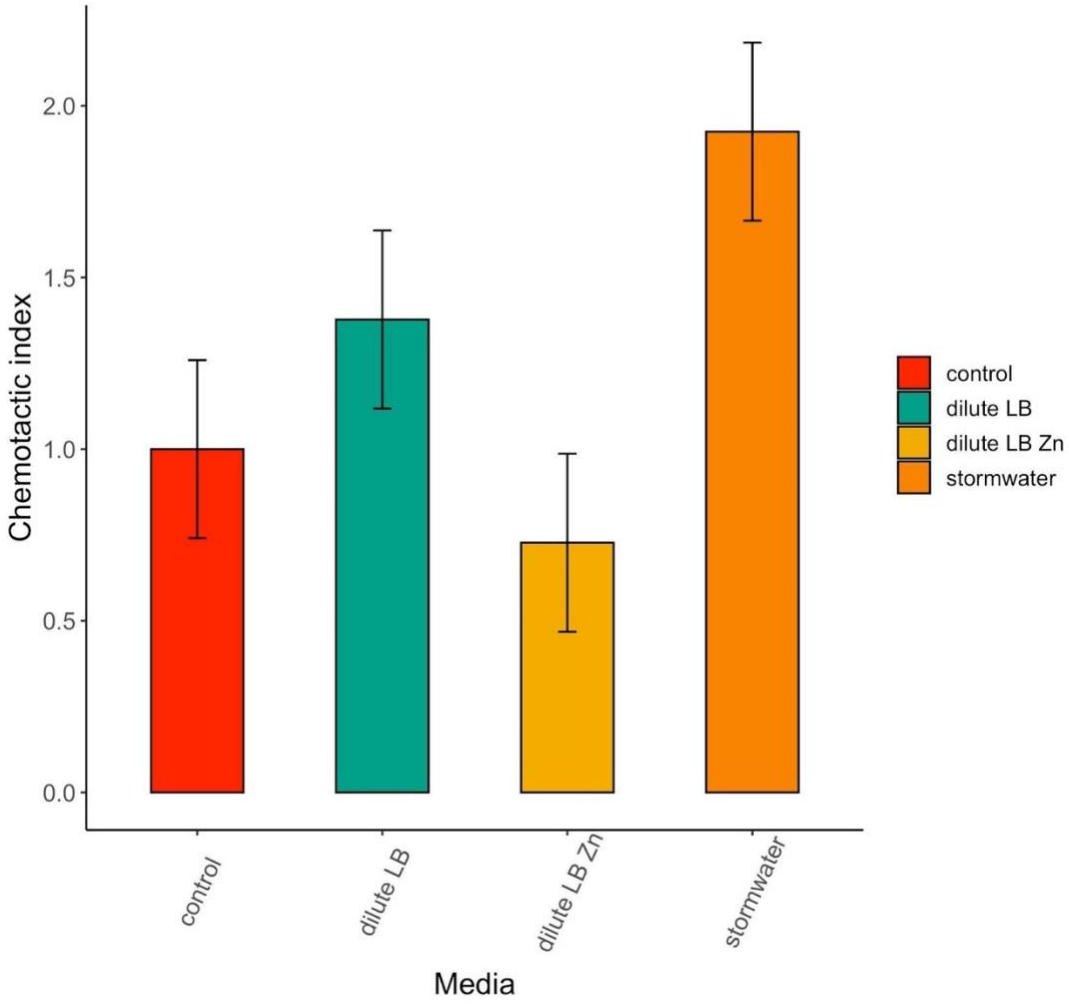

Figure 13. Chemotactic index (ratio of treatment well cell count/µl to control well cell count/µl) for bacterial cells of a *Pseudomonas* isolate (CVBHM) retrieved from ISCA wells with 1% LB , 1% LB media amended with 1 mM Zn, and filtered SW media with 10 µM Zn. All media types were tested in 5 replicate ISCA wells. Cells were quantified by flow cytometry after a 60 minute deployment for a single ISCA assay (SE = 0.259).

IV: DISCUSSION

Bacterial and Fungal Community Assembly

We found that the microbial communities in stormwater samples were significantly different from the microbial communities in associated bioswales, for both fungi and bacteria. This is consistent with stormwater functioning as a dispersal mechanism, although the origin of the microbes being transported along the stormwater path is beyond the scope of this study. A fraction of the fungal and bacterial assemblies transported through stormwater may be derived from precipitation itself. Some taxa are able to survive ice nucleation in the atmosphere, and could be deposited by rainfall or other atmospheric events (Beall et al., 2021). However, it's more likely that the majority of the microbial cells in stormwater runoff come from the surface-associated communities it flows through. Stormwater is a dispersal event that occurs only periodically during heavy rainfall, so it isn't a habitat in the strictest sense, but microbes dispersed from their communities of origin must survive hydrodynamic and chemical stress in stormwater for at least a brief period in order to colonize new spaces, and there may be some degree of selection for microbial traits that are favorable specifically for dispersal via stormwater.

Fungi, bacteria and archaea can be assembled differently in the same study system (Orland et al. 2020), and this appears to be true of the Eugene urban stormwater system. We found evidence that fungal communities in Eugene were more structured by deterministic processes, suggesting that deterministic processes may be a more important assembly mechanism than stochastic processes for fungi at these sites, and that some environmental filtering and species sorting is probably occurring, with location-specific resource patches and priority effects result in niche separation and competitive exclusion (Adams, Crump & Kling, 2014). Site-specific differences in abiotic variables like the composition of engineered bioswale media can explain some of the heterogeneity in fungal communities, but priority effects from the founding

members of the initial inoculum present in the components of bioswales at the time of construction is another plausible explanation for fungal community assembly in this study (McGuire, et al., 2013).

There were a relatively small number of fungal ASVs in stormwater relative to the bioswale samples, suggesting that a small core group of fungal taxa is being dispersed via stormwater, and that strong priority effects at bioswales maintain niche differentiation between resident and dispersed community members. The most abundant fungal functional guilds were classed as animal pathogens and saprotrophs, with both groups being more diverse in bioswale samples. It's reasonable to expect that saprotrophs would be more diverse in bioswale soils, as the accumulation of a variety of organic matter in swales supports fungi with a variety of resource-use traits. The animal pathogen functional group in both stormwater and bioswale samples was dominated by the genus *Fusarium*, a multi-kingdom opportunistic pathogen that may be particularly hardy and well adapted to urban environments.

In bacterial communities, sample type was the only significant variable explaining differences in bacterial assemblies, indicating that bacterial communities in this system are assembled more by stochastic processes than deterministic processes, with mass effects and high dispersal rates being the most likely mechanisms (Adams, Crump & Kling, 2014). This finding is similar for many bacterial communities in engineered and aquatic systems. Bacterial community composition in other urban infrastructure systems has also been not entirely explainable by site-specific variables (Gill, Lee & McGuire, 2017). Mass effects and immigration have been identified as the most important assembly mechanism for the free-living bacterial communities in a river habitat, but deterministic processes were more important for sediment and biofilm communities in the same study system (Gweon et al., 2021). This suggests that it's possible deterministic processes become more important for bacteria as the length of residence time in a given location (sediment and biofilm would be more static) increases. For the bacterial communities in the Eugene urban stormwater system there was a larger number of ASVs detected in the stormwater samples than the bioswale samples, although the majority of these are most likely from rare taxa. Because sample type was the only significant variable explaining differences between communities, it's likely that there is a large group of relatively low-abundance, transient bacterial taxa dispersed via stormwater, and that frequent dispersal rates and mass effects homogenize bacterial communities between locations in this system.

Motile isolates and metals

Over half of the isolates identified by Sanger sequencing showed some form of motility. Motile bacteria may have an advantage in systems like this one, with taxa that can actively scavenge nutrient patches and avoid predation or unfavorable conditions being more likely to persist long enough to colonize new niches. There is some evidence that coexistence among bacterial populations is maintained by trade-offs between motility and growth. For example, small fast-moving populations may be able to impede the migration of large, fast-growing populations, and small, fast-growing populations may be able to push large, fast-moving populations out of the zone of contact (Gude et al. 2020). I selected bacteria with contrasting motility and growth traits to identify any growth/motility trade-offs potentially modified by the presence of elevated metal concentrations. In the Eugene urban stormwater system, concentrations of Zn are the most likely parameter to increase (City of Eugene, 2018). Bacterial responses to zinc and other metals are most relevant for the individual microbes isolated from stormwater in this system, and I chose to use Zn and Cu as the specific contaminants of interest for all tests with individual isolates.

Bacillus thuringiensis

The *Bacillus thuringiensis* strain isolated from high-metal treated stormwater inoculum was among the rarer taxa identified in the communities across sites, although it is highly likely to survive dispersal. *Bacillus thuringiensis* has been used as a surrogate for *Bacillus anthracis* in models of endospore dispersal via stormwater because the two species share similar electrostatic properties and are presumed to interact the same way with particles in stormwater (Mikelonis, Ratliff & Youn, 2020). A fast-growing, spore-forming and motile species might be expected to have an advantage for colonizing new habitat, but source populations of *Bacillus thuringiensis* may not be as abundant as other species near the study sites. Although this strain is motile, microscopic examination indicates that it moves relatively slowly compared to most of the other

motile isolates in this study, in contrast to its relatively fast growth rate. If the *Bacillus thuringiensis* strain, which prefers rich media for optimal growth, is outcompeted for nutrients by faster moving taxa, it may be unable to persist in new habitats already colonized by a diverse assembly.

Metal concentration data for the different sites were not available at the time of writing, but it would be interesting to see if there is any correlation between metal concentrations and sites where *Bacillus* ASVs originated. *Bacillus thuringiensis* is among the *Bacillus* species that can survive environmental stressors and remove or detoxify metals efficiently enough to be potential bioremediation agents (Alotaibi, Khan & Shamim, 2021). The initial MIC of both Zn and Cu for the *Bacillus thuringiensis* isolate was 2.5 mM. This remained unchanged throughout the study, even after treatment incubations in elevated metal concentrations. The growth of *Bacillus thuringiensis* under treatments with 2 mM Zn and Cu was somewhat enhanced by sub-inhibiting levels of both metals, but the difference was not statistically significant. So far, there have been no clear indications of changes in *Bacillus thuringiensis* motility under different metal treatments during live imaging, but observations from compound microscopy in brightfield suggest that elevated levels of zinc may slow movement relative to controls and induce modified aggregation behavior, so more investigation is needed. Interestingly, the colonies of this *Bacillus thuringiensis* strain resemble a fuzzy spreader *Bacillus thuringiensis* morphotype described in Lin et al. (2022), so if this isolate has similar adaptations, it may be a very efficient colonizer of surface habitats, and warrants investigation to identify any tradeoffs between planktonic growth and biofilm formation under different conditions.

Brevundimonas sp.

The *Brevundimonas* strain isolated from high-metal treated stormwater inoculum was also among the rare taxa in this study. This strain grows slowly (around 72 hours to form colonies on R2A plates), and prefers nutrient-poor media. When grown on LB media, it can take up to a month for colonies to appear, if growth occurs at all. Microscopic observations of swimming motility in the *Brevundimonas* isolate indicate faster swimming speed during runs than the

Bacillus thuringiensis isolate, although this parameter has not yet been sufficiently quantified to report at the time of writing.

The preference for nutrient poor media and swimming motility suggest that this *Brevundimonas* is adapted to oligotrophic environments and may be at a disadvantage in bioswales. *Brevundimonas* species are found in a variety of terrestrial and aquatic habitats, but freshwater habitats seem to be among the more common where *Brevudimonas* are detected and isolated (Friedrich, 2021). This particular isolate produces a pale orange pigment, which may protect the cells from uv radiation. If so, it's possible that this *Brevundimonas* species is among the microbes adapted to dispersal through atmospheric precipitation.

Although metal-tolerant strains of *Brevundimonas* have been identified from other systems (Sharma et al., 2022), the strain from the current study was among the less metal-tolerant of the isolates from high metal incubations, implying that it originated from a source population that is not normally exposed to elevated metal concentrations. The initial MIC was 0.125 mM for Zn and 0.08 mM for copper. This result is consistent with microplate growth assays, where Zn at sub-inhibitory levels slightly enhanced growth, but Cu at the same concentration impeded growth after 20 hours, and the difference between the treatments was significant. After incubation in sub-lethal concentrations of Zn and Cu, the MIC for both metals improved in the strains that had been incubated in metal treated media, but also (from 0.08 mM to 0.125 mM) to Cu for the strain incubated in untreated media. This finding suggests that exposure to sublethal metal concentrations can induce an adaptive response in microbes that are not initially very tolerant, but also that some variation in tolerance to metals occurs with no environmental stimulus. Although no detectable changes to motility have been observed under different treatment conditions for this isolate so far, it may be that cell size and density are the more relevant parameters that change when this isolate experiences stress from elevated metal concentrations.

ISCA optimization

Microbes can adapt to environmental stressors either by physiological adaptation or by migration to more optimal environments (Kliminenko et al., 2021). Results from an initial ISCA

deployment with a highly motile *Pseudomonas* isolate from metal-treated bioswale inoculum shows evidence of negative taxis to a concentration of Zn at about half its MIC (data not shown). Cell concentrations in the wells of control synthetic freshwater media were higher than the wells with dilute growth media and Zn. It's possible that random migration or some physical disturbance during the incubation could have caused cells to be more concentrated in the control wells, but cell concentrations were higher than the control well concentration for all other media types, indicating that this isolate did actively migrate to wells with growth media, but was repelled to an extent by the presence of Zn at 1mM. This result needs to be reliably repeated in replicate ISCA deployments, but it would be reasonable for motile cells to actively avoid areas with elevated metal concentrations that do not enhance growth if they are able to. Of the different media tested during the initial deployment, cells were more concentrated in wells with filtered stormwater media amended with a concentration of 10µM of Zn. Further deployments will include filtered stormwater media with a range of Zn concentrations to verify that negative taxis in response to a relatively high concentration of Zn is actually occurring, and whether positive taxis is a response to organic nutrients in stormwater media or to optimal concentrations of Zn.

Conclusions

The fungal and bacterial communities in the Eugene urban stormwater system are structured by different assembly mechanisms, and these assembly mechanisms may determine which traits allow the microbes in this system to survive dispersal via stormwater runoff and colonize new niches. The identification of sensitive taxa, and the characterization of specific assemblies can enhance biomonitoring strategies, where relative concentrations of key contaminants like metals can be correlated with gradual shifts along the path of stormwater flow (Ancion et al., 2014). Of the isolates described in this study, the *Brevundimonas* species in particular shows promise as a potential indicator species, pending more thorough characterization of some specific responses to metal stresses. A major limitation of this study is the absence of information about either the concentrations of metals in environmental samples at different sites or the 16S and ITS sequence data from experimental incubations at the time of

writing. Although that data will be available soon, this thesis cannot address any direct associations between assembly and contaminants. The fact that some of the isolated taxa are among the rarer members of this system is potentially a limitation as well, but because the community assembly data represents a single time point, it would be necessary to compare data from other heavy rainfall events during different seasons to have a better sense of whether some taxa are truly transient, or persistently localized.

Understanding microbial community dynamics and the mechanisms that shape microbial community assembly in this system is an important part of management strategies for stormwater runoff mitigation. These communities provide a number of ecosystem services like intercepting and mitigating contaminants in stormwater and enhancing the function of the plants and soils in the network of bioswales. The preliminary research undertaken in this study suggests that distinct patterns in assembly processes coupled with in-depth characterization of microbes isolated from this system can be leveraged to inform improvements to green infrastructure systems in this region.

REFERENCES CITED

Adams H.E., Crump, B.C. & Kling G.W. Metacommunity dynamics of bacteria in an arctic lake: the impact of species sorting and mass effects on bacterial production and biogeography. *Front Microbiol*, 5:82, (2014).

Alotaibi, B. S., Khan, M., & Shamim, S. Unraveling the Underlying Heavy Metal Detoxification Mechanisms of *Bacillus* Species. *Microorganisms*, *9*:8 (2021).

Ancion, P. Y., Lear, G. & Lewis, G. D. Three common metal contaminants of urban runoff (Zn, Cu & Pb) accumulate in freshwater biofilm and modify embedded bacterial communities. *Environmental Pollution* 158, 2738–2745 (2010).

Ancion, P. Y., Lear, G., Neale, M., Roberts, K., & Lewis, G.D. Using biofilm as a novel approach to assess stormwater treatment efficacy.*Water Research* 49, 406-415 (2014).

Beall, C.M., Michaud, J.M., Fish, M.A., Dinasquet, J., Cornwell, G.C., Stokes, M.D., Burkart, M.D., Hill, T.C., DeMott, P.J., & Prather, K.A. Cultivable halotolerant ice-nucleating bacteria and fungi in coastal precipitation. *Atmospheric Chemistry and Physics*, (2021).

Birrer, S. C., Dafforn, K. A., Simpson, S. L., Kelaher, B. P., Potts, J. & Scanes, P. Interactive effects of multiple stressors revealed by sequencing total (DNA) and active (RNA) components of experimental sediment microbial communities. *Sci. Tot. Environ.* 637–638 (2018).

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J., & Holmes, S. P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nature methods*, *13*:7 (2016).

Caporaso, J., Kuczynski, J. & Stombaugh, J. *et al.* QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7, 335–336 (2010).

City of Eugene. *Year 2019 Stormwater Annual Report* (2019).

City of Portland Bureau of Environmental Services. *City of Portland 2006 Stormwater Management Facility Monitoring Report* (2006).

City of Salem. *Oregon national pollutant discharge elimination system (NPDES) Municipal Separate Storm Sewer System (Ms4) Permit (Permit Number 101513, File Number 108919) Annual Report Fy 2017-18* (2018).

City of Seattle. *Seattle's Source Control Plan for the Lower Duwamish Waterway (2015 – 2020) Volume 3 : Appendices*, (2016).

Clerc, E. E., Raina, J. B., Lambert, B. S., Seymour, J. & Stocker, R. In situ chemotaxis assay to examine microbial behavior in aquatic ecosystems. *Journal of Visualized Experiments* 2020, (2020).

Cyriaque, V.G.A., Billon, G., Nesme, J., Werner, J., Gillan, D., Sørensen, S. & Ruddy, W. Metal-induced bacterial interactions promote diversity in river-sediment microbiomes. *FEMS microbiology ecology*, 96 (2020).

Environmental Protection Agency. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms: Fifth Edition. (2002).

Friedrich, I., Klassen, A., Neubauer, H., Schneider, D., Hertel, R. & Daniel, R. Living in a Puddle of Mud: Isolation and Characterization of Two Novel *Caulobacteraceae* Strains *Brevundimonas pondensis* sp. nov. and *Brevundimonas goettingensis* sp. nov. *Appl. Microbiol.*, 1 (2021).

Gbeddy, G., Goonetilleke, A., Ayoko, G. & Egodawatta, P. Transformation and degradation of polycyclic aromatic hydrocarbons (PAHs) in urban road surfaces: Influential factors, implications and recommendations. *Environmental Pollution*, 257 (2019).

Gill A.S., Lee A. & McGuire K.L. Phylogenetic and functional diversity of total (DNA) and expressed (RNA) bacterial communities in urban green infrastructure bioswale soils. *Appl Environ Microbiol*, 83 (2017).

Gude S., Pinçe E., Taute K.M., Seinen A.B., Shimizu, T.S. & Tans, S.J. Bacterial coexistence driven by motility and spatial competition. *Nature*, 578 (2020).

Gweon, H.S., Bowes, M.J., Moorhouse, H.L., Oliver, A.E., Bailey, M.J., Acreman, M.C. & Read, D.S. Contrasting community assembly processes structure lotic bacteria metacommunities along the river continuum. *Environ Microbiol*, 23 (2021).

Hobbs, W., B. Lubliner, N. Kale, & Newell, E. Western Washington NPDES Phase 1 Stormwater Permit: Final Data Characterization 2009-2013. *Washington State Department of Ecology, Olympia, WA*. Publication No. 15-03-001 (2015).

Klimenko, A., Matushkin, Y., Kolchanov, N., & Lashin, S. Leave or Stay: Simulating Motility and Fitness of Microorganisms in Dynamic Aquatic Ecosystems. *Biology*, *10*:10 (2021).

Lambert, B.S., Raina, J.B., Fernandez, V.I., Rinke, C., Siboni, N., Rubino, F., Hugenholtz, P., Tyson, G.W., Seymour, J.R. & Stocker, R. A microfluidics-based in situ chemotaxis assay to study the behaviour of aquatic microbial communities. *Nature Microbiology* 2 (2017).

Langenheder, S. & Lindström, E. Factors influencing aquatic and terrestrial bacterial community assembly. *Environmental Microbiology Reports* 11 (2019).

Liguori, R., Rommel, S. H., Bengtsson-Palme, J., Helmreich, B. & Wurzbacher, C. Microbial retention and resistances in stormwater quality improvement devices treating road runoff. *FEMS Microbes* 2 (2021).

Lin, Y., Xu, X., Maróti, G. *et al.* Adaptation and phenotypic diversification of *Bacillus thuringiensis* biofilm are accompanied by fuzzy spreader morphotypes. *npj Biofilms Microbiomes* 8, 27 (2022).

Low-Décarie E., Kolber, M., Homme, P., Lofano, A., Dumbrell, A., Gonzalez, A. & Bell, G. Community rescue in experimental metacommunities. *Proc Natl Acad Sci USA.*, 112 (2015).

McGuire K.L., Payne S.G., Palmer M.I., Gillikin, C.M., Keefe, D., Kim, S.J., Gedallovich, S.M., Discenza, J., Rangamannar, R., Koshner, J.A., Massmann, A.L., Orazi, G., Essene, A., Leff, J.W. & Fierer N. Digging the New York City Skyline: soil fungal communities in green roofs and city parks. *PLoS One*, 8, (2013).

Mikelonis, A. M., Ratliff, K. & Youn, S. Laboratory results and mathematical modeling of spore surface interactions in stormwater runoff. *Journal of Contaminant Hydrology* 235**,** (2020).

Murínová, S., & Dercová, K. Response mechanisms of bacterial degraders to environmental contaminants on the level of cell walls and cytoplasmic membrane. *Int J Microbiol* 873081 (2014).

Ni, B. R. Colin, H. Link, Endres, R.G. & Sourjik, V. Growth-rate dependent resource investment in bacterial motile behavior quantitatively follows potential benefit of chemotaxis. *Proceedings of the National Academy of Sciences*, 117 (2020).

Orland, C., Yakimovich, K.M., Mykytczuk, N.C., Basiliko, N., & Tanentzap, A.J. Think global, act local: The small scale environment mainly influences microbial community development and function in lake sediment. *Limnology and Oceanography*, 65 (2020).

Prabhakaran, P., Ashraf, M., Wan Mohd N. & Wan Syaidatul A. Microbial Stress Response to Heavy Metal in the Environment. *RSC Adv*. 6 (2016).

Pulschen, Andre A., Bendia Amanda G., Fricker Ashwana D., Pellizari Vivian H., Galante Douglas & Rodrigues F. Isolation of Uncultured Bacteria from Antarctica Using Long Incubation Periods and Low Nutritional Media. *Frontiers in Microbiology*, 8 (2017).

Qian, Q., Caixia, H., Jiahui L., Wang, X., Tang, C., Dai, Z. & Xu, J. Contamination with multiple heavy metals decreases microbial diversity and favors generalists as the keystones in microbial occurrence networks. *Environmental Pollution* 306 (2022).

Sharma, P., Chaturvedi, P., Chandra, R. & Sunil Kumar, S. Identification of heavy metals tolerant Brevundimonas sp. from rhizospheric zone of Saccharum munja L. and their efficacy in in-situ phytoremediation. *Chemosphere*, 295 (2022).

Wesener, F. & Tietjen, B. Primed to be strong, primed to be fast: modeling benefits of microbial stress responses, *FEMS Microbiology Ecology*, 95: 8 (2019).

Wiles, T. J., Schlomann, B. H., Wall, E. S., Betancourt, R., Parthasarathy, R., & Guillemin, K. Swimming motility of a gut bacterial symbiont promotes resistance to intestinal expulsion and enhances inflammation. *PLoS biology*, *18*:3 (2020).

Wolff, B.A., Clements, W.H, & Hall, E.K. Metals Alter Membership but Not Diversity of a Headwater Stream Microbiome. *Appl Environ Microbiol*, 87 (2021).

Xiao, Y., Xiao, C. & Zhao, F. Long-term adaptive evolution of *Shewanella oneidensis* MR-1 for establishment of high concentration Cr(VI) tolerance. *Front. Environ. Sci. Eng.* 14, 3 (2020).