ASSESSING BACTERIAL COMMUNITY ASSEMBLY AND FUNCTION FOR IMPROVED BIOLOGICAL REMOVAL OF PATHOGENS AND CONTAMINANTS IN STORMWATER FILTRATION SYSTEMS

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Abstract

Pathogens and nutrients are consistently top pollutants of waterbodies around the world. Stormwater runoff is a major source of these pollutants, though with proper treatment, such as engineered filtration, water quality can potentially be improved for safe infiltration, discharge or reuse of runoff. Microorganisms are ubiquitous in stormwater, thus microbial community development on filtration based remediation systems requires consistent maintenance, which is far from optimized in practice. Previous work has demonstrated that biofilm microorganisms that colonize stormwater filters can lead to biofouling, as well as differ substantially in their remediation potential. However, few studies have investigated either the variation of the community in stormwater, or tested remediation ability with natural communities that are representative of variation from different potential treatment locations. Here we assessed the natural bacterial community structure variability of urban stormwater with 16S rRNA gene sequencing at a variety of runoff locations. We inferred the presence of potential pathogens and organisms associated with remediation functions (e.g. denitrification) from their sequence classification. Overall, we found high variability in stormwater bacterial community structure across rooftop, roadway, and Municipal Separate Storm Sewer outfall samples, but substantially less variability in potential for contaminant remediation. We also tested whether microbial community functional potential (e.g. pathogen presence and nitrate removal) in experimental filtration systems was sensitive to inoculum community composition, deposition and drift during biofilm assembly in experimental filtration

columns. Potentially pathogenic and denitrifying organisms increased in total abundance in experimental filtration columns over a one month growth period. Additionally, inoculation of filters with stormwater microbial communities provided significantly better pathogen removal than single isolate, sand, and control columns. Filters inoculated with stormwater communities performed similarly despite substantial taxonomic differences in inoculum communities taken from different runoff locations. Model pathogen initial removal performance had significant correlation with inoculum community diversity while biofilm presence was anti-correlated with the amount of *E. coli* remobilized in a subsequent simulated storm event. A similar approach could be used to investigate other pathogens of concern, varied chemistry and environmental conditions associated with stormwater or drinking, waste and other water treatment systems.

Committee: Sarah Preheim, Harihar Rajaram, Grace Brush, Jocelyne DiRuggiero, Genee Smith, and Edward Bouwer, may he rest in peace.

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Preface

Let us be reminded…

“Nature does not strive to classify things; humans do.

Despite many gray areas where classification of organisms is not easy (and sometimes does not seem to make much sense), classification is essential for our organization of knowledge and for communication among scientists, practitioners, and others...

The principles of engineering lead to quantitative tools while the principles of microbiology often are more observational. Quantification is essential if processes are to be reliable and cost-effective. However, the complexity of the microbial communities involved in environmental biotechnology often is beyond quantitative description; unquantifiable observations are of the utmost value.”

Rittmann, Bruce E., and Perry L. McCarty. Environmental biotechnology: principles and applications. Tata McGraw-Hill Education, 2012.

Thank you Dr. Ed Bouwer and Dr. Liza Wilson Durant for connecting me to microbiology with this book.

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Thank you Baltimore City for providing me with a safe and lovely home.

Thank you Newark, NJ and all my mentors for sparking my focus on Environmental Health and Civil Engineering on my way to Community Driven Public Health and Environmental Justice.

This thesis was sampled and completed on unceded Piscataway-Conoy and other indigenous territory, on a former Calvert family slave plantation, Johns Hopkins University in Baltimore, Maryland. The brutality that has occurred on this land will never be forgotten, as the University continues to pursue a private police department accountable to the University President and the State, not the community that will be surveilled and brutalized. The University continues to make bombs and weapons at the Applied Physics Lab and benefit from global war, colonization, destruction of the environment, and the death of innocent people. The University continues to forcibly displace, break agreements with, pollute and experiment on residents of Middle East Baltimore.

I am dedicated to holding this University accountable and building a liberated world against these generations of offenses. As the University gains attention during the coronavirus pandemic, this same University has created the militarized and financially stratified world and medical industrial death machine that is currently raging and fueling the COVID19 and escalating social pandemic. This claimed private/ non-profit institution has shown itself to me and I have seen administrators from the very top to the very bottom abuse and intimidate marginalized people and government actors to attempt to gain institutional profit, power and control. Let my graduation spur escalated direct action against Johns Hopkins University atrocities.

Thank you for reading

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Chapter 1. Introduction, Motivation and Objectives

1.1 Introduction

Microorganisms are ubiquitous in the environment, specifically in stormwater runoff. Research suggest that microorganisms present in stormwater can greatly influence the functions of stormwater management technologies by colonizing and forming biofilms on the filters, with the potential for biofilms to improve biological removal of contaminants in the system [1], [2]. Stormwater biofilms have been credited with the removal of numerous contaminants in a variety of treatment setups. However the taxonomic composition of biofilm organisms within stormwater management systems is not well described, even though these microorganisms can help to remediate top pollutants like pathogens, nutrients, and other bio-remediable compounds [3].

1.1.2 Fecal pollution

Fecal pathogens contaminate urban stormwater runoff, endanger human health, and are a major impediment to stormwater reuse. They enter runoff from pet and animal waste as well as sewage leaks and human sources. Pathogens are not consistently removed from stormwater using current best management filtration practices, and can remobilize in subsequent rain events [3], leading to concerns of human exposure and degradation of receiving waters. Pathogens associated with fecal contaminated stormwater are typically in found at higher concentrations in places with inadequate sanitation and sewerage service and is associated with diarrheal illness and associated malnutrition across the world [4].

Fecal pathogens have been detected in studies sampled under varied locations and conditions. Higher concentrations of fecal indicators *E. coli* and

Enterococcus spp. during wet weather events compared to dry weather event (baseflow) have been detected for both inoculum sources [5]. Opportunistic pathogens were detected in up to 57.9% of samples collected at rain barrels collecting rooftop runoff [6]. Additionally, stormwater ponds and basins are widely used in agriculture where pathogen loads in runoff are significant [7]. Therefore, pathogen pollution must be addressed with a range of land uses.

With these risks, there is a significant opportunity as well for reuse of this water source [8]. Some concerns about pathogen contamination have been addressed in direct potable reuse technology, which has been implemented in a number of places worldwide using wastewater [9]. There are significant differences in stormwater harvesting compared to wastewater reuse, as the systems will be operated under drastically different parameters. These must be addressed specifically for the removal of pathogens as they are considered the main concern for stormwater reuse [8]. Pathogens are part of a larger microbial community that influences the performance of filtration systems. In depth assessment of these communities is made possible with increased accessibility of genetic sequencing, so that we can begin assess pathogen risks and microbial community function to predict and control biologically-associated removal of pathogens and other contaminants.

1.1.3 Microbial community processes in stormwater filtration systems

Physical and chemical processes in stormwater filtration systems are broadly investigated, while microbiological processes in these systems are understudied

[1], [2], [10]. Microbiological processes inside stormwater filters are widely considered a “black box" because traditionally the specific microorganisms involved in removal or remobilization have not been investigated in detail even though they are known to be crucial to contaminant removal from runoff.

1.1.3.1 Variability in stormwater quality

Land use can have an important influence on runoff water quality, which results in a range of stormwater contaminants, such as suspended solids, carbon, nitrogen, phosphorous, heavy metals, oil, grease, and pathogenic bacteria [2], [3], [11]. These water quality parameters can influence biofilm characteristics by impacting environmental conditions and nutrient concentrations in the filter. Stormwater quality varies in chemical composition and microbial community structure, both of which will impact the composition of biofilm that forms on surfaces within filtration systems [12], [13]. As biofilms colonize the filter surface, they will alter the physical and chemical properties of the filtration media such as charge and roughness [14]–[17]. This will impact the hydrodynamics of the column and the deposition rate of pathogens and other contaminants [16], [18]. This could also influence the proliferation of pathogens in the filter that can remobilize in a subsequent rain event [1].

The variation of fecal indicators and overall bacteria cell concentrations in urban stormwater runoff associated with land use and seasons has been well documented using culture-based methods. Yet differences in the total diversity and taxonomy of microbial communities associated with different filter inocula are understudied [19]. It is not well established how variability in biofilm composition that form within the same and between different filters impacts pathogen

attachment and removal. It is also not well established how the removal rates in filters is correlated to complex community biofilm activity.

1.1.3.2 Variability of filter biofilm-associated removal of *E. coli*

The main mechanisms for bacterial removal in porous media are physical straining and adsorption [1], [20]. However, straining will not be a significant factor for typical, pristine sand columns used in stormwater filtration systems and many bacterial pathogens where the cell diameter is under 5% of the sand particle diameter [20]. Adsorption is determined by a number of predictors including hydrophobicity of the filter surface, roughness, charge, and characteristics of biofilm extracellular polymeric substances [14], [15]. Both sand and bacteria are negatively charged, which is not conducive to attachment of negatively charged colloids, such as pathogens and *E. coli* [21]. As the media surface is colonized by bacteria, the surface properties are altered, which can influence attachment. Studies have investigated adsorption of pathogens in porous media for many water quality scenarios [17], [22]–[25]. Representative stormwater microbial communities are under-investigated and few studies have evaluated how significant the influence of stormwater community input variability is to biofilm formation and the removal of pathogens.

Most studies that investigate pathogen removal from stormwater are conducted to predict properties that create variable efficiency for greater control. The following examples highlight developments made in understanding physical-chemical properties of pathogen removal and the promise of investigating

biological parameters to explain removal and understand biofouling and filter maintenance.

Zhang *et al.* (2010) compared *E. coli* removal in conventional sand media to iron coated sand and calculated 82% and 99% removal efficiency, respectively. However, conventional sand resulted in 99.9% die-off of pathogens in the first week where as the iron coated media had only 52% die-off. Any biofilm that formed on the filters during the study was not directly investigated but they explained the variability as a result of attractive electric double layer interactions between the collectors and bacterial cells [20].

Chandrasena *et al.* (2012) tested the removal efficiency of *E. coli* with and without abiotic turbidity and in the presence of different plant species. They found both turbidity and plant species had a significant influence on removal and determined that adsorption was the primary removal mechanism over straining [26]. Biofilms can alter surface properties and influence adsorption in the filters thus altering removal of pathogens [1], though this wasn't investigated in these biological columns. Parker *et al.* (2017) recently analyzed the results from this study to explain the observed removal rates. They used a combination of a mechanistic, theoretical model (CBFT) along with statistical models to explain 66% of the removal. Clean bed filtration theory (CBFT) accounted for the largest amount of removal, at 31%, followed by antecedent dry period (14%), study effect (8%), biofilter age (7%), and the presence or absence of shrubs (6%). As in many cases, biofilm formation and function was noted as important. It was not investigated but hypothesized to impact clogging [27].

Chandrasena *et al.* (2014) also investigated the influence of temperature, moisture content, sunlight exposure and presence of other microorganisms in the filer on the retention and remobilization of *E. coli*. Sunlight exposure and moisture content were important in the top of the filter while temperature and other microorganisms were most important at the top and within the filter. The concentration of *E. coli* in the inflow is an important component related to removal efficiency [28]. Most recently, Chandrasena *et al.* (2017) investigated the influence of vegetation, rhizosphere microorganisms and antimicrobial filter media on the removal of *E. coli* [29]. Root exudes and microbes, combined with the antimicrobial properties of the filter discouraged the survival of *E. coli* in the filter. Other plant extracts also showed potential antibacterial activity. Still, the influence of the microbial community present is not well assessed despite being associated to *E. coli* and pathogen presence and removal.

Mohanty *et al.* (2013) investigated engineering solutions to improve the removal of fecal indicator bacteria by bioinfiltration systems during intermittent flow of stormwater on iron oxide coated sand. Saturated columns released less fecal bacteria than gravity-drained columns under intermittent flow conditions. The increased presence of natural organic matter increased remobilization of pathogens [30]. The increase in organic matter should influence biofilm formation on the filter but this aspect wasn't investigated in the study design.

To investigate biofilm parameters, Nabial Arfooz *et al.* (2016) tested *E. coli* removal in biochar-modified biofilters. *Pseudomonas* biofilms were grown on biochar-blended media columns and tested for *E. coli* removal. They used an

ATP based system to quantify the amount of biofilm biomass. They found the presence of biofilm was significant to removal and dependent on specific surface area and hydrophobicity of the filter [31]. Thus, biofilms growing on biofilters significantly impact removal efficiency under typical stormwater conditions.

Zhang *et al.* (2011) conducted a critical study on the long-term sustainability of *E. coli* removal in conventional media. By testing replicate columns, they found that the initial mean removal started at 72% and increased to >97% after an 18-month period. They also found *E. coli* concentrations rapidly decreased between each *E. coli* test. Decreased porosity and increased hydrodynamic dispersion as filters age created more favorable conditions for removal. Temporal changes in surface charge were not a key factor. They detected growth of native protozoa which is believed to play an important role in predation of trapped *E. coli* [32], however bacterial community abundance of the biofilm was not investigated.

In summary, studies conducted to investigate the removal efficiency of *E. coli* in stormwater sand filters have not been controlled to predict the influence of bacterial communities within the system that contribute significantly to biofilms that colonize the filters and change their physiochemical properties. It is understood for sand filtration that controlling the biofilm will influence the hydrologic conditions that lead to better removal.

1.1.3.3 Remobilization of pathogens from a subsequent rain event is an important concern for overall removal of pathogens in the filter

Some studies have observed significant remobilization, [26], [33] while under other conditions it did not have a significant impact in overall sustainability [32]. Cells trapped in the secondary energy minimum are not irreversibly attached to the filter and can remobilize [14]. Additionally, biofilm that forms on the filter can become dislodged given flow conditions and increase pathogens in the effluent [34]. Intermittent flow characteristics of stormwater have been shown to influence remobilization [8], [35]. This again suggests controlling the biofilm will influence the hydrologic conditions that lead to remobilization. Complex biofilms using stomrwater communities have not been investigated in connection to this phenomenon, [8] but are believed to be influential under certain conditions [1], [24].

1.1.3.4 Filter Performance and Biological Metrics

Important metrics to evaluate filter performance have been developed from physical and chemical process as well as microbial ecology theory and practice. These are used to quantify physical straining, biofilm adsorption, and the influence of bacterial biofilms in removal processes [20], [36]–[38]. These mechanisms will influence the overall removal efficiency which is the key variable being tested between different biofilms. The implications of larger porous media theory to overall experiment design are included as Appendix D.

Biofilm Activity and Presence

Biological activity of the biofilm may be an important predictor for pathogen removal, however it is under-investigated. The activity of the biofilm is directly related to the amount of biomass and the number of cells present and can be measured by quantifying adenosine tri-phosphate (ATP). This method is applied by operators in a number of water quality filtration scenarios [31], [36], [39], [40]. Results from injecting *E. coli* onto biochar modified columns colonized by pseudomonas biofilm determined that the presence of ATP and biomass was directly related to the amount of *E. coli* that deposited on the column [31]. Additionally, the added pathogen in this study did not contribute a significant amount of ATP compared to the biofilm. These methods will be applied to mixed community columns and conventional sand media.

Biofilm Taxonomy

Biofilm taxonomy has importance in water quality treatment, however limited connections have been made in stormwater quality scenarios. It has been determined that protozoa are important in *E. coli* predation however a limited number of additional biofilm taxonomic groups have been identified as important to removal [41]. For example, *Methylobacterium* is important in drinking water biofilm processes as its presence encourages the formation of aggregates [42]. Synchronized population dynamics have been identified in anaerobic wastewater digesters [43]. Variable *E. coli* removal was detected between different *P. aeruginosa* strain biofilms grown on glass beads, suggesting variable assemblies of organisms in a mixed biofilm could influence removal variability [17]. The

assembly and interaction of organisms within stormwater biofilms with pathogens is largely unknown [1], [41]. There are many genomic and culture based methods available to investigate this microbial black box that have yet to be applied [4], [44]–[47].

1.1.3.5 Summary

Pathogen contaminated stormwater is a global problem. Removal efficiency of pathogens from stormwater using sand and engineered filtration systems is inconsistent; we cannot effectively control and predict retention and remobilization. Physical filter properties are a known contributor however, the contribution of the complex bacterial microbiome is largely unknown and considered and important black box to investigate. Stormwater quality is biologically variable temporally and spatially, which could impact pathogen presence and microbial filter composition. Previous studies suggest biomass presence and bacterial community assembly and activity will influence variable pathogen removal rates in complex community stormwater systems.

1.2 Motivation

1.2.1 The removal efficiency of pathogens from stormwater using engineered filtration systems is inconsistent

"Green infrastructure" systems implemented to sustainably treat stormwater are installed to reduce flooding and erosion and can remove pollutants from stormwater by using filtration media such as sand. These systems replace "gray infrastructure" systems that have been used traditionally

such as combined sewer systems and municipal separate stormwater sewer systems [3]. Green infrastructure stormwater systems are generally designed specific to location, therefore design specifications can vary significantly between installments [2]. Research has been conducted to evaluate water quality treatment suitability of the filters for various contaminants. However, their performance for pathogen removal is highly variable, both temporally within a system, and between filter locations [2], [27], [33]. In general, all designs incorporate porous media that can be colonized by microbial biofilms and may allow for the proliferation of pathogens in the filter [20].

Many bioretention schemes can effectively remove pathogens, although results are highly situational. For example, fecal coliform mean removal in laboratory columns was 91.5% of influent concentration as studied by Rusciano and Obropeta [48]. Six bioretention facilities in Wilmington, NC were characterized at 70-98% removal [49]. A study by Zhang *et al.* calculates 80% and above efficiency for removal of *E. coli* O157:H7 strain B6914 from synthetic urban stormwater runoff [20]. These results show that pathogen removal can occur with these systems, but that the results are variable.

Remobilization of pathogens is an additional concern for remediation systems. Hathaway *et al.* (2009) evaluated two field bioretention systems in North Carolina and reported percentage removals ranging from -611 to 92% for *E. coli*, -132 to 86% for enterococci and 89% for fecal coliforms [26], [33]. Negative percentages represent an increase of pathogens in the effluent from initial concentration, which would be consistent with growth and remobilization of

pathogens from the systems. Removal rates as well as system designs are temporally and spatially variable, therefore it is difficult to compare results from the field. Between the two systems studied by Hathaway *et al.* the variable removal capacity was hypothesized to be due to design parameters such as depth and media type. However one crucial component that was not investigated here, and in many other cases, is the functional impact of bacterial biofilm that forms on the filter over time [2]. It has been shown that the removal rate of *E. coli* can stabilize over time as a stormwater filter ripens and that biofilms can eventually clog filters, though this mechanism is largely under investigated in physical-chemical removal studies.

1.2.2 Contribution of the microbiome to pathogen removal in stormwater filters is largely unknown

As bacteria are ubiquitous in stormwater, they can colonize the filter media to form a biofilm under favorable conditions. The process begins when a cell becomes attached to a filter surface. The cell begins to replicate and more cells from the environment begin to attach and aggregate on the surface. The multi-organism biofilm matures and eventually cells are released or detached from the biofilm and the cycle begins again on another surface [50]. This is a highly dynamic process with many environmental factors influencing how the biofilm community assembles [34].

Many known mechanisms of the biofilm have yet to be investigated in stormwater filters [2], [27]. Ecological factors that influence community assembly in other environments (e.g. selection, drift, dispersion, evolution) have not been investigated in stormwater filters [51]. Studies on the interactions of pathogens

and fecal bacteria within filters is rare [28], [52]. The processes that occur within these filters are considered a black box that must be explored to improve stormwater technology [1], [2].

1.3 Objectives

To better understand how variation in biofilm community composition influences contaminant removal, I investigated the natural variability of bacterial communities in urban stormwater and how variable inocula could influence pathogen and contaminant removal. First, a field investigation was conducted to look at the temporal and spatial variability of bacteria in urban water, which could serve as an inoculum to stormwater biofilters. Next I experimentally investigated how migration of different complex communities into biofilters affects community assembly and potential function. Lastly, laboratory columns were used to determine if different mixed community biofilms created significant differences in initial removal efficiency and subsequent remobilization of fecal indicator *E. coli*.

Overall objective: Assessing the influence of stormwater microbial ecology on pathogen and other contaminant removal in sand filtration systems.

Overall hypothesis: Stormwater microbial communities are heterogeneous across potential stormwater treatment locations (roadways, rooftops, etc), and biofilm community composition within sand filters has a significant influence on removal efficiency, specifically for pathogens.

Objective 1: Characterize the differences in urban stormwater bacterial community composition over time at different locations (rooftop, roadway and outfall) to determine the variability in dominant taxa and potential pathogens.

Objective 2: Assess the dynamic and functional potential of stormwater microorganisms colonizing experimental sand filters

Objective 3: Determine if *E. coli* retention and remobilization is significantly different between sand filters inoculated from different stormwater source locations and correlated to bacterial community diversity or biomass.

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Chapter 2. Bacterial community composition and functional potential associated with a variety of urban stormwater sources[1]

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Abstract

Pathogens and chemical contaminants in stormwater are a major environmental and public health concern. Characterizing the variability in composition, potential pathogens and bioremediation functions of the microbial community from different sources of stormwater will help with efforts to properly manage water after it rains. To improve understanding of the variability and composition of the microbial community in run-off from different sources, we sampled stormwater over time from an outfall and receiving stream, along with run-off from different locations (rooftop, roadway) to identify microbial components associated with these urban waters. Overall, community composition was variable in space and time for water samples from the same source, although outfall and roadway communities varied in taxonomic composition more than run-off from rooftops and an urban stream. Even with this variability, we found taxonomic and functional groups differentially distributed in water samples collected during wet vs. dry weather or collected from different sources. The differentially distributed taxonomic and functional groups represent the unique characteristics of the source, such as exposure to iron in pipe material from the outfall or hydrocarbons from road and rooftop run-off. Additionally, fecal indicator sequences from *Clostridiales* and *Bacteroidales* made up a larger fraction of the microbial community from water collected under dry conditions. This survey provides insight into the sources of taxonomic and functional variability in urban stormwater.

2.1 Importance

Rain flushes urban surfaces creating stormwater that is a conduit of disease and contributes substantially to the community composition and function of surface waters. While some research has been done to characterize microbial communities in stormwater, focus has largely been on changes to indicator organisms, communities associated with combined sewer overflows, or microbial communities associated with one locations or sample type. Our work characterizes the variability in microbial community composition, potential pathogens and functions from a variety of typical urban water sources, with specific attention to location types likely to be associated with stormwater remediation. This will aid our understanding of the impact of human activities in urban environments on the surrounding water system.

2.2 Introduction

Inadequately managed stormwater in urbanized areas deteriorate the quality of receiving waters resulting from pathogen contamination, eutrophication from excess nutrients, mobilization of toxins and other contaminants (1-3). Microbial contamination from human or animal fecal material increases the risk of disease transmission, which has been a long-standing concern in urban water management and infrastructure development. Chemical contaminants that arise from both point and non-point sources can be difficult to track and remediate. By identifying the nature and source of microbial and chemical contaminants, continued progress can be made towards reducing

the impact of water from the urban landscape on receiving water bodies and improving treatment of stormwater for reuse (4).

Pathogens are an important aspect of the microbial community in urban waters, originating from a variety of sources that may vary in space and time. An important source of pathogens derives from combined and sanitary sewage overflows associated with increased water volumes due to rain (5). These events contribute to the higher observed concentrations of *E. coli* and *Enterococcus spp.* indicator species during wet weather events compared to dry weather [e.g. (6-8)]. Management strategies have been moving away from combined storm and sewer systems to municipal separate storm sewer systems (MS4), with the hope of reducing sewage release during rain events. Yet, MS4 can still be a source of pathogens due to aging infrastructure and cross connections between the storm and sewer system (9, 10). For regulatory reasons, indicator organisms are typically monitored, such as of *E. coli* and *Enterococcus* (11-13), but there is often a poor correlation with these indicators and pathogens most associated with disease risk, such as *Salmonella spp*. *Campylobacter spp*., *Cryptosporidium*, *Giardia spp*., and human enteroviruses (13). This could be attributed to the many sources of fecal waste contributing to the signal of indicator organisms, including human sewage, pet waste, birds, and wildlife (14), each associated with a different level of risk to public health (15, 16). Better characterization of pathogens from non-point sources and a range of land use types was previously identified as a major research gap in stormwater management (17).

Stormwater flushing a variety of urban surfaces, such as roads, lawns, and buildings, contain a variety of chemical contaminants that could influence stormwater microbial community composition and functional capability (18). Pollutants from urban environments typically include nutrients [e.g. phosphorous and nitrogen (N)], hydrocarbons [e.g. PAHs, oil and grease] or other chemicals (e.g. pesticides) of concern to human health (19). Contaminants associated with stormwater can select for microbes with specific functions, such as genes associated with organic hydrocarbon degradation, metal resistance, and antibiotic resistance (20, 21). These communities will also serve as the incoula for many types of green infrastructure management systems that take advantage of their capabilities for remediation of pollutants, such as nitrogen (22). Genes associated with denitrification, a microbial pathway that contributes to nitrogen removal, have been observed in biochar amendments (23) and model stormwater systems (24). Functional capabilities of microorganisms coming in from stormwater could influence remediation function within green infrastructure projects, so an increased understanding of the variability of microbial community functional potential for contaminant remediation within stormwater would benefit stormwater management.

Researchers have attempted to identify microbial signatures of degraded or impacted water, but variability in community composition could hamper these efforts. Bacteria found to be associated with urban stormwater include *Oxalobacteraceae, Acinetobacter, Pseudomonas, Aeromonas, Tolumonas, Enterobacteriaceae, Pantoea* (25-27), yet it is unclear whether this signature

holds across all types of stormwater. Microbial signatures have also been used to identify the source of fecal contamination that could benefit stormwater management [e.g. (6, 28)]. Different surfaces could also contain unique signatures of microbial communities related to the specific contaminants or surfaces that are flushed. More work is needed to determine if there are microbial taxa or functions consistently associated with urban stormwater, given its various sources.

Here we investigate the microbial community composition and potential function associated with run-off and water samples from rooftop, roadway, MS4 outfall, and stream locations. Our primary objective was to characterize the spatial and temporal variability of urban water microbial communities, focusing on fecal indicators, potential pathogens and functions of interest for biogeochemistry of receiving bodies and bioremediation within bioretention systems. We determined that there is a significant difference in microbial community composition, taxa and associated functions between samples collected under wet and dry conditions and between different run-off sample types. While our results show some overlap with results from previous work, the differences between samples of the same type and between our results and previous work largely demonstrate that microbial community composition and function of urban water is highly variability in space and time with few well-defined characteristics.

2.3 Results

Microbial community taxonomic variability within and between sample types

To better understand the taxonomic and functional variability of microbial communities from urban stormwater sources, we analyzed the microbial community from stormwater run-off from two surface types (road and rooftop), water from a stormwater outfall (a separate stormwater and sewer system; MS4) and water samples from an urban stream (Table S2.1). Large changes were observed in the relative abundance of class-level taxonomic groups both within and between sample types (Figure 2.1). Roadway and outfall samples had striking differences in taxonomic composition between samples of the same type. Dominant phyla identified in outfall samples changed between Firmicutes (6/9/16), Bacteroidetes (6/15/16) and Proteobacteria (6/2/16, 6/30/16, 8/16/16 and 9/29/16), which could not be attributed to changes based on sampling during wet or dry weather alone. Taxonomic composition of run-off also varied across roadway locations, with two locations (Brentwood Ave. [BW] and Maryland Ave [MD]) dominated by Alphaproteobacteria, and another location (flowing into an engineered infiltration system on Wyman Park Drive) dominated by Betaproteobacteria. Run-off samples from all rooftop locations and time-points were dominated by Gammaproteobacteria and stream samples collected during both wet and dry events were dominated by Betaproteobacteria. Average β diversity (Weighted and Unweighted Unifrac) was significantly larger (p-value < 0.01) for biological replicates than technical replicates for all samples types except stream samples, highlighting the variability in relative abundance

microbial community composition across the sampling categories (Fig. S2.1, S2.2).

Along with changes in dominant taxa, we also looked for the presence of taxonomic groups previously associated with urban stormwater [i.e. *Oxalobacteraceae*, *Acinetobacter*, *Pseudomonas*, *Aeromonas*, *Tolumonas*, *Enterobacteriaceae*, *Pantoea* (1)]. All previously identified taxa were present in our database except *Pantoea*. *Oxalobacteraceae, Acinetobacter, Pseudomonas,* and *Enterobacteriaceae* were found in all environmental samples (both wet and dry). *Tolumonas* was present in all outfall and stream samples, but was occasionally absent from road and rooftop samples. *Aeromonas* was found in only two wet and three dry samples. Thus, taxa previously identified as urban stormwater-associated were not specific to stormwater collected in wet weather or a specific source of run-off in our sample set, but more generally associated with many different sources of urban water.

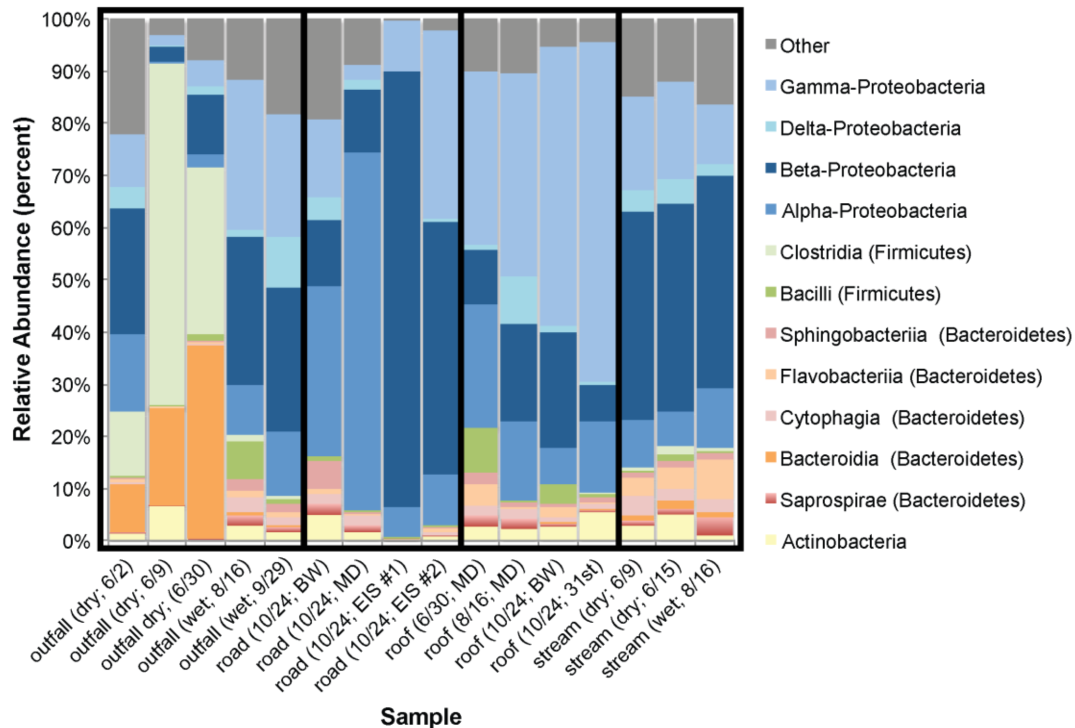

Figure 2.1. Relative abundance of class level taxonomic assignments of observed sequences across wet and dry water samples. Black lines separate sample types (outfall, road, roof and stream). Class level taxonomic assignments (with corresponding Phylum in parentheses where appropriate) of the same phylum are represented with similar colors (Proteobacteria, blue; Firmicutes, green; Bacteroidetes, orange and red; Actinobacteria, yellow). Sample labels indicate sample type along with weather type (wet/dry), sampling date, and specific location as appropriate.

Taxa and functional groups characteristics of wet weather samples

Given that previously identified signature taxa associated with urban stormwater were also present in water collected during dry events, we sought to determine if any differences could be identified between microbial communities from road, rooftop, outfall and stream collected during wet and dry weather. Differences between negative (blank) and positive (mock community) control samples and environmental samples (**Fig. 2.2**) drove the largest variation in

community composition (along PC1; 19.27% of the variation), followed by differences between outfall/stream and road/rooftop (along PC2; 11.93% of the variation) using Unweighted Unifrac as the distance metric. Differences between wet, dry and control samples were similar with other distance metrics (e.g. Bray Curtis, Jaccard) that do not weight species presence by relative abundance (data not shown). Differences between a few road and outflow samples collected during dry weather explained the largest amount of variability in composition when considering changes in relative abundance (i.e. using Weighted Unifrac distance metric), but control, wet and dry samples did not cluster separately using this metric (Fig. S2.3). The overall community composition was found to be statistically significantly different (p-value < 0.01) between wet and dry samples with both distance metrics used (Weighted and Unweighted Unifrac Distances).

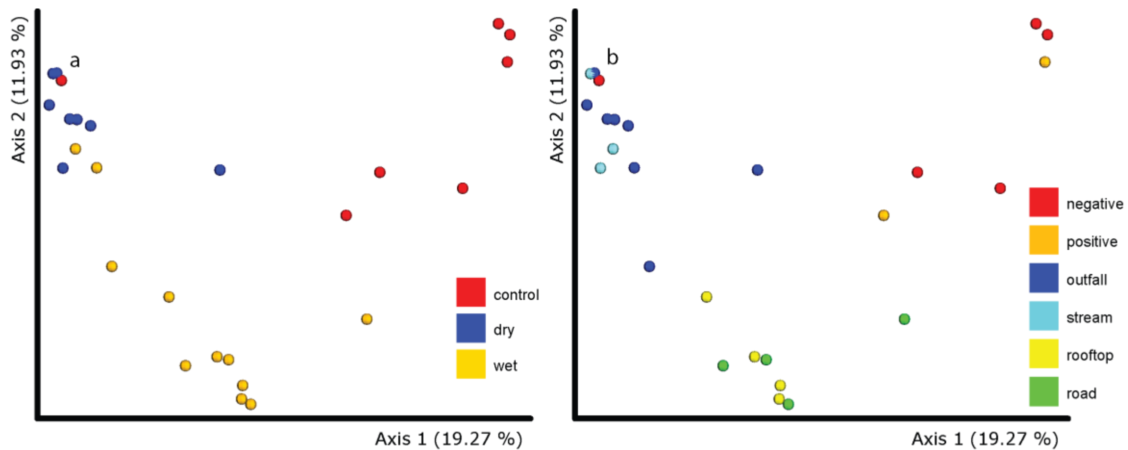

Figure 2.2. Principal coordinates analysis of microbial community composition using Unweighted Unifrac distance metric colored by a.) collection category (wet/dry/control) and b.) sample type. Ordination is identical between (a) and (b), but samples are colored according to different categories. a.) Samples colored according to collected period, either during rain events (wet; orange) or during dry periods (blue). Control samples (both positive, lab negative and field negatives) are shown in red. Negative sample clustering with dry samples likely represents contamination. b.) Samples largely cluster by type into three main groups by collection type; from roadway runoff (green) and rooftop (yellow) samples, water collected from an urban stream (light blue) and outfall (dark blue), and control (red, negative; orange, positive) samples.

To identify species and functional groups that were distributed differently between wet and dry samples, we used ANCOM (29) with functional predictions and species- and genera-level taxonomic predictions (Fig. 2.3). We identified unclassified species in the family *Geodermatophilaceae* (in the phylum Actinobacteria) and genus *Azohydromonas* (in the subphylum Betaproteobacteria) that were more abundant in wet samples (stream, outfall, road and rooftop) than dry samples (stream and outfall; p-values < 0.01). *Geodermatophilaceae* are often associated within sandy or rocky surfaces (30) or soils (31) and could be indicative of water flushing from lawns and roadway surfaces. We also identified potential functions within the microbial community

associated with the wet and dry sample types, as predicted by FAPROTAX (32). Aromatic and aliphatic non-methane hydrocarbon degradation were identified as differently abundant between wet and dry samples with ANCOM (p-value < 0.01). These functions were more abundant in samples collected during wet weather. Though hydrocarbons are difficult to degrade, there are a wide range of bacteria and microorganisms that are capable of using both saturated and unsaturated forms (33). The differential abundance of this function within wet samples could be related to the specific land use of flushed surfaces, such as the presence of hydrocarbons in asphalt from road or roof material and gasoline or oil leaked from cars.

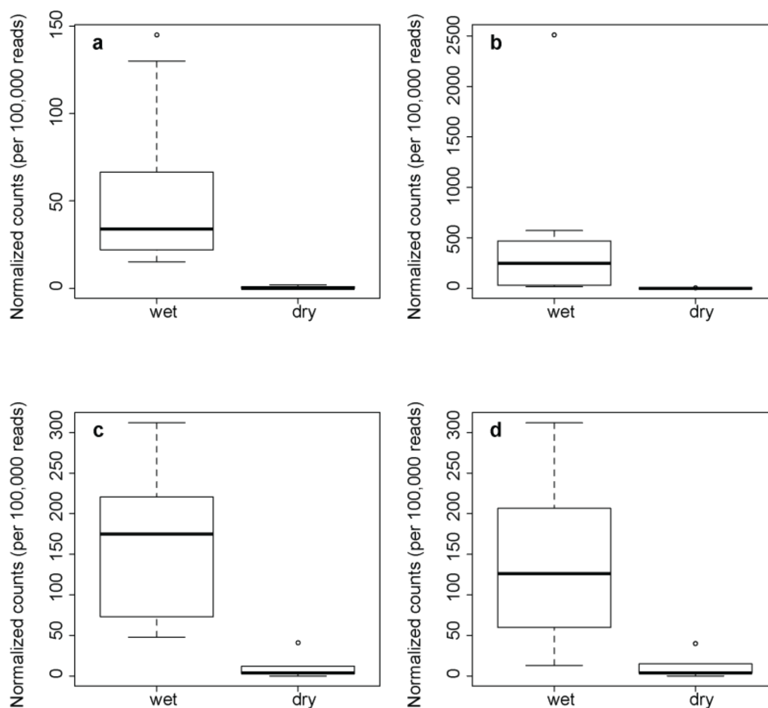

Figure 2.3. Distribution of significantly differentiated taxa and predicted functions across wet and dry sample types. All of the predicted functions were relatively more abundant in sampled collected under wet weather

(wet), as compared samples collected during dry weather (dry). Relative abundance (presented as read counts normalized per 100,000 reads) across wet and dry samples of a.) an unclassified species of *Geodermatophilaceae*, b.) an unclassified species of *Azohydromonas*, c.) aliphatic non-methane hydrocarbon degradation and d.) aromatic hydrocarbon degradation are shown. All differentially abundant taxa and functions were identified with ANCOM and had a p-value less than 0.01 by Wilcoxon rank sum test.

We also determined whether samples contained signature sequences of fecal material. Fecal material often enters waterways through combined sewer overflows during rain events, although transitioning to separate sewer and stormwater systems could reduce the release of fecal material during heavy rainfall. To determine if and where fecal material was present across urban water samples, we used FORENSIC (34) to identify specific fecal indicators within the samples. This source tracking method uses a random forest classifier to predict contamination based on the presence of host-specific *Bacteroidales* and *Clostridiales* sequences. Sewage contamination was predicted with high confidence for stream and outfall samples, while sewage contamination of rooftop and roadway run-off was predicted with low confidence (Table S2.2, S2.3). Roadway and rooftop samples had the smallest fraction of fecal contamination, while stream and outfall samples had the largest amount. This likely contributed to a significantly higher fraction of contaminating reads in dry compared to wet samples (Fig. 2.4, p-value < 0.01), although stream and outfall samples collected during wet weather had a smaller fraction of contaminating reads than dry stream and outfall samples (Table S2.2, S2.3) consistent with this trend. More work is needed to determine whether the fraction of signature fecal sequences within the total community is consistently smaller within this system

during wet weather given the small sample size and skewed distribution of sample types between wet and dry categories.

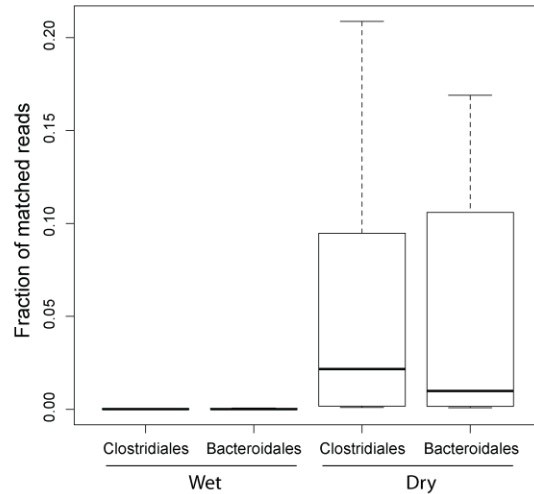

Figure 2.4. The fraction *Clostridiales* and *Bacteroideales* fecal indicator sequences, determined by FORENSIC, is significantly larger within urban water collected during dry weather than wet weather. "Dry" samples include water from outfall and stream collected during dry weather (no rain within at least 24 hours) while "wet" samples include water collected from road, roof, stream and outfall during rain events. This suggests that a substantial fraction of water flowing from the outfall into the stream during dry weather could come from illicit sewage connections or broken sewage pipes.

Potential pathogens and functional capabilities found across sample types

We were also interested in how the landscape influences the microbial community associated with urban water by determining whether there is a unique taxonomic or functional signature associated with each sample type. Community composition from different sampling types, except between road and rooftop samples, were also significantly different (q-value < 0.05) using a distance metric not weighted by relative abundance (Unweighted Unifrac). Sample types were not statistically significantly different using an abundance-weighted metric (Weighted Unifrac distance).

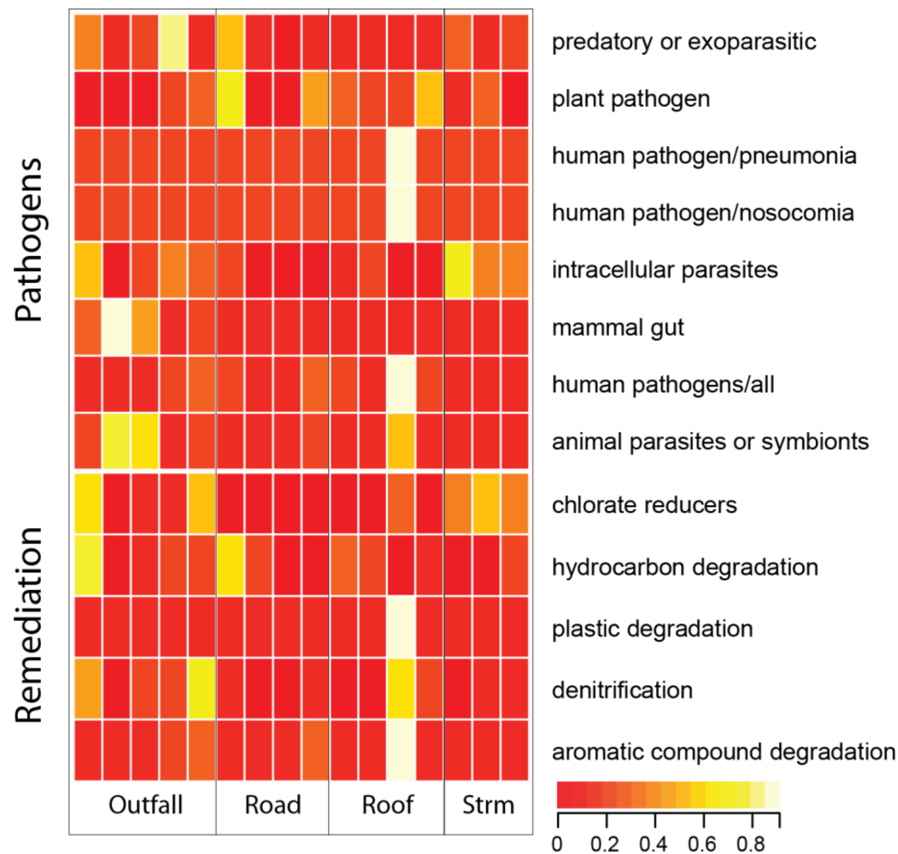

Figure 2.5. Distribution of pathogens and potential functions of interest in bioremediation across samples from different urban water sources. Potential pathogens and functions, as predicted with FAPROTAX from 16S rRNA gene sequence taxonomic classifications, are divided by sample type (columns) and functional categories (rows). The counts of each pathogen or function are normalized by sample (column), then by function (row), ranging from 0 (red) to 1 (light yellow). Only the most abundant or significantly differentiated functions are shown, but results are similar for other types. The potential pathogens and functions are highly variable between samples, even of the same type. Abbreviation: Strm, Stream.

All sample types shared chemoheterotrophy as the dominant potential function, except for the roadway samples where ureolysis was the dominant functional category. Ureolysis is the process bacteria use to breakdown urea into ammonia and carbon dioxide, associated with bio-cementation of calcium carbonate to improve the engineering properties of soil (35), a potential pathway

that could be utilized in soil and filtration based stormwater remediation. However, these shifts were not statistically significant between sample types (p-value > 0.05). Dark iron-oxidation was the only predicted function that was statistically significantly different between samples types (p-value < 0.01). It was found to be relatively more abundant in outfall samples, compared to other sample types (Fig. 2.5a), possibly associated with the corrosion of stormwater infrastructure containing iron (pipes, values, grates etc). Other potential functions related to biogeochemistry (Fig. S2.4), remediation or potential pathogens (Fig. 2.5) were found at relatively high abundance within specific samples, but were not differentially distributed across sample types. This demonstrates the high variability of the microbial community associated with potential functions and pathogens of interest across samples of the same type.

Five taxonomic groups were significantly differentially distributed across sample types (p-values < 0.01; Fig. S2.5b-f). Uncharacterized species from the genus *C39* (*Rhodocyclaceae* family) were significantly more abundant in the stream samples, similar to previous observations (21), along with *Leadbetterella* and *Zymomonas*. Two taxa, *Woodsholea maritima* and uncharacterized species in the genus *Rhodoferax*, were significantly more abundant in the outfall and stream, as compared to the road and rooftop, samples. These taxa do not have well characterized functions associated with bioremediation, pathogenesis or unique biogeochemical processes of interest, making it difficult to interpret the factors that influence their abundance across water types.

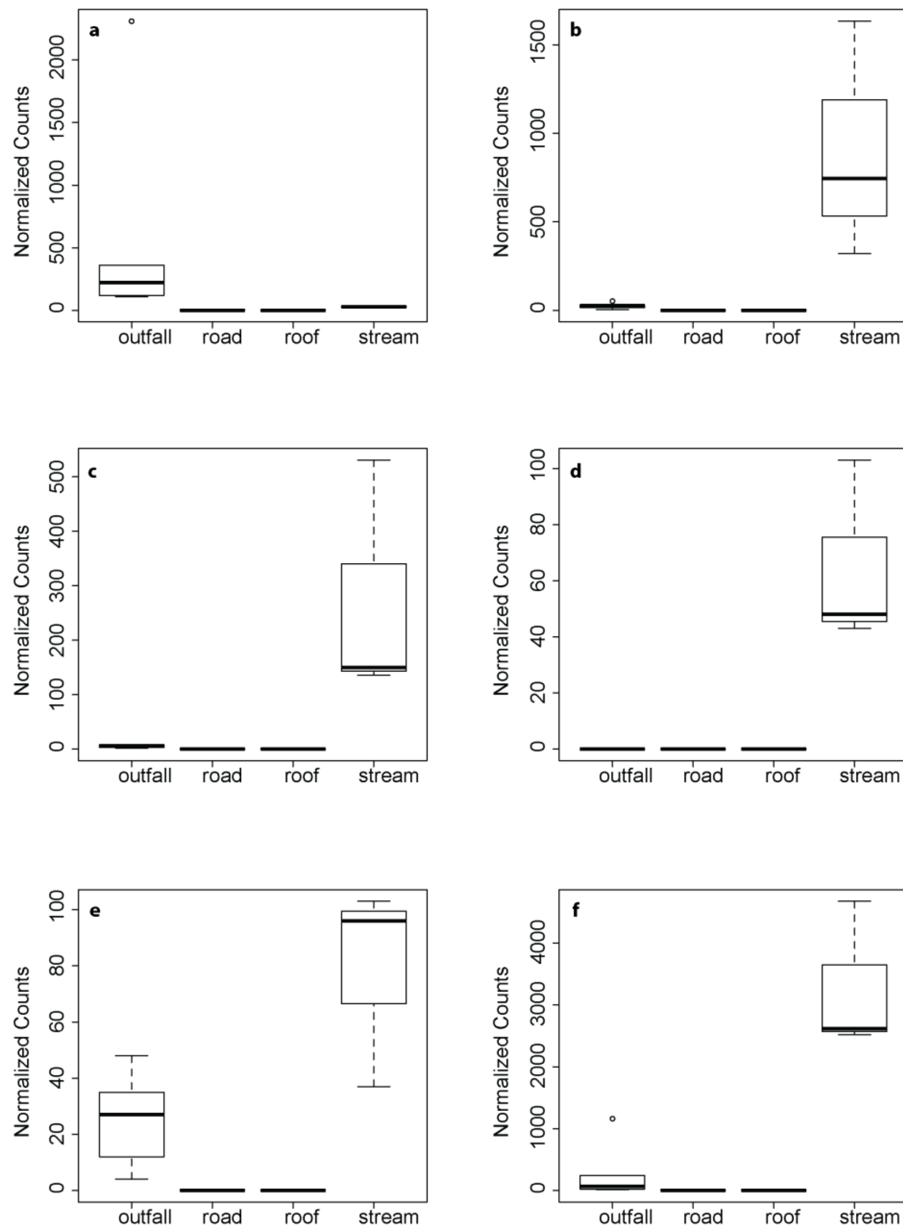

Figure 2.6. Distribution of significantly differentiated taxa and functions across sample types. Normalized counts (rarefied to 100,000 reads per library) of a.) dark iron oxidation, b.) *C39* (*Rhodocyclaceae* family) c.) *Leadbetterella* d.) *Zymomonas* e.) *Woodsholea maritima* and f.) *Rhodoferax* across sample types. All differentially abundant taxa were identified with ANCOM and p-value found to be less than 0.01 by Kruskal-Wallis test.

2.4 Discussion

Our results demonstrate that there is a substantial amount of functional and taxonomic variability between wet and dry samples of stormwater runoff, outfall, and stream, but that certain taxa and functions are differentially distributed. Differences between sample types drive the largest amount of overall variability in OTU composition, but there is substantial variability of the relative abundance of OTUs between some samples of the same type. While we observed taxa known to be associated with stormwater in our samples collected during wet weather, they were also present in samples collected during dry weather. Fecal indicators of sewage made up a larger fraction of the community in water from a stream and MS4 outfall collected during dry weather than across all wet weather, suggesting cross connections or leaks in these systems. The taxa and potential functions differentially distributed between both wet and dry samples and between sample types in this dataset, such as hydrocarbon degradation and dark iron oxidation, suggest that land use and infrastructure leave a unique signature on the microbial community in stormwater.

Table 2.1. Differentially abundant taxa and potential functions associated with samples types

Category	Taxonomic Group or Functional Category	Implication
Wet[1]	Aromatic and aliphatic non-methane hydrocarbon degradation	Adaptations to hydrocarbons washed from streets and rooftops
Wet	Geodermatophilaceae and Azohydromonas	Geodermatophilaceae isolated from soil, sediment and stone, possibly signal from roadway material
Dry[2]	Clostridiales and Bacteroidales fecal indicators	Potential influence of sewage on outfall and stream during dry conditions
Outfall	Dark Iron Oxidation	Associated with corrosion of stormwater infrastructure containing iron
Stream	*C39* (Rhodocyclaceae family), *Leadbetterella* and *Zymomonas*	Rhodocyclaceae previously associated with urban streams
Stream/Outfall	*Woodsholea maritima* and *Rhodoferax* spp.	NA

[1] Samples collected during rain events
[2] Samples collected during dry weather

Previous studies have documented high variability in microbial taxa associated with urban water. Fisher et al. 2015 (26) identified *Pseudomonas, Flavisolibacter, Sphingomonas*, and unclassified members of the families *Oxalobacteraceae* and *Enterobacteriacea* as more abundant in stormwater (from outfall samples) than sewage and natural aquatic communities. Chaudhary et al. 2018 (21) found significant differences in the relative abundance of a number of different taxa before and after the rain event, such as increases in *Legionella*, *Pseudomonas*, and *Arcobacter*, which were all found to be associated with contamination of urban waters (1). We found a substantial amount of fecal contamination in the urban stream and outfall, even during dry weather,

suggesting contamination from sewage may strongly influence community composition regardless of stormwater system (combined or separate) or weather (wet or dry). Chaudhary et al. 2018 (21) also found a substantial amount of variation in community composition between sampling time points, along with differences under baseflow and stormflow conditions, similar to the variability between dominant taxa in our outfall samples. Baral et al. 2018 (6) found larger temporal variability than spatial variability in microbial community structure between storm events, although their sites fall within a few kilometers of each other along the flowpath of a stream. We observed substantial spatial variability in the dominant taxa across all samples between sites that are not hydrologically connected. This demonstrates the microbial community composition of urban stormwater is spatially and temporally variable, but that rain events can substantially affect the composition of microbial communities.

Few studies have looked at the functional differences between urban waters during wet and dry weather events. Chaudhary et al. 2018 (21) applied shotgun metagenomic analysis to investigate the changes within a stream before and after a rain event and found a number of genes were relatively more abundant after the rain, including degradation of organic pollutants and antibiotic resistance genes. We found microbes capable of aromatic and aliphatic non-methane hydrocarbon degradation were significantly enriched in wet weather samples, largely associated with run-off from road and rooftops. These findings suggest that the microbial community in run-off is influenced by pollutants that

are flushed from urban sources, potentially carrying with them the genes that could benefit remediation, if properly handled.

Previous studies also demonstrate substantial differences in microbial community structure and function between urban water sources. Studies have found that microbial community composition from a variety of sources are significantly different (6) and largely cluster by source (26, 36). Our results largely agree with previous findings, although we only find significant differences and clustering when using distance metrics not weighted by relative abundance. Additionally, rooftop and roadway samples are not significantly different with either type of distance metric. The taxonomic composition of rooftop run-off has most commonly been studied in the context of non-potable re-use in rain barrels, where they have found a number of potential pathogens, including *Campylobacter spp.*, *Salmonella spp.* and *Giardia lamblia* (17, 37) not observed in rooftop run-off in our samples. Proteobacteria have been observed as the dominant phyla in rooftop rain barrels (38) and street sweepings (6), similar to our observations. Fisher et al. 2015 (26) used discriminant analysis (LEfSE) to identify hundreds of taxa associated stormwater (outfall), as compared to aquatic and sewage communities. This was a much larger set of discriminating taxa that was found with our analysis using ANCOM and our sample set. Baral et al. 2018 (6) looked at an urban stream (Antelop Creek) not influenced by CSO or WWTP, to determine the major sources of the microbial community composition between wet and dry weather. They found that the dominant microbial community source shifts from the lake to the outfall, with street sweepings contributing substantially

to the outfall community. Our observations, similar to previous work (36), to not suggest substantial contribution of the roadway run-off to outfall and stream microbial communities during wet weather, although our samples are not hydrologically connected and we have many fewer samples from the stream and outfall during wet weather. Few studies have explicitly investigated the functional potential associated with stormwater and urban communities from different sources. We found that iron-oxidation was associated with outfall samples, likely related to the influence of iron-containing stormwater infrastructure. Since our investigations assess microbial community structure through DNA analysis and infer function through taxonomic assignments, more work will be needed to verify the activity of the observe members, especially potentially pathogenic organisms, and verify the identified functions are active within the community. It must also be noted that changes in relative abundance do not necessarily translate into increases in absolute abundances because of inherent biases in 16S rRNA amplicon analysis (39) and the changes in the total number of cells per mL, which was not determined. This would be particularly important when considering the absolute abundance of sewage fecal indicators between wet and dry sample types, when changes in the volume of water flowing would contribute substantially to the absolute amount of each cell type.

Better characterizing the potential functional capabilities and risks associated with potential pathogens in stormwater will be a key aspect of urban stormwater management. Stormwater microbial communities will be a major source of microbes colonizing engineered stormwater treatment systems, such

as green infrastructure infiltration and bioretention systems. The stormwater will also bring signature contaminants and pathogens that the systems may be designed to remediate. Numerous studies demonstrate that the microbial communities on biofilter alter their function (40-42) and a number have identified organisms and properties correlated with improvements in removal processes (43-46). However biological processes that occur within stormwater filters are largely considered a black box (47, 48). Increased understanding of factors that drive filter performance, including the availability and stability of microorganisms with specific functions, will be a crucial step in predicting and controlling the bioremediation of stormwater in filtration systems. More work is needed to understand the variability in microbial community composition in stormwater and to directly tie community composition or biofilter composition to function.

2.5 Conclusion

Increased understanding of taxonomic and functional changes in urban waters can improve the quality of receiving water bodies through improvements to stormwater and sewage infrastructure management practices. As combined sewer systems are being phased out for MS4 systems, it will be important to identify the chemical and biological contaminants of stormwater and design cost-effective and efficient treatment systems. Our results expand knowledge of how the composition and functional potential of stormwater microbial communities change as water flushes from different surfaces and moves through the urban environment.

2.6 Materials and Methods

Sampling locations and protocol

Outfall and baseflow samples were collected from a MS4 stormwater outfall that empties into Stony Run, a tributary to Jones Falls (1983 Remington Ave, Baltimore, MD 21211, USA; latitude N 39°19′36.172″, longitude W 76°37′32.355″) during baseflow events and storm evens. Upstream samples were taken 50 ft upstream of the outfall. Rooftop samples were collected from gutter spouts as they emptied into alleys. Roadway water samples were collected at sites as water was flowing into MS4 systems or bioretention facilities (see Table S3.1 for collection dates and locations). Except for the stream samples that were collected near the outfall, and the two roadway samples flowing into the same bioretention facilities, other sampling sites are not connected hydrologically. Events at the stormwater outfall were considered dry events if there was no precipitation for at least 24 hours prior to sampling. Events are considered wet events when they were collected during rain events, typically while runoff was flowing. Triplicate water samples were collected in 50mL Falcon tubes or washed and autoclaved glass bottles and stored on ice while being transported to the lab (within 4 hours). In the lab, samples were filtered through a 0.22 μm polyethersulfone filter (MilliporeSigma, Inc., Burlington, MA, USA) using a peristaltic pump. Filters were stored at −80 °C until DNA extraction.

DNA extraction and 16S rRNA gene sequencing

DNA was extracted from the water samples using the PowerWater DNA extraction kit (Qiagen, Hilden, Germany). The 16S rRNA gene was amplified using primers U515F and E786R (49) modified with overhangs to facilitate the addition of barcodes and adapters for sequencing as previous described (50). Multiple control samples were amplified and sequenced, including a completely characterized mock community and negative (water or blank) controls during field sampling, DNA extraction and PCR steps. DNA sequencing was performed at the Genetic Research Core Facility at Johns Hopkins University on an Illumina MiSeq. Raw sequence reads have been submitted to the National Center for Biotechnology Information Sequence Read Archive under BioProject accession number PRJNA599168.

Sequence analysis

QIIME2 was used as previously described (24) to process the sequences for all analyses except source tracking. Briefly, sequences were demultiplexed, quality filtered and trimmed with DADA2 (51). Unique sequences, identified from DADA2, were used as operational taxonomic units (OTUs) and classified using the 99% identity GreenGenes database (52) formatted for the sequenced region. Samples were subsampled to 100,000 reads per sample before calculating beta diversity metrics using Unweighted and Weighted Unifrac distances. Ordination with principal coordinates analysis was used to identify major factors influencing OTU composition. FAPROTAX (32) was used to predict potential functions from

species level taxonomic assignments. ANCOM (29) was used to identify differentially distributed taxa and potential functional categories (from FAPROTAX). Significance values were calculated from the rarefied OTU table for taxa identified with ANCOM and ureolysis using the Wilcoxon rank sum test (wilcox.test) for wet/dry comparisons and Kruskal-Wallis (kruskal.test) for run-off type (outfall, stream, road, roof) comparison in R (53). PERMANOVA (54) was used to determine whether community composition was statistically significantly different, using an empirical significance test with 999 permutations. Technical replicates for each biological sample were combined with mean-ceiling using QIIME2, where the mean OTU abundance across all technical replicates is rounded to the nearest integer. Additionally, all statistical analyses for either wet/dry or sample categories were repeated with sequence run as the category to determine if differences between sequencing runs could have contributed to the result. None of the significantly differentiated taxa or functional categories reported were found to be significantly differentially distributed across sequencing runs.

For source tracking with FORENSIC (28), raw sequences were processed following the recommended pipeline for the V4 region. Cutadapt (55) was used to remove primers and adapters and PEAR (56) was used to overlap paired-end reads. The on-line tool FORENSIC (https://forensic.sfs.uwm.edu/) was used to determine the confidence level and percent of matching reads per library. Replicate libraries from each water sample were concatenated and processed together. The Wilcoxon rank sum test (wilcox.test) was used to determine the

whether the percent of matching reads was significantly different between wet and dry samples.

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Chapter 3. Dynamics and Functional Potential of Stormwater Microorganisms Colonizing Sand Filters[2]

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Abstract

Stormwater management is increasingly relying on engineered infiltration systems (EIS) to reduce the volume and improve the quality of managed stormwater. Yet, EIS in the field will be colonized by a diverse array of environmental microorganisms that change the physiochemical properties of the EIS and provide a habitat for microorganisms with harmful or beneficial qualities. Understanding factors influencing the composition and stability of microbial communities could open strategies for more efficient management of stormwater. Here, we analyzed the potential pathogenic and metabolic capabilities of stormwater microorganisms colonizing idealized EIS (i.e., sand columns) under laboratory conditions over time. The diversity of microbial communities was analyzed using 16S rRNA gene sequencing, and potential pathogens and denitrifying microbes were identified from taxonomic match to known species. Denitrification potential as determined by *nosZ* abundance was also assessed with quantitative polymerase chain reaction PCR. Our findings demonstrate that replicate microbial communities colonizing sand columns change in a similar way over time, distinct from control columns and the source community. Potential pathogens were initially more abundant on the columns than in the stormwater but returned to background levels by 24 days after inoculation. The conditions within sand columns select for potential denitrifying microorganisms, some of which were also potential pathogens. These results demonstrate that a diverse suite of stormwater microorganisms colonize sand filters, including a transient population of potential pathogens and denitrifiers. Manipulating the inoculating

microbial community of EIS could prove an effective mechanism for changing both potential pathogens and denitrifying bacteria.

3.1 Introduction

Urbanization has significantly increased the area of impervious surfaces that prevent natural groundwater recharge, resulting in large volumes of stormwater that need to be managed [1]. Stormwater can transport pathogens, nutrients, such as nitrogen, and other contaminants from these surfaces to surrounding water bodies if not properly managed [2]. Engineered infiltration systems (EIS) can promote groundwater recharge and reduce the concentration of contaminants through physical filtration, chemical reactions and biological transformations [3,4]. Biotransformation of nutrients and removal of pathogens is influenced by microbial communities colonizing engineered infiltration systems (EIS), and these processes are not well understood [5]. Microbial biofilms, or microorganisms attaching to the surface of the media which typically secrete a protective extra polysaccharide layer, are an important aspect of biotransformation and contaminant removal [6]. Understanding the factors that promote efficient and effective contaminant removal in EIS will aid stormwater management efforts and improve surface water quality in surrounding areas.

Nitrogen and nitrate removal in EIS is variable, and often below ideal efficiencies [2,7,8]. Along with physiochemical properties of the media promoting nutrient removal, the microbially mediated process of denitrification transforms bioavailable nitrogen (nitrate) into various gaseous forms (N_2, N_2O) that are removed from the stormwater. Denitrifying bacteria must either be present within

the EIS during installation or colonize EIS from the environment. Denitrification potential within EIS will be impacted by the colonization, growth, and dynamics of these microorganisms. Colonization of a robust denitrifying community within the filter will depend on the presence of microorganisms in the biofilm with these capabilities from the inoculating water. Conditions promoting denitrification provide a selective advantage to microorganisms with this potential function, increasing the potential for denitrification over time. For EIS to be maximally effective at nutrient removal, it is important to understand the factors that influence the presence of denitrifying bacteria.

Pathogens are an important contaminant of stormwater that can be managed effectively with EIS, although their fate within EIS is not well understood. The fate of pathogens within EIS can be influenced by colonizing microorganisms in many ways. Physical factors, such as mechanical filtering and water velocity have a strong impact on the initial retention of pathogens within the EIS [9]. Biofilms created by colonizing microorganisms within EIS can alter the physical environment to further influence pathogen retention [10]. Pathogen retention efficiency within EIS may change over time, as the properties of the EIS are altered by the dissolved material, particles, and microorganisms that pass through [3]. Once trapped on the EIS, pathogen survival is also impacted by predators and other organisms competing for nutrients [5,11]. Conditions, such as the presence of a protective biofilm or suitable energy and nutrients for growth, can also promote their survival. These pathogens could eventually be

transported out of the EIS during the next storm if survival is high, negating the short-term beneficial effects of retention with the EIS.

Given the importance of the colonizing community in the biotransformation of many pollutants in stormwater, it is important to understand the factors that influence EIS microbial community assembly and succession. Within any ecosystem, both selective (“niche”) and neutral factors can impact microbial community assembly [12,13]. Selective factors will be highly variable in the field, as EIS configurations and environmental conditions experienced will vary over time and from site to site [3]. Neutral processes influencing microbial community composition in EIS include random fluctuations in populations abundances (i.e., drift) and movement of organisms into the EIS from other areas (i.e., dispersal; [13]). Both drift and dispersal could have a large impact on community composition but are often overlooked as compared to selective factors. Some studies indicate that neutral factors could have a large role in shaping the microbial community. For example, historical contingency, or the order in which microorganisms arrive, has been shown to play a large role in the resulting community [14,15] and has also been shown to impact interactions [16]. Both niche and neutral factors have been shown to impact microbial community assembly on sand filters [17]. Understanding the influence of selection versus neutral factors will be important for engineering the microbial community of EIS to enhance biotransformation since efforts could be thwarted if drift or dispersal drive the community away from a desired state. Thus, while studies have focused on how environmental conditions impact the resulting community [18], few have

investigated the impact of drift and dispersal alone on the resulting community composition.

Given the importance of the colonizing microbial community in determining the fate of nutrients and pathogens within EIS, we examined the potential for denitrification and pathogen survival in experimental EIS initiated with stormwater inoculum. Additionally, we investigated the successional dynamics of the microbial community within idealized EIS under experimental conditions, from inoculation through 24 days post-colonization, to determine whether historical contingency has a sustained impact on microbial community composition. Sand is the most common EIS media, so sand filters were used as the model experimental EIS. Here, we use microbial community analysis of the 16S ribosomal RNA gene (16S rRNA) as a proxy for microbial community composition and infer potential functions from taxonomic predictions, including potential pathogenicity and denitrification, and validated denitrification potential by quantifying gene abundance for a key gene in the denitrification pathway (*nosZ*).

3.2 Materials and Methods

Sampling Site and Protocol

Water was collected along Stony Run (1983 Remington Ave, Baltimore, MD 21211, USA; latitude N 39°19′36.172″, longitude W 76°37′32.355″) during a storm event on 29 September 2016. The air temperature at time of collection was 65 °F (18.3 °C), and the total rainfall in the previous 48 h was 3.1 inches (7.87 cm) (Baltimore-Washington International Airport weather station). A storm drain outfall empties directly into Stony Run at this location.

Two types of samples were collected; water for inoculation of experimental columns and water for analysis of the microbial community in the stream and outfall discharge. For inoculation of experimental columns, 1 L of water was collected directly from the outfall. Stormwater from the outfall was not filtered, collected in a 1 L carboy, transported back to the lab on ice, stored at 4 °C until use two days later.

Water samples for microbial community analysis were taken from the outfall and approximately 50 ft upstream and downstream from the outfall and from the inoculum immediately before being added to the column. After collection, 50 mL of water was filtered through a 0.22 μm polyethersulfone filter (MilliporeSigma, Inc., Burlington, MA, USA) using a peristaltic pump. Filters were stored at −80 °C until DNA extraction. The microbial community composition of water used for inoculation and in the stream nearby provides a comparison to determine how much the column communities deviate from the original community structure.

Column Description, Set-Up, and Operation

A 24-day study was designed to investigate the dynamics of microbial communities colonizing sand columns inoculated with stormwater. Disposable polypropylene chromatograph columns (14 cm depth, 20 mL bed volume, 1.5 cm end fitting) including a 30 μm polyethylene filter at the bottom (BioRad, Inc., Hurcules, CA, USA) were rinsed with deionized water and autoclaved. Fifty to seventy mesh sand (SiO_2, 212–300 μm) was rinsed with sterile, deionized water three times and dried for about 24 h (105 °C) and autoclaved as previously described [19]. 9.4 g (approximately 6 cm depth) of sand was packed into each column. All of the columns were autoclaved again before inoculation to ensure a sterile environment inside the columns.

Twenty columns were initiated on day one, grouped into four sampling time-points with five columns per time-point (Figure S3.1). Each time-point consisted of three stormwater columns (A, B, and C), non-inoculated control column, and one *Pseudomonas aeruginosa* positive control column. Columns were inoculated with an approach velocity of 15 cm/h for 3 h using a 24-channel peristaltic pump resulting in approximately 78 mL total volume added to each column. This simulates a common storm of 0.75 cm/h intensity and 3 h duration (return period < 1 year [20]) concentrated by a factor of 20, which resembles a typical bioretention area sized at 5% of the drainage area [11,21]. The top of each column served as the inlet and was uncovered. During the inoculation, *P. aeruginosa* overnight culture, sterile synthetic stormwater (SS), and

approximately 78 mL stormwater were added by recycling liquid from 1 L bottles. After inoculation, the first group of columns (Day 1) was collected.

Sterile synthetic stormwater was added to the columns under the same simulated storm velocity and duration on days 3, 6, 10, 13, 17, and 20. During simulated storm events, a total of 78 mL of sterile synthetic stormwater was pipetted directly into each column intermittently to evenly wet the surface and avoid contamination. Columns were sacrificed on days 10, 17, and 24 before each simulated storm event.

Media and Culture Conditions

Synthetic stormwater (SS) was used to simulate storm events. SS was formulated from a previous recipe [22]. The media consisted of 5 mM NaCl, 0.75 mM $CaCl_2$, 0.075 mM $MgCl_2$, 0.30 mM Na_2SO_4, 1 mM $NaHCO_3$, 0.15 mM $NaNO_3$, 0.07 NH_4Cl, and 0.02 mM Na_2HPO_4 (pH ca. 7).) Carbon was added in the form of yeast extract (3 g/L) as well as 0.0015% (by weight) peptone, 0.0011% meat extract, and 0.0003% urea. All media was filter sterilized through a 0.2 μm polyethersulfone filter (MilliporeSigma, Inc., Burlington, MA, USA) and stored at 4 °C until use.

Biofilm-forming *Pseudomonas aeruginosa* served as a positive control. *P. aeruginosa* was stored in 10% glycerol (*v/v*) at −80 °C until use. Glycerol stocks were regrown on Luria Broth (LB) agar plates and incubated at 37 °C overnight. A single colony was picked to inoculate 1 L LB. The culture was allowed to grow overnight at 37 °C before inoculation onto the columns.

DNA Extraction and 16S rRNA Gene Library Protocol

All water samples collected in the field were filtered through 0.22 μm polyethersulfone filters (MilliporeSigma, Inc.) and stored for DNA extraction. The inoculation sample was filtered and stored for DNA extraction as described previously. The DNA was extracted using the PowerWater kit (Qiagen, Hilden, Germany). The sand from each column was poured into a sterile 50 mL falcon tube and votexed. Three replicate three-gram sub-samples were added to 15 mL sterile falcon tubes. The columns were homogenized to remove the effect of depth when sampling replicates, as organisms can deposit differently throughout the length of the column [23]. Tubes were immediately frozen at −80 °C until DNA extraction. DNA was extracted using the PowerSoil kit (Qiagen), following the manufacture's protocol plus 20 μL proteinase K and a 65 °C incubation step before bead-beating to promote additional cell lysis. The 16S rRNA gene was amplified using primers U515F and E786R [24] modified as previously described [25]. Modification provided overhanging adapters used as the primer-binding site for a second step PCR reaction, adding sample- specific barcodes and adapters appropriate for Illumina MiSeq sequencing. Sample indices and binding sites are added in the second step. A mock community positive control [26] and PCR negative controls were also amplified and sequenced. Replicates from group A stormwater columns were sequenced twice to control for sequencing batch variability. DNA sequencing was performed at the Genetic Research Core Facility at Johns Hopkins University. Illumina data has been submitted to the

National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under study accession number PRJNA482666.

Sequence Analysis and Quality Control

Samples were processed with the bioinformatics platform QIIME2 [27] using the program DADA2 [28] to remove sequencing artifacts and chimeras. We analyzed the composition of a positive control (mock community) to ensure the resulting processed sequence data represented the input community as accurately as possible. The mock community was comprised of purified DNA templates of known sequence and concentration, as previously described [26]. We compared the resulting sequence read count for each mock community template to the expected read count for samples without mismatches in the primer binding site (**Figure S3.1**). We expected the input concentration of template to explain a large proportion of the variation in the resulting read count for the mock community templates without primer binding site mismatches (R^2 = 0.74). However, with the default DADA2 parameters in QIIME2, one mock community template was flagged as chimeric and removed. Additionally, a number of DNA sequences found in this library were not mock community sequences, including some non-16S rRNA gene sequences. We changed the DADA2 parameters to require chimeras to be 10-fold less abundant than parent sequences. We also used mothur [29] to align operational taxonomic unit (OTU) representative sequences to the Silva alignment [30] subset to the sequenced region. To remove non-16S rRNA sequences, any sequence shorter than 250 bp or missing data within the first 5 bp of the alignment was removed from the final

analysis. With these changes, the relationship between observed and expected mock community templates improved to R^2 = 0.88. We also used the OTU calling program dbOTU plug-in in QIIME2 to create operational taxonomic units (OTUs) from closely related, similarly distributed sequences [31]. The greengenes classifier distributed with QIIME2 was used for taxonomic classification. Multiple sequence alignment and phylogenetic trees were generated with the programs MAFFT [32] and FastTree2 [33], respectively within QIIME2. Bray-Curtis, Jaccard, Unweighted and Weighted Unifrac distances were calculated in QIIME2 subsampled to 49,950 counts (lowest non-negative sample library read count). Principle coordinate analysis plots were visualized using EMPeror [34]. Bray-Curtis distances were used in the analysis, but the results were similar with other distance metrics. OTU tables collapsed by taxonomy created with QIIME2 were used as the input for the program FAPROTAX [35] to predict functional information and potential pathogens. OTU tables were normalized to the total read count for each library before running FAPROTAX.

Negative controls were included at every step of processing, from DNA extraction through the library preparation. A subset of samples was sequenced in both sequencing runs to verify that methodological errors did not impact our results. Negative and positive controls samples were distinct from the majority of environmental samples (**Figure S3.2a**). Clustering was not driven by batch effects, as replicates from the same samples processed in different batches clustered together (**Figure S3.2c,d**).

Statistical Analysis

Biological columns replicates (A, B, C) and their technical replicates (1–7) were analyzed. Statistical significance of distances between column samples from day 17 and 24 plus the initially sampled and inoculated outfall samples was carried out with permanova and analysis of similarity program ANOSIM [36] analysis in QIIME2. Data from the last two time-points were aggregated because positive and non-inoculated columns did not have multiple biological replicates per time-point. Exported Bray-Curtis distance matrices were used to test the average distances between technical replicates, biological replicates, and source community using Welch two sample *t*-test and Wilcoxon rank sum test in R [37].

Quantitative Polymerase Chain Reaction (qPCR)

A quantitative Polymerase Chain Reaction (qPCR) protocol was developed to quantify the number of 16S rRNA and *nosZ* gene copies within the columns. 16S rRNA templates were created as previously described [26] from 16S rRNA gene amplicon from a freshwater lake sample cloned into *Escherichia coli* with TOPO Blunt End cloning kit (Invitrogen, Carlsbad, CA, USA). *NosZ* templates were created by amplifying with *nosZ* primers (*nosZ* Forward: 5′-CGYTGTTCMTCGACAGCCAG-3′; *nosZ* Reverse: 5′-CATGTGCAGNGCRTGGCAGA-3′) using DNA extracted from a *Pseudomonas aerugniosa* culture, purified with Zymo PCR clean-up kit. Templates were quantified using the High Sensitivity DNA assay on a Bioanalyzer (Agilent; Santa Clara, CA, USA). A standard curve was made to determine the relationship between concentration and the threshold value (Cq). PCR was carried with

SsoAdvanced Universal SYBR® Green Supermix (Biorad; Hercules, CA, USA) according to the manufacture's protocol on the RealTime PCR thermocycler (BioRad).

3.3 Results

Stormwater-Inoculated Column Communities Are Distinct from Positive and Non-Inoculated Control Column Communities.

Bacterial Growth on Columns

To determine whether bacteria could successfully colonize the sand columns, the change in 16S rRNA copy number, which corresponds to bacterial concentration, over the 24-day experimental period was determined by qPCR (16S rRNA gene copies/μL). Non-inoculated control columns, positive control *P. aeruginosa* columns, and replicate stormwater-inoculated columns all showed an increase in 16S rRNA copy number over the experimental period (**Figure 3.1**). Although microorganisms were not intentionally added to the non-inoculated columns and media and tubing were sterilized before use, some level of contamination was expected. The non-inoculated columns represent the microbial community coming from the equipment, reagents or laboratory environment. *Pseudomonas* columns also became contaminated with a different set of microorganisms that could have been the same as the non-inoculated control or come from within the *Pseudomonas* culture itself if it had low levels of contamination. The non-inoculated control had the lowest measurable cell concentration on day 1 (1.47×10^7 copies/g), but increased to levels slightly

exceeding the stormwater columns by day 24. In

contrast, *Pseudomonas* columns on Day 24 had the highest measurable cell concentration for the entire experimental period (5.9 × 10^{11} copies/g). The high cell concentration on the *Pseudomonas* columns likely results from the high initial loading of *Pseudomonas* cells from the culture. Overall, this demonstrates that microbial growth, rather than just deposition of dead or dormant cells, influences the community composition on all columns.

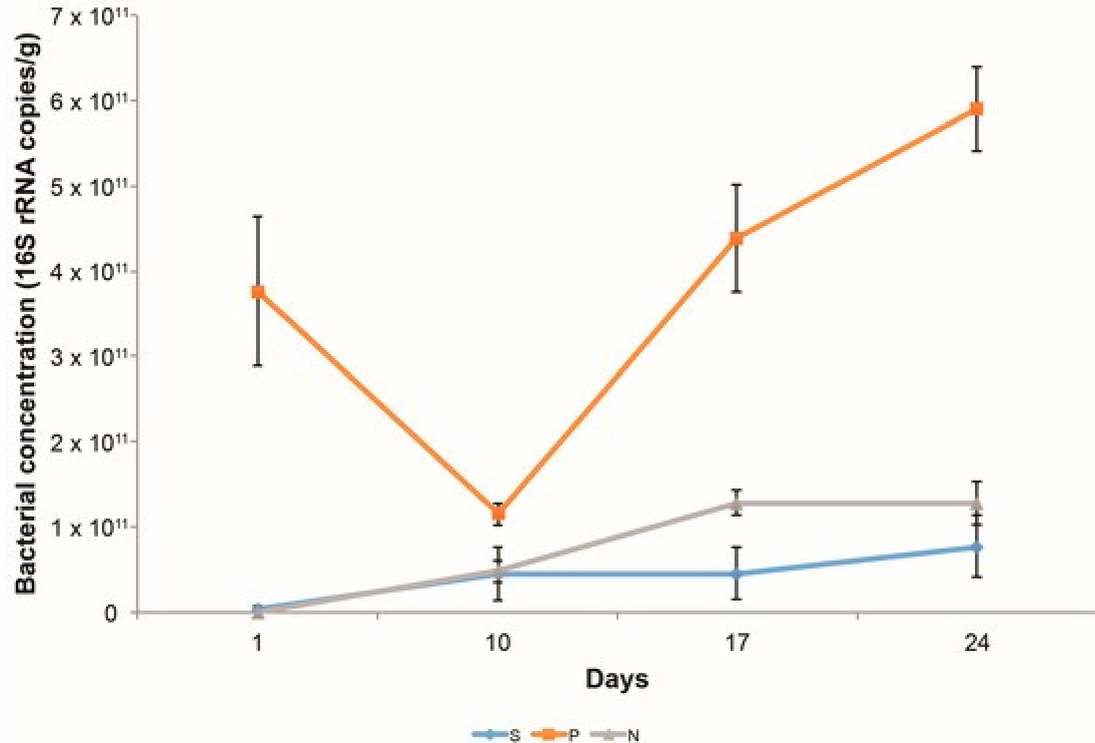

Figure 3.1. Change in bacterial cell concentration within environmental and control columns over time, as estimated by 16S rRNA gene copy number/μL. Abbreviations: S (blue), stormwater-inoculated columns; P (orange), *Pseudomonas aeruginosa* -inoculated columns; N (gray), non-inoculated columns. Significantly more cells were added in the *Pseudomonas* columns on Day 1 than in the non-inoculated or stormwater columns. *X*-axis; Days-days of the experiment from 1 (first day) to 24 (last day). *Y*-axis; cell concentration as measured by quantitative PCR of 16S rRNA gene copies per gram of sediment in the columns.

Bacterial Community Composition on Columns

Columns were colonized by a diverse range of microbial taxa (Figure 3.2). Water sampled up-stream from the stormwater outfall contained a majority of *Proteobacteria* (45%) and *Saccharibacteria*, formerly Candidate division TM7, (26%). Outfall samples used as inoculum for stormwater columns had a higher percentage of *Proteobacteria* (76%) and less *Saccharibacteria* (4%). Samples downstream from the outfall were variable, with some samples more similar to

stream and others more similar to outfall samples. Initially, stormwater columns were more similar to the outfall community but diverged by the end of the 24-day experimental period. Stormwater-inoculated columns still had a large percentage of *Proteobacteria* (51%), but more *Bacteroidetes* (18%) and *Firmicutes* (9%), and less *Saccharibacteria* than the outfall community. Non-inoculated column samples were dominated by *Firmicutes* (42%), *Proteobacteria* (34%), and *Bacteroidetes* (11%). In contrast, *Pseudomonas* columns were dominated by *Proteobacteria* (71%) and *Bacteroidetes* (25%). Negative PCR samples had more *Actinobacteria* and *Planctomycetes* than other samples, along with common contaminant genera *Halomonas* and *Shewanella*.

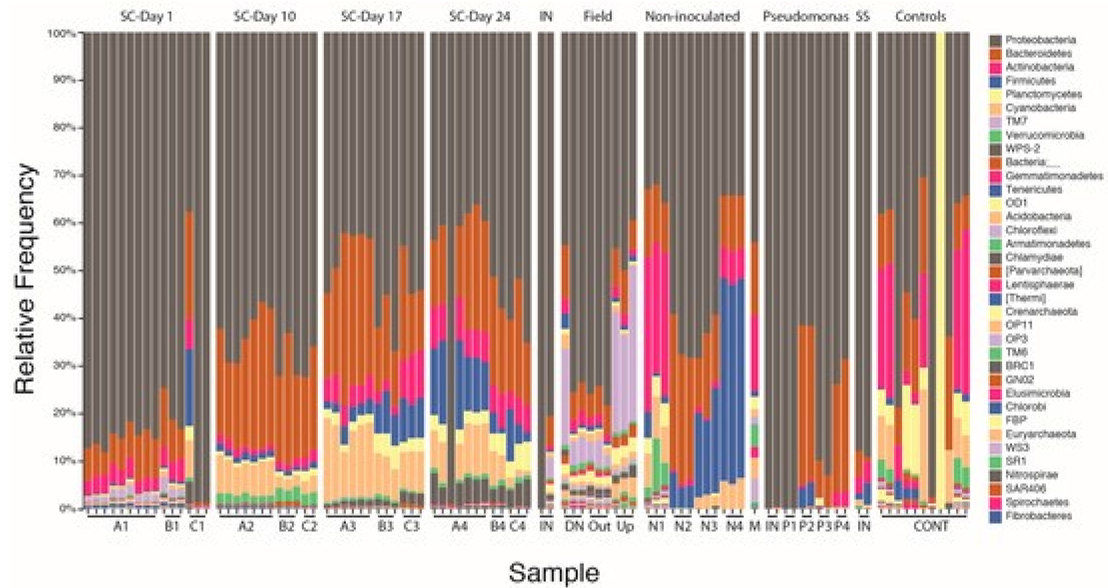

Figure 3.2. Taxa plots (phylum level) of microbial communities across replicate stormwater columns, *Pseudomonas* columns, non-inoculated columns, field samples, and controls. Legend provides phylum level classification although phyla comprising less than 1% of the samples overall are not listed. Sample type is listed about each group of samples (SC, Stormwater inoculated columns by day; IN, Stormwater column inoculum; Field, Field samples; Non-inoculated, Non-inoculated columns; Pseudomonas, *Pseudomonas* columns; SS, Synthetic Stormwater media; Controls, PCR control samples). Sample names (*X*-axis) include column replicate (A, B, C) and time-point (1–4) for stormwater columns, inoculum type (N, non-inoculated; P, *Pseudomonas*) and time-point (1–4) for non-inoculated and *Pseudomonas* columns, and short descriptors for other samples (DN, Downstream of outfall; Out, Outfall; Up, Upstream of outfall; M, Mix9 positive control; CONT, negative controls; IN, inoculum). Technical replicates for the same column are displayed individually.

A statistical analysis was used to determine whether the microbial communities in the stormwater-inoculated columns, non-inoculated columns, and *Pseudomonas* columns were significantly different after 17 days of incubation. The Bray-Curtis distance between microbial communities that developed on the non-inoculated columns was significantly different (permanova and analysis of similarity with ANOSIM p-value ≤ 0.005) from the community that developed on the stormwater- and *Pseudomonas*- inoculated columns (**Figure**

3.3). Additionally, the stormwater columns were significantly different (permanova and ANOSIM p-value ≤ 0.005) from the field and initial inoculum samples. Only the *Pseudomonas* columns and field samples were not significantly different, likely because they lack statistical power from the small sample set (sample size = 9). The complex community that developed on the stormwater-inoculated columns did not resemble either the initial inoculum or the non-inoculated columns in either phylum-level composition (Figure 3.2) or specific taxa.

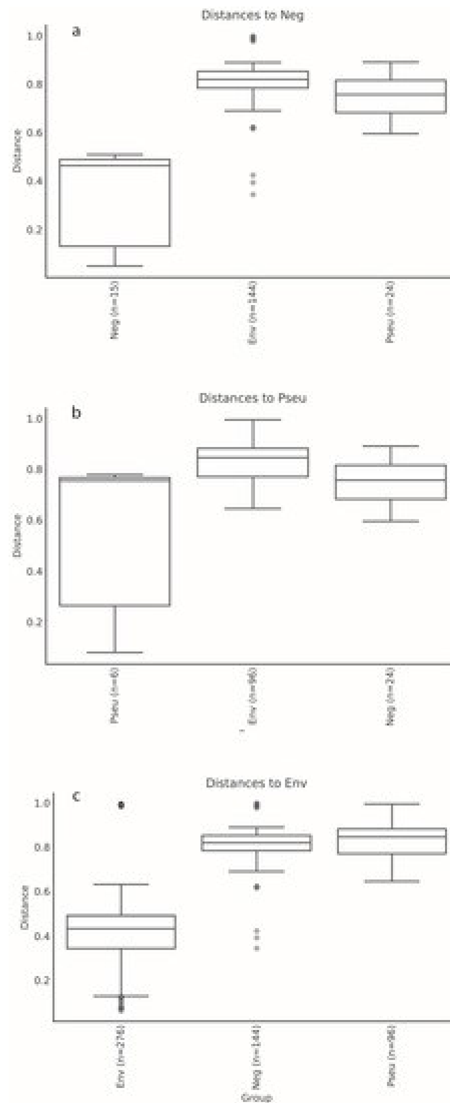

Figure 3.3. Bray-Curtis distance between different inoculum types from the last two time-points combined. Median, interquartile range and outliers distances between all column samples and (**a**) non-inoculated (Neg) column distance (**b**) *Pseudomonas aeruginosa* (Pseu), and (**c**) Stormwater inoculated columns (Env). For each group, the left most comparison represents the within-group distances, and other comparisons are between-group comparisons. All pairwise comparisons between groups were statistically significantly different with both permanova and ANOSIM (p-value ≤ 0.005).

Microbial Community Succession on Stormwater Columns: Technical Variability Is Less Than Biological Variability between Replicate Columns

To understand the influence of drift on microbial community structure variability, we compared the variability across biological replicates of stormwater-inoculated columns to the variability across technical replicates. The median Bray-Curtis distance between biological replicates (i.e., samples from different columns incubated for the same amount of time with the same inoculum) was greater than the median distance between technical replicates (i.e., different DNA extractions or libraries from the same column; **Figure 3.4**). The average Bray-Curtis distance between biological replicates was greater than the average distance between technical (*p*-value < 0.001) at all weeks, demonstrating that a portion of variability between biological replicates cannot be explained by technical reproducibility. Additionally, the communities shifted away from the inoculum community by day 10 and changed slowly after that point. The average distance between biological replicates was significantly lower ($p < 0.001$) than the distance to the inoculum community between days 10 to 24 (**Figure 3.4**), although the difference was not significant at day 1. Thus drift, as measured by the distance between biological replicates, is significant, but small compared to the difference between the source community and the communities later time-points.

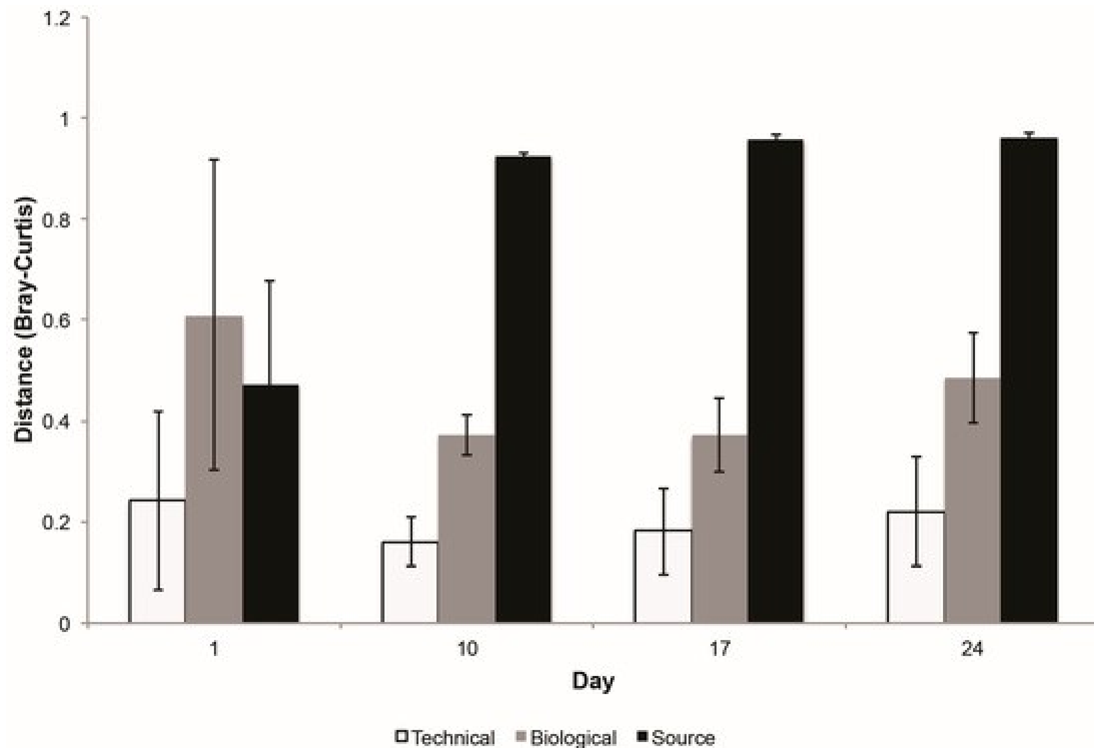

Figure 3.4. The average Bray-Curtis distance between technical replicates (T), biological replicates (B) and the distance it diverged from the starting inoculum community (S) each day. Numbers following single letter comparison group designations indicate the day of the experiment (e.g., S24 is the distance between 24-day columns and inoculum community). More similar communities have a lower Bray-Curtis distance. The average distance between technical replicates is significantly different than the average distance between biological replicates. Average distances between biological and technical replicates are statistically significantly different (*t*-test and Wilcoxon rank sum *p*-value < 0.001) for all days. Average distances between biological replicates and the inoculum community are statistically significantly different from average distances between technical and biological replicates ($p < 0.001$) for all days, except for the day 1 samples.

Stability of Stormwater-Inoculated Columns over Time

The community structure in stormwater-inoculated columns became more stable over time. The mean Bray-Curtis distance between samples from day 1 and samples from other time-points was large (0.88–0.93), suggesting a rapid change in community structure by day 10. Between day 17 and 24, the average

distance between samples from different time-points (0.44) became similar to the average distances between biological replicates from the same time-point (0.37–0.49) (**Figure 3.5**). If all columns types (stormwater inoculated, Pseudomonas-inoculated and non-inoculated) were becoming more similar to each other over time, this would suggest that contamination from reagents or equipment resulted in the similarity observed between replicate columns (e.g., high dispersal resulting in homogenization). However, the statistically significantly different community structure between columns with different inoculum-types (**Figure 3.3**) demonstrates dispersal of the lab environment to the columns is not high enough to cause the observed the similarity between replicate columns.

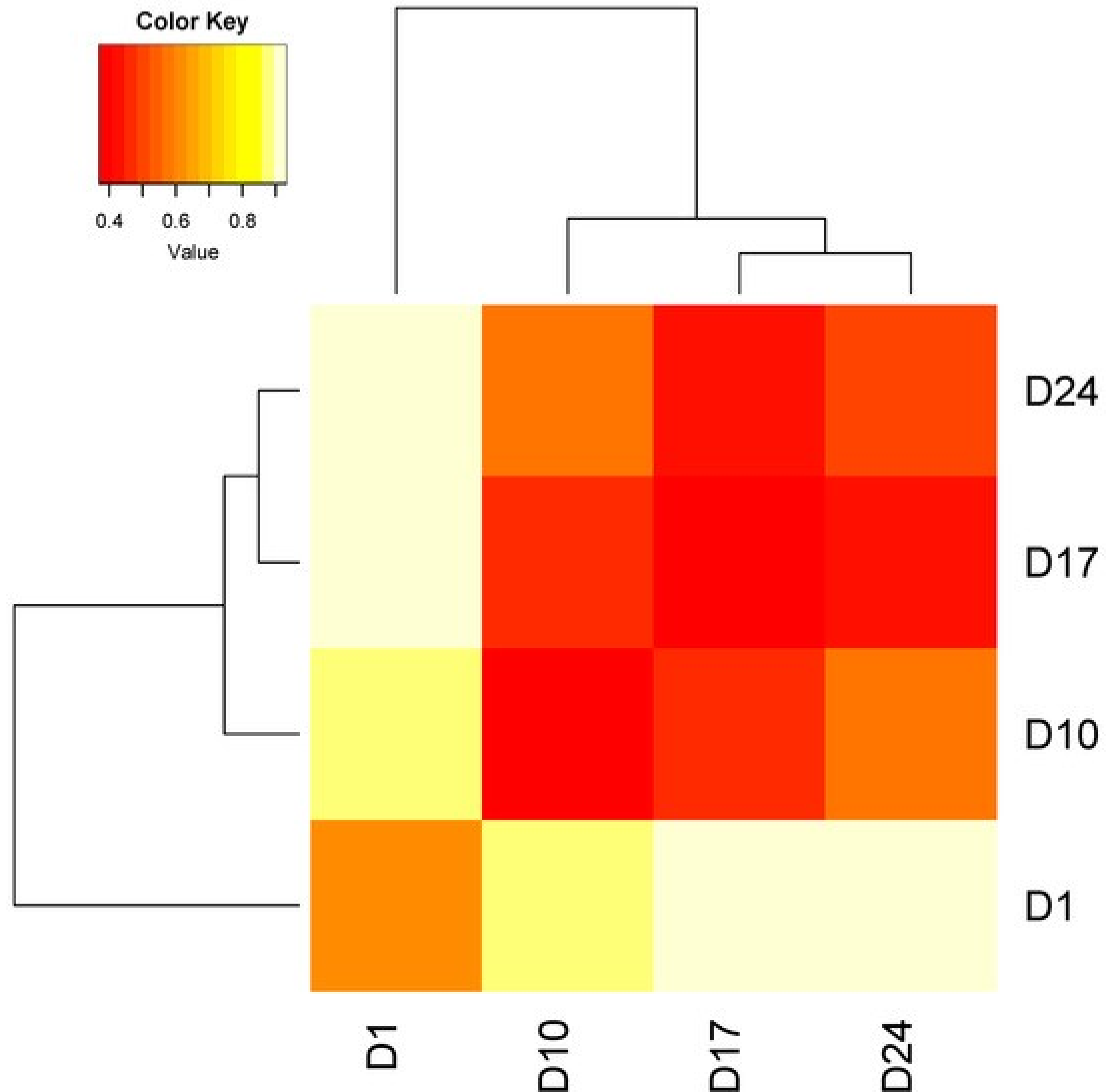

Figure 3.5. Heatmap of mean Bray-Curtis distance between time-points from stormwater inoculated columns. Colors indicate mean distances between biological replicates (diagonal) or all sample comparisons between different time-points, with red indicating more similar and yellow indicating more different. The last two time-points are as similar between time-points as within time-points, suggesting that the community is stabilizing. Labels indicate Day (D) in experiment 1, 10, 17, and 24.

Dynamics of Potential Pathogens and Denitrifying Bacteria

We also investigated changes in the functional potential of microbial communities on columns after inoculation. Using the taxonomic classification from the 16S rRNA gene sequences and a database linking taxonomy to function

(software program FAPROTAX [24]), we found 10 potential denitrifying taxa. All potentially denitrifying taxa were from *Alpha*-, *Beta*-, and *Gamma-Proteobacteria*.

Using the same method, we identified 24 potentially pathogenic taxa within the dataset. *Stenotrophomonas acidaminiphila* [38] was the most abundant potential pathogen. Interestingly, this species also has denitrification capabilities, although it was not flagged as a potential denitrifier, but rather only nitrate-respiration. Although the pathogenicity of this species has not been evaluated, some of its closest relatives are opportunistic pathogens [39,40]. *Acinetobacter johnsonii* was the second most abundant potential pathogen identified in the stormwater columns, which was on average 1.85-fold more abundant on columns than in the inoculum samples. *A. johnsonii* can be found in environmental samples [41], on the human skin [42], and associated with disease [43,44]. Other relatively abundant potentially pathogenic taxa were also classified as *Stenotrophomonas* or *Acinetobacter*. The potential pathogen OTU composition was slightly different between stormwater inoculated, and non-inoculated samples but the same *S. acidaminiphila* was the most abundant potential pathogen OTU in both (**Figure S3.3**). This suggests that this potential pathogen could have come from the lab. Other OTUs are also found in both samples but at different relative abundances.

Both potentially pathogenic and denitrifying microorganisms initially increased in relative abundance on the columns but declined from the peak by day 24 (**Figure 3.6**). The relative abundance of potential pathogens on stormwater columns was high on day 1 and was maintained through day 10

(**Figure 3.6**c). Potential pathogens increased on the non-inoculated columns at day 10 as well. In contrast, the relative abundance of potential denitrifying taxa was high on day 1 in stormwater columns but decreased immediately (**Figure 3.6**a). Non-inoculated columns showed a peak at day 10 in denitrifying taxa. Both potentially pathogenic and denitrifying microorganisms decrease in relative abundance from their peak by day 17 and 24 (**Figure 3.6**a). This suggests that these microorganisms are initially selected for under the conditions within the column, but that this selection pressure is decreased as the community stabilizes by day 24.

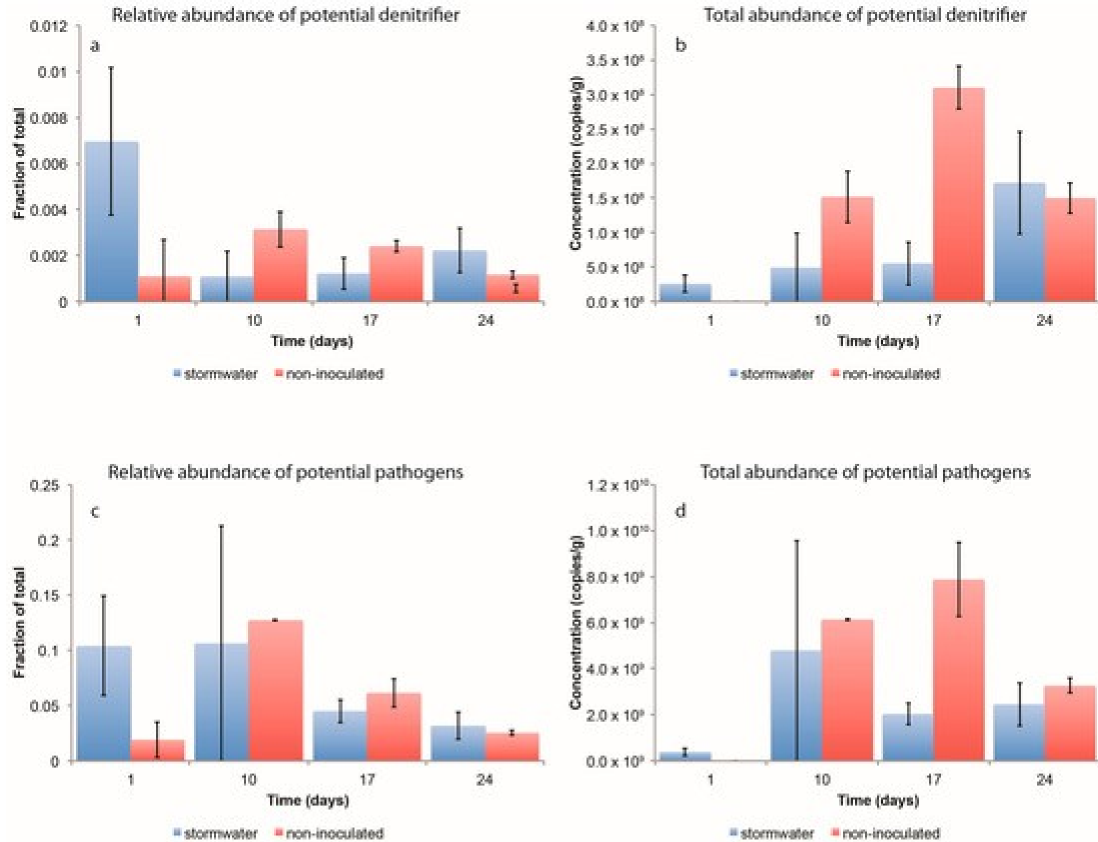

Figure 3.6. Relative (**a**,**c**) and total (**b,d**) abundance of potential denitrifying (**a**,**b**) and pathogenic (**c**,**d**) microbial taxa over time within non-inoculated, (red) and stormwater inoculated (blue) columns. (**a**) The relative abundance of potential denitrifying taxa within the community over time. (**b**) The abundance of denitrifying taxa within the sand columns over time. The total number of 16S rRNA gene copies per gram was multiplied by the fraction of the total community to provide a quantitative measure of changes of potential through time. (**c**) The relative abundance of potentially pathogenic taxa within the community over time. (**d**) The abundance of potentially pathogenic taxa within the sand columns over time. The total number of 16S rRNA gene copies per gram was multiplied by the fraction of the total community to provide a quantitative measure of changes of potential through time.

The relative abundance of potential pathogens and denitrifying microorganisms was transformed by the concentration of 16S rRNA copies to provide a quantitative estimate of total abundance. While potential pathogens made up a relatively large proportion of the total input community on stormwater columns on day 1 (Figure 3.6c), the overall bacterial cell concentration was

lower than at later time-points (**Figure 3.6**d). However, both potential pathogens and denitrifying taxa expanded within the column on day 10, reaching a maximum between day 10 and 17 (**Figure 3.6**b,d). This initial increase was not maintained, and both types decreased from their peak by day 24.

Changes in the Abundance of Denitrification Potential over Time

The concentration of the *nosZ* gene, a key enzyme in the denitrification pathway, was assessed with quantitative PCR (**Figure 3.7**). *nosZ* is the gene encoding nitrous oxide reductase, capable of mediating the conversion of nitrous oxide (N_2O) to N_2 as the final step in denitrification. The number of copies of *nosZ* increased throughout the experiment in both non-inoculated columns and stormwater-inoculated columns. The non-inoculated control samples start out with few copies of *nosZ* but become colonized with organisms containing *nosZ* genes. By day 24 after inoculation, the concentration of *nosZ* gene copies in the non-inoculated columns is greater than the stormwater-inoculated samples. While the potential denitrifying microorganisms predicted from the taxonomic classification show a decrease in the abundance of denitrifying taxa by the 24-day time-point, the trend in the *nosZ* signal shows a continuous increase in the potential for denitrification over time.

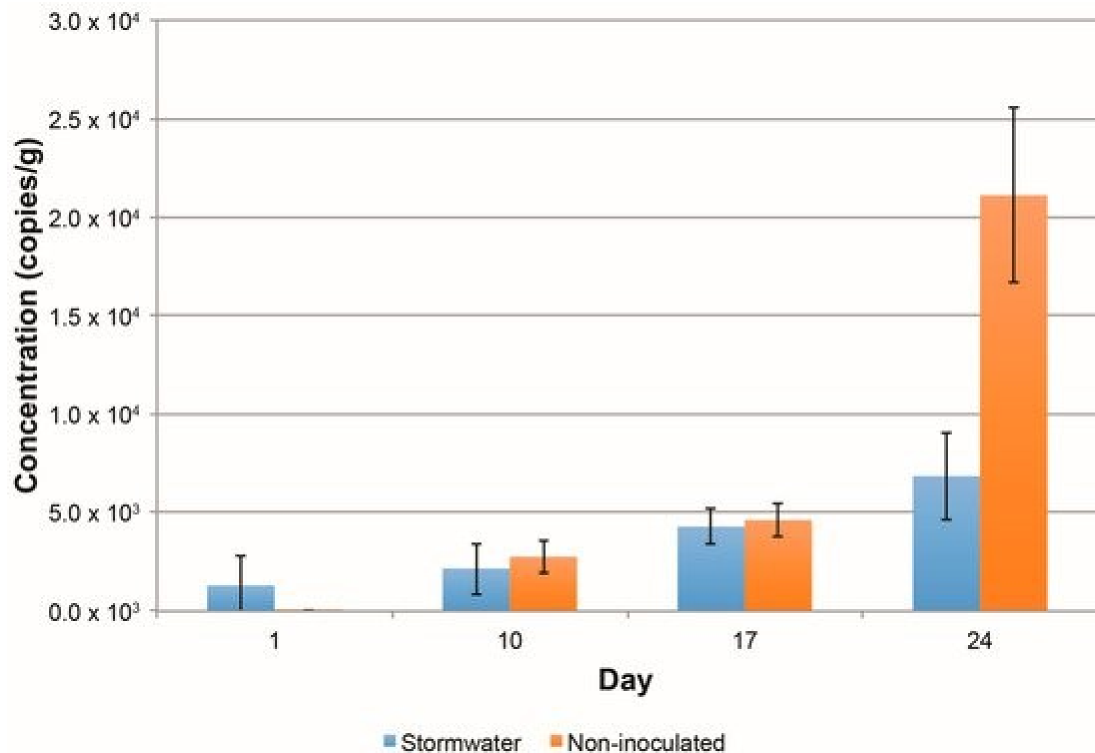

Figure 3.7. Total abundance of *nosZ* over time within non-inoculated (Neg) and environmental columns. Env-stormwater inoculated columns; Neg-Non-inoculated columns.

3.4 Discussion

This work demonstrates how neutral factors, such as drift and initial inoculum, shape the microbial community composition within idealized EIS systems in the absence of other factors influencing the microbial community. A portion of the variation in community composition across replicate columns cannot be explained by technical variability, suggesting that drift has a significant impact on community structure. However, this difference is small in comparison to the differences observed between communities on columns with different inocula. Biological replicates became more similar to each other over time, and were distinct from the source community, demonstrating that selection by the

unique conditions of the experiment allowed for the expansion of the same subset of stormwater taxa in each column. The conditions in the column transiently selected potentially pathogenic taxa but resulted in a decrease in abundance by the 24-day time-point. Column conditions continuously selected for taxa that were capable of denitrification over the 24-day experiment.

While the experimental conditions do not mimic the environmental conditions experienced by microbial communities in EIS in the field, the controlled conditions provide insight into the factors that impact community assembly. We found that neutral processes, including historical contingency and drift, can significantly influence the resulting community structure. While there has been a great deal of discussion about whether niche (e.g., environmental conditions) or neutral factors (e.g., drift, migration) dominate community assembly processes [12], both are likely to have some influence on the resulting community. A previous study of the microbial community on slow sand filters found evidence for both niche and neutral processes impacting the microbial community structure [45]. Here, we focused on the impact of neutral processes on the resulting community under identical environmental conditions. Since the distance in the microbial community between biological replicates was greater than the distance between technical replicates, drift between identical columns influenced the resulting community structure. Drift between biological replicates could be due to random fluctuations in population abundances between replicate columns or introduced by chance during inoculation. But these distances were small compared to the differences in community structure between columns with

different inoculum types. Previous work has found that environmental conditions influence the community structure of sand filters in drinking water treatment [18], although they did not separate the impact of historical contingency and migration from influent water on filter community composition, or have the opportunity to investigate replicates. Drift and historical contingency could undermine efforts to engineering specific communities to improve desired biotransformations, such as denitrification or pathogen retention. While we demonstrate that drift is not an important factor over the short term, it could become more important over the lifespan of the EIS. Future work should focus on the relative contribution of variable environmental conditions on shaping the microbial community structure compared to neutral processes over a typical life-span of EIS.

The microbial community on the EIS over time was determined by the initial inoculum, suggesting that initial seeding could be an effective mechanism for altering the resulting microbial community on EIS. The seeded microbial community can change both the chemistry and the hydrology within the system, which could have a feedback mechanism on the resulting community. Seeding the microbial community with specific microorganisms has been successful in altering the resulting community with nitrifying communities in drinking water sand filtration [46]. However, our results suggest it will be difficult to control the direction the community takes within the environment without more understanding about the selective conditions of the EIS since all experimental communities in our experiment diverged substantially from their initial state. To successfully manipulate the community, seeding the sand with a culture or

consortium with the desired function, such increased denitrification or pathogen retention and removal, would likely result in loss of the majority of the seeded microorganisms from the community. This could limit the potential impact specific strains could have within EIS. Future work is needed to determine whether the communities eventually become similar regardless of inoculum over the normal operation period of a typical EIS in the field or with the migration of other microorganisms on to the filters, as would be expected under normal operating conditions. Microbial seed cultures, like the *Pseudomonas* and stormwater communities seeded in this experiment, were an important determinant of the final microbial community structure in this experimental system. More work is needed to determine whether seed cultures could be an effective mechanism for manipulating the resulting microbial community within EIS as compared to selective pressures or high dispersal rates into the system.

Pathogenic taxa were transiently selected for within our experimental system. In this case, the most abundant potential human pathogen, *Stenotrophomonas acidaminiphila*, could also denitrify, demonstrating that conditions promoting beneficial functions for one type of pollutant (nitrogen) might negatively impact other pollutants (pathogens). The initial expansion then contraction of this population on sterile sand media suggests its role as an early colonizer in primary succession of sand surfaces.

The conditions of the column selected for the expansion of potentially denitrifying taxa, as assessed by both the presence of the *nosZ* gene and potentially denitrifying taxa. Gene abundance has been shown to correspond to

denitrification rates within certain environments but not others [7,47,48]. We did not measure the removal of nitrate within our columns with this experiment to connect denitrification potential and nutrient remediation. Denitrification is not as phylogenetically conserved as other metabolic processes [49], which might have caused the prediction tool we used based on phylogeny to miss the dynamics of *nosZ* on the non-inoculated columns. The potential for denitrification, as assessed through the concentration of *nosZ* genes in the columns, increased in both the environmental and non-inoculated samples. Interestingly, the non-inoculated columns had a higher final concentration of *nosZ* than the stormwater columns, demonstrating the importance of inoculum in shaping the structure and potential function of the microbial community. The high organic carbon content of our synthetic stormwater media could have selected for of potential denitrifiers, as denitrification potential and denitrifying populations increased with organic carbon concentrations [7]. More work is needed to determine whether this copy number difference results in a measurable change in nitrogen removal from the columns and how to influence the community toward greater denitrification under EIS conditions.

3.5 Conclusions

This work shows that replicate sand filters inoculated with the same community and incubated under controlled laboratory conditions change in a similar manner over a 24-day period. The largest changes to the community composition occur within the first 10 days, then the community changes slowly, even as growth remains constant. Columns inoculated with nothing or with a single isolate maintained a distinct community from the stormwater inoculated column communities. Potential pathogens and denitrifying microorganisms become more abundant on the columns as compared to both the inoculum and the day 1 communities, suggesting specific growth within the columns. Potential pathogens decrease by the end of the 24-day experiment as other microorganisms become more abundant. Denitrifiers continued to increase in abundance over the entire 24-day period. This work demonstrates that neutral processes of drift, historical contingency and migration have a significant impact on the resulting microbial community structure, although the impact of drift is small compared to historical contingency over 24 days in the absence of additional migration. Future work needs to be done to determine the relative importance of these processes as compared to selective pressures imposed by different chemical and physical environments in shaping the community colonizing EIS. Our results suggest that management strategies manipulating inoculum could promote lasting change to microbial community structure and function, although it may be difficult to maintain a specific community composition

within these systems unless the community is well adapted to the conditions within the EIS.

Supplementary Materials

The following are available in the SI section as well as online at https://www.mdpi.com/2073-4441/10/8/1065/s1.

Author Contributions

Conceptualization, A.N.F. and S.P.P.; Formal analysis, A.N.F., Y.Z. and S.P.P.; Funding acquisition, S.P.P.; Methodology, A.N.F. and Y.Z.; Supervision, A.N.F., E.G.S. and S.P.P.; Validation, S.P.P.; Visualization, A.N.F. and S.P.P.; Writing—original draft, S.P.P.; Writing—review and editing, A.N.F., Y.Z., E.G.S. and S.P.P.

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Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 4. Diverse stormwater communities correlated with improved initial *E. coli* removal during sand filtration[3]

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Abstract

Pathogens contaminate urban stormwater runoff, endanger human health, and are a major impediment to stormwater reuse. Pathogens enter runoff from pet and animal waste as well as sewage leaks and human sources. Pathogens are not consistently removed from stormwater using current best management filtration practices, and can remobilize from filtration systems in subsequent rain events. Microbiological processes inside stormwater filters are widely considered a “black box" because traditionally they have not been investigated. Yet, microbial processes are known to be crucial in contaminant removal from runoff. Here we investigate how variation in complex microbial community composition of water inoculating filters contributes to inconsistent pathogen removal. Specifically, we focused on whether the alpha diversity of the initial inoculum or biomass proxies of biofilms within columns are correlated to removal efficiency and subsequent remobilization of fecal indicator *Escherichia coli*. We used sand columns as the model filtration media, and synthetic stormwater to simulate rain events. Results show that filters inoculated with stormwater microbial communities with different taxonomic composition resulted in no significant difference in removal and remobilization of pathogen under the same initial chemical and physical conditions. Based on total number of *E. coli* removed, the mixed environmental communities performed better than biofilm forming isolate, *Pseudomonas aeruginosa* used as a model biofilm in many lab studies, and better than clean sterilized sand that would be found in an unripened filter. Shannon diversity index as a representative of alpha diversity showed strong

anti-correlation with initial *E. coli* removal and subsequent remobilized *E. coli*. Subsequent remobilization was significantly correlated to biofilm presence, more than biofilm diversity. These results suggest that biofilm variability can be a driver of pathogen removal efficiency variability, although factors that influence removal in complex communities are not well understood.

4.1 Introduction

Pathogen contaminated stormwater is a global problem

Fecal waste pollution degrades the environment during stormwater runoff events. There are many sources of waste including human sewage, pet waste, birds, and wildlife [1]. Pathogen concentrations are highest in places with inadequate sanitation and sewerage service and is associated with diarrheal illness and associated malnutrition across the world [2]. Fecal pathogens have been detected in water collected from a variety of urban locations and conditions, including wet and dry weather events, roadway, outfall, and rooftop locations [3]–[6]. In areas with persistent pathogen contamination, there is a significant opportunity to reduce environmental damage and potentially reuse this water source if pathogens can be consistently removed [7][8].

Inconsistent removal of pathogens from stormwater using engineered filtration systems

Green infrastructure and sand filters are implemented to reduce flooding and erosion and can remove pollutants from stormwater. Green stormwater systems are generally designed specific to location, therefore design specifications can vary substantially between installments [9]. Research to evaluate water quality treatment suitability of the filters for various contaminants

has revealed that performance for pathogen removal is highly variable, both temporally within a system, and between filter locations [9]–[11]. Remobilization of pathogens is an additional concern, meaning, an increase of pathogens in the effluent from initial concentration has been observed due to proliferation of the pathogen in the filter [12] and could contribute to inconsistent removal. There are many potential explanations for inconsistent performance, including differences in physical design properties like filter media size and filter length, and chemical properties like the presence of organic material [12]–[14]. One crucial component that is largely under-investigated but could contribute substantially to performance variability is the functional impact of bacterial biofilms that forms on the filter over time [9], [15].

Studies demonstrate biofilter microbial communities alter pathogen removal

As bacteria are ubiquitous in stormwater, they can colonize the filter media to form a biofilm under favorable conditions [16]. Biofilms can form robustly on the filters to alter performance, known as biofouling. The biofilm diversity is likely to be different across filters in the environment, contributing to performance variability. We don't know much about which biofilm taxa would be the best at removal or conditions that result in a better or worse performing biofilm [15]. Physical, chemical and biological factors influence how the biofilm community assembles [17]. Fecal pathogens can experience many interactions with other biofilm organisms in the filter which can lead to removal, growth or negligible changes depending on the interaction [15]. Many known mechanisms of the biofilm have been studied generally for colloid removal and in other water

treatment scenarios, but have yet to be investigated in stormwater filters, [9], [11] specifically with widely used sand media [18]–[20]. The processes that occur within these filters are considered a black box that must be explored to improve stormwater technology [9], [15]. Thus, the relationship between pathogen removal and biofilm biomass has not been conclusively demonstrated. It is believed that as the biofilm develops, it will not alter removal properties until the media surface is covered and the pore space between the filter media starts to be restricted [11], [15].

Biofilm microbial community composition has importance in water quality treatment, however limited connections have been made in stormwater quality scenarios. It has been determined that protozoa are important in *E. coli* predation [21], and the influence of a few other biofilm functional mechanisms have been identified as well. *E. coli* removal efficiency was different between *P. aeruginosa* strain biofilms grown on glass beads [22], suggesting that mixtures of microorganisms in a biofilm with different characteristics could be a factor in removal. Though single isolate studies [15], [22] and larger field studies with complex microbial communities have been conducted [9], [23], the assembly of organisms within stormwater biofilms and interactions with pathogens is largely unknown [15], [21]. Additional information about the biofilm, exopolymeric substance secretions by bacteria, and the influence of physical and chemical processes are included in APPENDIX D. There are many genomic and culture based methods available to investigate this microbial black box that have yet to be applied [2], [24]–[26], [6]. The differential presence and abundance of taxa in

stormwater filters can be measured using alpha diversity metrics [27] as well as biomass quantification to determine if these values correlate with removal efficiency. Major differences in pathogen removal are known to be related to physical conditions in the filter and much of the biological influence has been unknown [9]–[11], [15].

Here we show that stormwater bacterial communities from different locations grown under the same conditions do not have significant differences in removal after the communities have assembled on sand filters under simulated stormwater physical-chemical conditions. The stormwater communities removed more *E. coli* overall than a biofilm forming isolate or clean sand alone. Increases in *E. coli* remobilization in the subsequent storm event were observed but were not statistically significant. The concentration of *E. coli* in the column effluent from initial removal had a stronger absolute correlation with the alpha diversity metric Shannon Diversity Index than biomass proxies. The concentration of *E. coli* in the column effluent from the subsequent remobilization event had the strongest absolute correlation with biomass. Results connect biofilm bacterial diversity with pathogen removal and show that initial bacterial inoculum may not have as strong an influence on pathogen removal variability as community diversity and biomass.

4.2 Methods

Sampling locations and protocols

Two roadway and one rooftop communities were sampled October 24, 2017 in Baltimore, MD, USA (Roadway: N 39°19′33″, W 76°36′51″; N 39°19′34″, W 76°37′26″; Rooftop: N 39°19′06″, W 76°37′04″) and were selected as

representative communities after 16s rRNA sequencing. These communities were previously presented in Chapter 2 and were sampled to investigate bacterial community composition and functional potential associated with a variety of urban stormwater sources. Rooftop samples were collected from gutter spouts which emptied into alleys and roadway samples were collected flowing into MS4 systems. None of the locations are connected hydrologically.

Triplicate water samples were collected in 50mL Falcon tubes with no headspace and stored on ice while being transported to the lab (<1 mile drive, samples did not freeze and were able to be filtered immediately). After collection, samples were filtered through a 0.22 μm polyethersulfone filter (MilliporeSigma, Inc., Burlington, MA, USA) using a peristaltic pump. Filters were stored at −80 °C until DNA extraction. Glycerol stocks (1:1 50% glycerol: stormwater) of samples were immediately prepared at the lab after sampling and stored in -80 °C for column studies.

Column description and set up

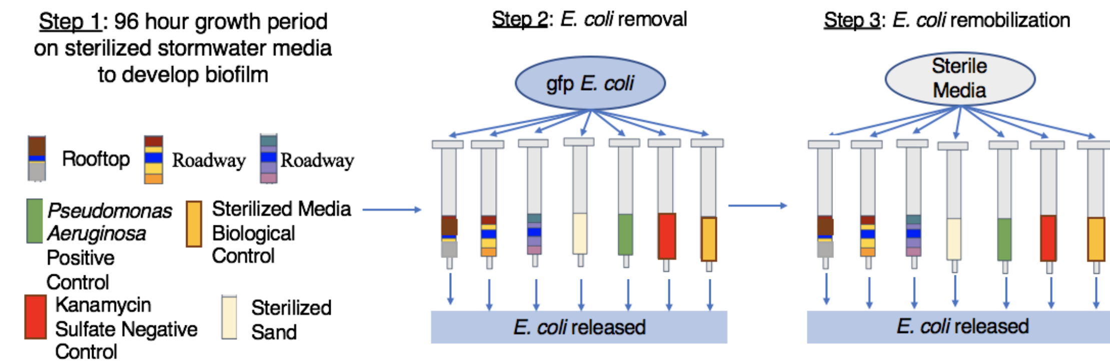

Figure 4.1 Column set up and controls. Each column type was run as triplicate, individual columns. Step 1 is the growth phase, Step 2 is the initial *E. coli* removal test, Step 3 is the *E. coli* remobilization from a subsequent storm. Green fluorescent protein (GFP) labeled *E. coli* are quantified using flow cytometry.

Columns were assembled as in previous work and a storm was simulated using the same parameters [6]. Briefly, disposable polypropylene chromatograph columns (14 cm depth, 20 mL bed volume, 1.5 cm end fitting) including a 30 µm polyethylene filter at the bottom (BioRad, Inc., Hurcules, CA, USA) were rinsed with deionized water and autoclaved. Fifty to seventy mesh sand (SiO_2, 212–300 µm) was rinsed with sterile, deionized water three times and dried for about 24 h (105 °C) and autoclaved as previously described [28]. 9.4 g (approximately 6 cm depth) of sand was packed into each column. The columns were autoclaved again before inoculation to ensure a sterile environment inside the columns.

The stormwater growth media used was the same as previous study [6], however 1g instead of 3g of yeast extract was added to encourage growth. The column types and controls include: one rooftop, two roadway communities, biofilm forming isolate *Pseudomonas aeruginosa* positive control (*Pseudomonas aeruginosa* PAO1) [29], sterile stormwater media negative control column to detect level of contamination from the lab, kanamycin sulfate antibiotic media

column to quantify *E. coli* removal with no biofilm, clean sand columns to represent a fresh sand filter. 12 columns were prepared for each column type– 3 to be sacrificed and rapidly measure biomass development through ATP quantification [30], 3 to measure the mobility of an unreactive tracer, NaBr, 3 to quantify the initial removal of model pathogen *E. coli*, and 3 to quantify the remobilization of the model pathogen in a similar, subsequent rain event without *E. coli* present. This resulted in a total of 84 columns.

Conditions for sacrificial growth study to assess biofilm development

The runoff communities and positive control were grown overnight from glycerol stocks as liquid cultures using the stormwater growth media described above. ATP quantification was used to estimate the amount of active biomass in each culture and standardize deposition onto the columns. Liquid cultures and sacrificed columns were also stored for DNA extraction. The 12 columns for each group were inoculated at the same time by the same storm simulation parameters previously used [6].

More specifically, all columns, besides the sterile sand columns, were inoculated, grouped into five sampling time-points with one column per inoculum group for time points 0, 48, and 96 hours in growth, and 9 columns per inoculum group for testing tracer removal, and *E. coli* removal and remobilization in triplicate for each inoculation type. See **Figure 4.1**. Each time-point leading up to 96hrs consisted of three stormwater columns (rooftop1, rooftop2, and roadway), non-inoculated control column of stormwater growth media, non-inoculated

control column of stormwater growth media with kanamycin sulfate antibiotic at 0.01mg/L concentration, and *Pseudomonas aeruginosa* positive control column. Paraphrased and adapted from previously described study [6], columns were inoculated every day for 4 days with an approach velocity of 15 cm/h for 1.5 h using a 24-channel peristaltic pump resulting in approximately 39 mL total volume added to each column. This simulates a common storm of 0.75 cm/h intensity and 1.5 h duration (return period < 1 year) [31] concentrated by a factor of 10, which resembles a typical bioretention area sized at 2.5% of the drainage area [23], [32]. To simulate a unit layer in a filter and ensure even biofilm coating, inocula was added to the columns then pumped forward and reverse every 10 minutes to grow an even biofilm as used previously [22] and tested in previous experiments. The top of each column served as the inlet and was uncovered. After inoculation, the first group of columns (0hrs) was collected. Sterile synthetic stormwater was added to the columns under the same simulated storm velocity and duration every day. During simulated storm events, a total of 39 mL of sterile synthetic stormwater was pipetted directly into each column intermittently to evenly wet the surface and avoid contamination. Columns were sacrificed after 48 and 96hrs before each simulated storm event to measure biomass presence before removal. Methods for measuring biofilm uniformity throughout inoculation and homogenization and subsampling columns for future analysis were preveiously tested and validated using the same methods. Through depth, the biomass varied by one degree less standard deviation than the average between top, middle and bottom of the column (SI 4.1).

Removal and remobilization conditions

After the 96 hr sacrificial growth study, the remaining 9 columns for each group were tested either by tracer to examine hydrodynamic properties, or removal and remobilization of indicator pathogen E. coli. This was conducted using the same storm parameters for growth, however only in the down-flow direction. The E. coli strain, *Escherichia coli* K12 MG1655, contains a green fluorescent plasmid so that it can be quantified in the column influent and effluent, and is resistant to the kanamycin sulfate used in our control column [33], [34].

Using a step tracer test method, the remaining replicate columns will be tested for the initial removal of *E. coli* from stormwater. The same storm flow parameters used to grow the biofilms was used for the test. Sterile stormwater media was added until at least 1 pore volume of media has been collected in the effluent Then stormwater media mixed with non-pathogenic *E. coli* strain, labeled with green fluorescent protein (gfp) plasmid at environmentally relevant concentrations (average 74 *E. coli* cells/uL) was added simultaneously to all the columns for 5 pore volumes. The gfp labeled *E. coli* was quantified using FACSCanto flow cytometer at the Johns Hopkins University Integrated Imaging Center. Conservative tracer NaBr was added in with the pathogens to determine the impact of the biofilms on the hydrodynamics of the columns [35]. Columns were frozen to be analyzed for biomass quantity and, taxa relative abundance at a later date.

Twenty-four hours after testing with *E. coli*, sterile stormwater media without E. coli or tracer was fed to the columns using the same established storm conditions. The amount of *E. coli* was quantified using cytometry (gfp). Columns were frozen to be analyzed for biomass quantity and, at a later date, taxa relative abundance.

Flow cytometry

Sample preparation: For flow cytometric analyses, samples were first fixed in 10% formalin (at least 10 min contact time). Gently vortexed CountBright^{TM} absolute counting beads (Molecular Probes^{TM}, Inc., Eugene, OR) were then mixed with the samples.

Sample analysis: Samples were analyzed on a BD FACSCanto^{TM} flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ) using a 488-nm 20-mW solid state laser for excitation. BD FACSDiva^{TM} Cytometer Setup and Tracking beads (CS&T Research Beads, BD) were used for automatic characterization, tracking, and quality control. A threshold was set on side scatter (SSC), and GFP fluorescence was detected through a 530/30 nm bandpass filter. Bacteria and beads were gated separately according to differences in light scatter, and at least 1,000 gated bead events were recorded per sample. Absolute numbers of GFP-positive *E. coli* were calculated by comparing the ratio of bead events to fluorescent cell events. All cytometric data were acquired using BD FACSDiva^{TM} software version 8.0 and analyzed using FlowJo software version 10.6.1 (BD).

DNA Extraction and 16S rRNA Gene Library Protocol

For the initial stormwater community samples, DNA extraction and 16S rRNA protocol was replicated from previous study [6]. DNA was extracted using the PowerWater kit (Qiagen). The 16S rRNA gene was amplified using primers U515F and E786R. A mock community positive control [36] and PCR negative controls were also amplified and sequenced. DNA sequencing was performed at the Genetic Research Core Facility at Johns Hopkins University.

Sequence Analysis

As previously described [6], QIIME2 platform and DADA2 were used to process the sequences. OTUs were called using dbOTU in QIIME2 for distribution based clustering. Greengenes classifier is used by QIIME to identify taxonomy. Beta diversity was measured using Unweighted and Weighted Unifrac distances were calculated in QIIME2, subsampled to 100,000 reads. PERMANOVA [37] in QIIME2, was used to determine whether community composition was statistically significantly different, using an empirical significance test with 999 permutations.

Quality Controls

As described in previous chapters, mock community of known sequence composition (positive control) was used to evaluate PCR amplification, sequencing and bioinformatics biases. Additionally, water collected from the outfall was filtered onto three different 0.2 μM filters and extracted with each

batch of environment samples to ensure DNA extraction date was not a substantial source of community variability.

Negative controls (sterile water) were extracted (during DNA extraction step) or amplified (during PCR steps) every time samples were processed. Sterile water was also flushed through the peristaltic pump between samples and collected for processing at the beginning and end of the sampling to determine the extent of contamination in the tubing or between samples.

Technical replicates for each biological sample were combined with mean-ceiling using QIIME2, where the mean OTU abundances across all technical replicates is rounded to the nearest integer. Additionally, all statistical analyses for either wet/dry or sample categories were repeated with sequence run as the category to determine if differences between sequencing runs could have contributed to the result. None of the significantly differentiated taxa or functional categories reported were found to be significantly differentially distributed across sequencing runs.

Quantification of Biomass Abundance with qPCR cycle number as a proxy

DNA extraction samples were quantified using qPCR in triplicate to best estimate proper relative cycle number which can then calculate the fold difference in the targeted gene. We calculated relative quantity of the 16S rRNA gene using primers U515F and E786R. The qPCR program is as follows: Heat, 98°C – 30 seconds; Amplify, 98°C – 30 seconds; 52°C – 30 seconds; 72°C – 30 seconds; Cool, 4°C – continuous.

Values for cycle number (Cq) value were calculated using a standard cycle time number at the bottom of curves, not mid-log, to determine relative concentration of the 16S rRNA gene. The difference in cycle time (delta Ct) was calculated compared to the lowest cycle time in the sample set for each sample. The fold difference was calculated as 1.75^(delta Ct) assuming each cycle increases with 75% efficiency as opposed to complete doubling every time.

Quantification of Biomass with ATP as a proxy

Luminultra Deposit Surface Analysis kit was used as an additional biomass proxy for rapid quantification as it uses firefly enzyme Luciferase in the presence of Oxygen gas to interact with ATP and ultimately produces light as a product [38]. Samples were sacrificed after initial deposition of microbial communities and controls, 24, 48, and 96hrs during growth, after *E. coli* removal test at 96hrs, and after *E. coli* remobilization test 24 hours after the removal test. Samples were homogenized as previous results showed uniform biofilm growth throughout the top and bottom of column (SI 5.1).

Statistical Analysis

We want to know if the differences in average removal and remobilization between treatment types is significant between the mixed communities, as well as between the mixed community columns and control columns. Without assuming normality, Wilcoxon-Mann-Whitney non-parametric test [39] was used to asses if the differences in average removal and remobilization between

treatment types is significant between the mixed community columns and control columns and between each of the treatment types.

As biomass and microbial community composition have been reported as significant to *E. coli* removal performance [15], [22], Pearson correlation analysis was performed in R to correlate quantity of *E. coli* present in the column effluent to the amount of biomass in the column and alpha diversity metric of the inoculate community.

4.3 Results

Differences between microbial community inoculum for sand filters

In order to determine whether differences in microbial communities contribute to pathogen removal variability, we chose substantially different stormwater microbial communities determined from previous investigations to be used as inocula. Two roadway samples and one rooftop sample were selected as representative communities after 16S rRNA gene sequencing. These communities were presented in Chapter 3. Roof and roadway run-off microbial communities were significantly different when using a diversity metric that was weighted by the relative abundance of each species (Weighted Unifrac). It should be noted that these samples types were not significantly different (q-value <0.05) using a distance metric not weighted by relative abundance (Unweighted Unifrac). Three samples were selected from these stormwater categories based on the dominance of different orders within the samples (**Figure 4.2**). Roadway locations were selected as they are typical locations for filtration units. Rooftop runoff filtration treatments are also utilized in some scenarios and was used as

another example of a likely location for stormwater filtration instalment. The first roadway sample is dominated by the order *Pseudomonadales*, while the second roadway sample is dominated by *Enterobacteriales*. The rooftop samples are dominated by *Rhizobales*. Samples from these stormwater categories with different dominant taxa at the order level were deemed as an appropriate set of inocula to investigate whether bacterial community composition associated with a variety of urban stormwater sources can contribute to inconsistent removal of pathogens from biofilters.

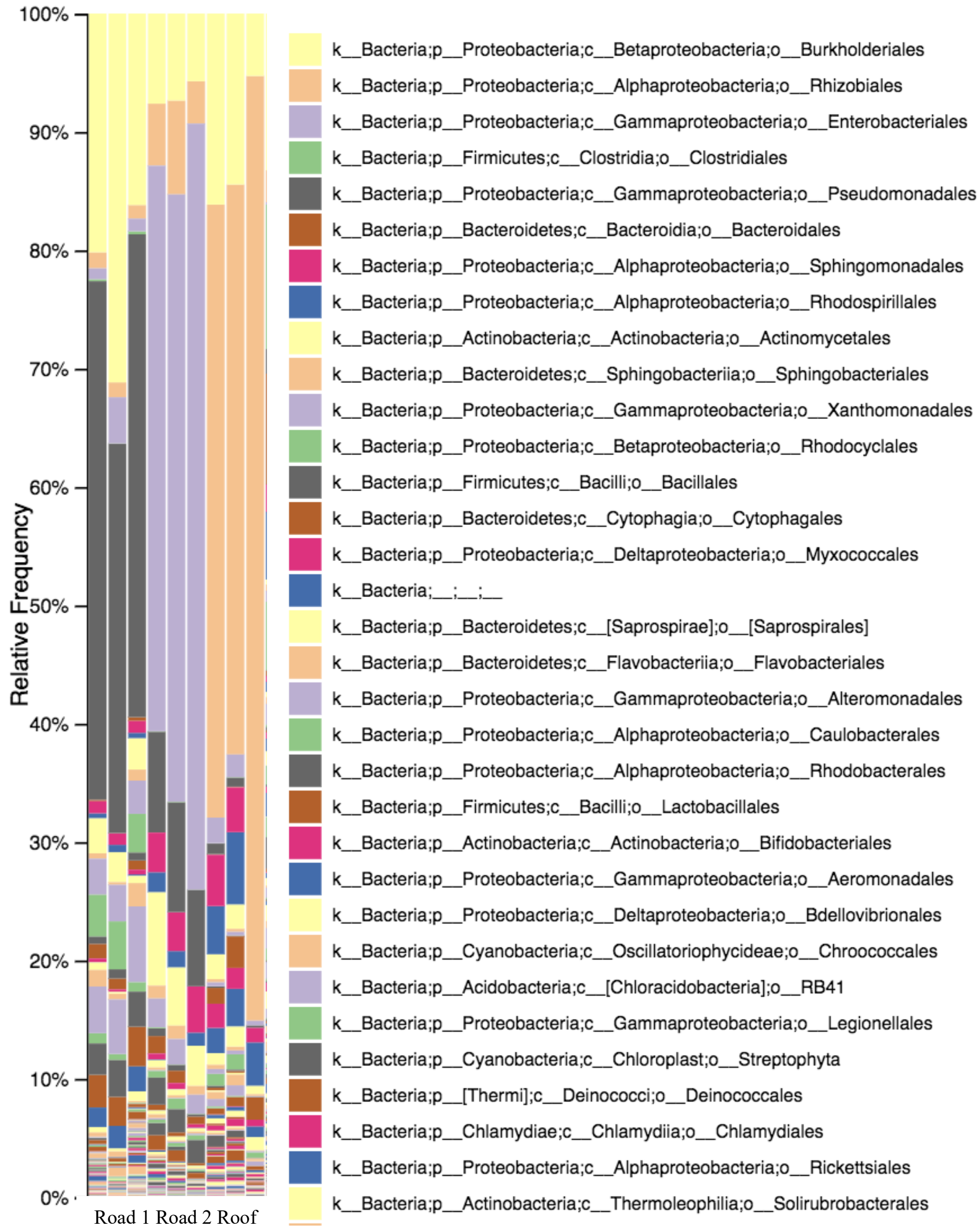

Figure 4.2 Relative abundance of order-level taxonomic assignments of OTUs from roadway and rooftop samples used as the inocula in experimental columns. These communities were stored as glycerol stocks and regrown with sterile synthetic stormwater media to simulate biofilms that colonize sand filters. Within these water samples, the dominant order of taxa between locations varies and are *Pseudomonadales*, *Enterobacteriales*, and *Rhizobales*, for Brentwood Ave, 31st St, and Maryland Ave rooftop locations, respectively.

Changes in biomass as measured through ATP quantification over time in sand columns

Microbial community growth or decay over time demonstrates the state the biofilms are in for comparison during transport tests. Significant differences in biomass or the activity of the biomass at the time of the transport tests could be other aspects of the biofilm that influence transport. During the growth phase on the columns, biomass development was monitored through ATP quantification (**Figure 4.3**). In the 96 hours of growth, biofilm development remained under 0.05g ATP/g sand, outside of one roadway sample that peaked at 0.25g ATP/g at 48 hours then fell below the initial deposited amount from liquid culture. These trends were validated by quantifying the total number of 16S rRNA gene copies in the column through quantitative polymerase chain reaction (qPCR). Despite diluting liquid cultures to standardize the amount of biomass initially added to columns, the communities were different after 96 hrs of growth, as measured by 16S rRNA gene quantification using qPCR quantitative cycle (Cq) value. These values, along with ATP as biomass proxy and initial diversity indices before growth are presented in **Table 4.1**. Shannon diversity index represents the diversity of the taxa present in the initial inoculum [27], [40], [41], while the observed number of operational taxonomic units (OTUs) in each sample represent OTUs identified by DADA2 and classified using the 99% identity GreenGenes database (52), formatted for the sequenced region. These parameters were hypothesized to influence *E. coli* transport based on previous studies [11], [15], [21], [22]. Based on volumetric porosity measurements before

removal testing and tracer test results (SI 5.3) no significant difference in porosity was detected between columns and over the week of biofilm growth on the columns.

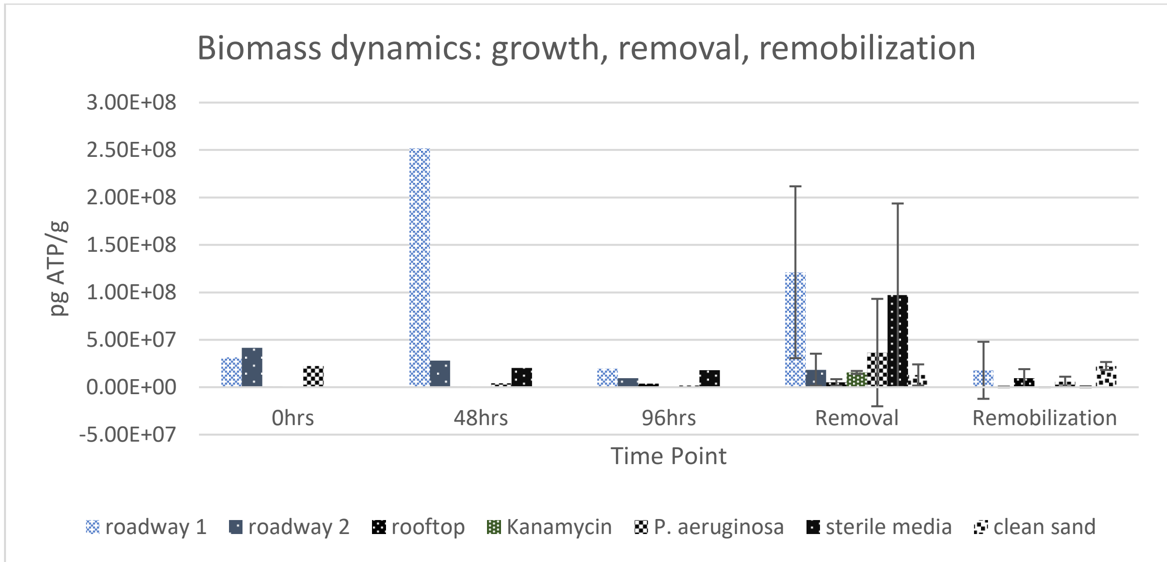

Figure 4.3 ATP measured as biomass proxy over time to rapidly quantify growth on the columns. Each point represents a single column. While there were fluctuations in these values, they appear to have stabilized after 96 hrs, before the removal test. Variability in biomass estimated by ATP concentration during removal and remobilization was related to the *E. coli* added and column homogenization process and not significantly different across column type.

Table 4.1 Biofilm parameters after 96 hours of growth, immediately before the *E. coli* removal test.

96 hr time point	Roadway 1	Roadway 2	Rooftop	*P. aeruginosa*	Stormwater media	Antibiotic media	Clean sand
Qubit ng/ul	0.377	0.163	Below detection	0.148	Below detection	Below detection	Below detection
qPCR (Cq value)	17.744 ± 0.129	20.966 ± 0.150	29.06 ± 0.115	21.253 ± 0.0878	23.398 ± 0.0420	32.39 ± 0.0723	33.1089 ± 0.469
Fold difference clean sand*	5421.005	893.361	9.639	760.759	229.116	1.495	1
Fold difference rooftop	562.393	92.680	1	78.923	23.769	0.155	0.103
Biomass (pg ATP/g sand)	19520000.00	9470720.72	3895089.28	1691176.47	17773809.52	837.66	54347.83
Shannon diversity**	10.14	5.52	7.41	1.80	3.27	0.13	0.014
Observed OTU	7165	2828	1555	43	74	18	7

*The fold difference was calculated using the difference in Cq value between samples as 1.75^(delta Cq) assuming each cycle increases with 75% efficiency as opposed to complete doubling every time.
**Values for Shannon diversity and observed OTU come from values obtained in Chapter 1 as well as [6].

E. coli Removal and Remobilization by biofilms formed from different inocula

In order to determine if communities from different locations grown under the same environmental conditions have significant differences in removal and retention of a model pathogen, we measured initial *E. coli* removal and subsequent remobilization from columns colonized by the biofilms from different inocula. The columns inoculated with stormwater from the environment (both roadway and rooftop) had the highest initial removal of all columns (**Figure 4.4**). The columns inoculated with stormwater communities compared to clean sand from a freshly installed or antibiotic control filter resulted in less total *E. coli*

remobilized during a subsequent storm event than the controls on average. Even though the rooftop community had greater than 10-fold lower 16S rRNA gene copy number abundance (high Cq value), it still provided better removal than the positive *Pseudomonas* and sterilized media control. For the initial removal, the stormwater inoculated columns released fewer *E. coli* cells than the control columns. For remobilization, there is no discernable difference between the stormwater inoculated columns and the control columns. However, the negative controls (clean sand and antibiotic media) and the columns had more remobilization than columns where biofilms were allowed to grow (stormwater community columns, *Pseudomonas*, and sterilized media biological control).

To assess the differences in removal and remobilization between the mixed community columns and control columns, the non-parametric Wilcoxon-Mann-Whitney test was used, as the small dataset with extreme high and low values does did not result in normally distributed data. The difference between the stormwater community columns and control columns was significant for initial removal ($m = 8$, $n = 1$, p-value= 2.646E-5). However, the difference between the stormwater community columns and control columns was not significant for subsequent remobilization ($m = 9$, $n = 12$, Exact p-value= 0.46391317). Pairwise comparison between the three mixed stormwater communities using the Wilcoxon-Mann-Whitney test did not detect any significant difference between the stormwater inoculated column *E. coli* removal or remobilization.

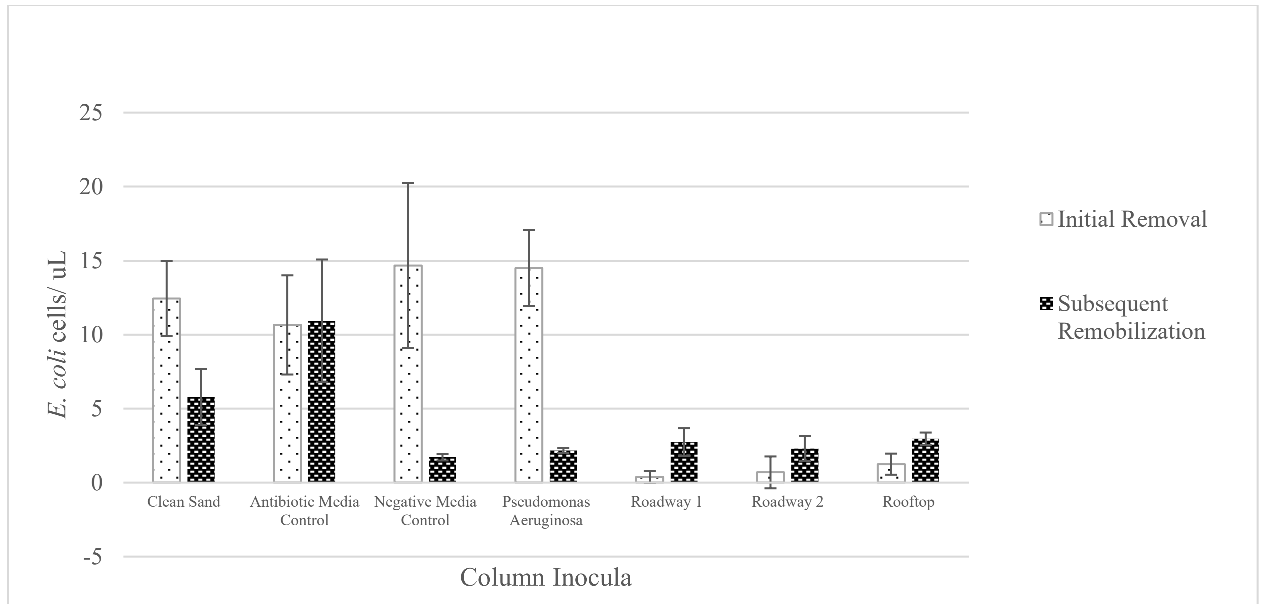

Figure 4.4 Initial removal of *E. coli* between columns show stormwater communities had significantly lower removal than controls. There was an increase in effluent *E. coli* concentration between initial removal and subsequent remobilization for the stormwater communities.

Log removal efficiency is an important parameter for measuring water treatment efficiency and regulation compliance for pathogen removal. Only the roadway locations removed over 1 log of *E. coli,* when comparing removal efficiency of each treatment (**Table 4.2**). None of the columns remobilized significantly more *E. coli* from the first removal event in the subsequent rain event, though the average quantity remobilized did increase from the initial concentration *E. coli* released for the stormwater columns.

Table 4.2 Average percent removal for each column type shown the only columns that performed at least 1 log reduction of pathogen were the stormwater innocula.

	%removed	Stdev	%remobilized	Stdev
Clean Sand	88.145	2.802	5.505	0.019
Antibiotic Media Control	89.846	3.415	10.415	0.041
Negative Media Control	86.020	5.571	1.626	0.003
Pseudomonas Aeruginosa	86.176	2.942	2.063	0.003
Roadway 1	99.645	0.399	2.600	0.010
Roadway 2	99.344	1.030	2.194	0.009
Rooftop	98.813	0.693	2.827	0.005

Correlation of Biofilm Properties to E. coli *removal*

In order to determine biofilm properties that could contribute most to *E. coli* removal, we looked for correlations between the measured biofilm properties and *E. coli* removal. First, we analyzed relationships between measured parameters to determine if they are correlated. Both qPCR of the 16S rRNA gene and ATP are used as proxies to quantify biomass of the biofilm. The qPCR quantification cycle (C_q) is the cycle number at which the fluorescence in the sample crosses a threshold value. The C_q is inversely proportional to the number of copies in the original sample, along with other factors, such as reaction efficiency and day-to-day variation. ATP quantification and qPCR C_q values are hypothesized to be inversely related, although ATP is more representative of cell activity and C_q values are more representative of the total number of cells. Shannon diversity and OTU richness are related measures of diversity, with Shannon diversity index weighting the occurrence of taxa by their relative abundance where as OTU richness does not. Shannon diversity and observed

OTUs are used to describe the diversity of the bacteria in the biomass. As previously stated, these parameters were hypothesized to be associated with removal based on previous studies, as an increase in the number of taxa increases the likelihood that an organism will thrive within the filter, form a biofilm and improve removal.

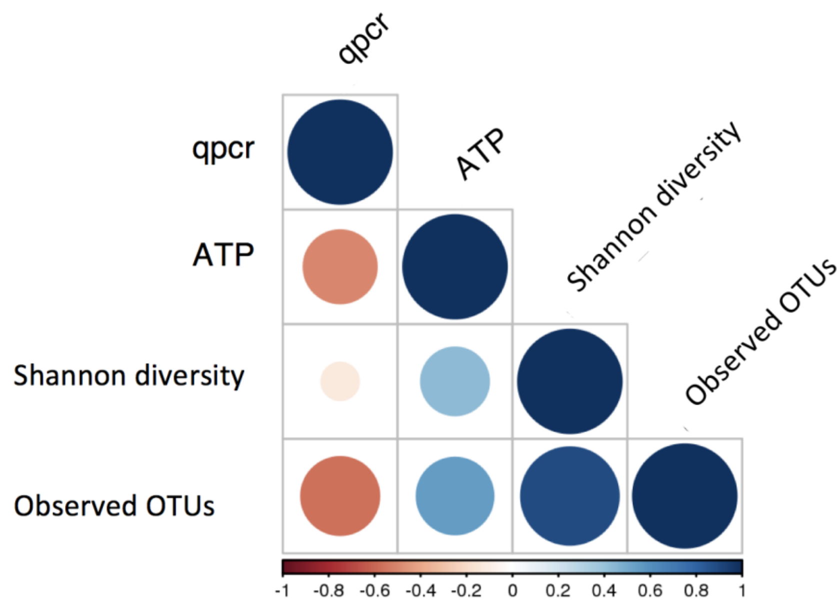

Figure 4.5 Pearson correlation between diversity and biomass parameters. We are analyzing the influence of biomass and diversity on *E. coli* removal, therefore the least related measures for both parameters were selected. The relationship between Shannon diversity and qPCR (Cq value from Table 4.1) were least related (Pearson product-moment correlation value = -0.13) and therefore selected for correlation analysis with *E. coli* initial removal and remobilization.

C_q values and Shannon diversity were selected as the least associated metrics to measure biomass quantity and diversity to help explain the relationship between biofilm biomass or diversity and *E. coli* removal (**Figure 4.5)**. The Pearson product-moment correlation value comparing qPCR and Shannon Diversity Index is -0.1254 (t = -0.21889, df = 3, p-value= 0.841), therefore they

are the least correlated diversity and biomass metrics evaluated. Those variables were then correlated to the amount of *E. coli* present in the column effluent after initial removal and subsequent remobilization (**Figure 4.6**).

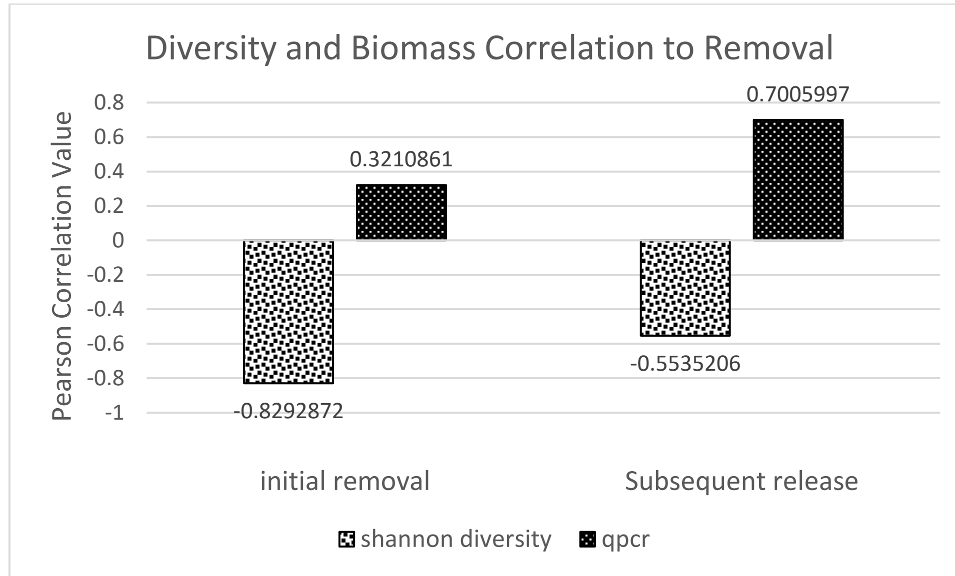

Figure 4.6 When comparing biomass proxy and diversity proxy to amount *of E. coli* initially released and the amount subsequently remobilized, Shannon diversity is significantly anti-correlated to initial *E. coli* removal whereas the amount of biomass estimated by qPCR is significantly correlated to subsequent remobilization. qPCR is represented by the Cq values presented in Table 4.1 where higher Cq values represent fewer copies of targeted DNA.

Representative metrics for biomass presence and biofilm community diversity were selected. For initial release, increased biofilm diversity was significantly anti-correlated with *E. coli* released from the columns (-0.8292872, t = -3.3183, df = 5, p-value = 0.021). Higher Cq values, which represent lower biomass quantification proxy were correlated with higher initial *E. coli* released (0.3210861, t = 0.75811, df = 5, p-value = 0.482) though the results are not statistically significant. A similar trend was shown for subsequent remobilization, though Shannon diversity was not significantly anti-correlated (-0.5535206, t = -

1.4861, df = 5, p-value = 0.1974) but Cq value was at a significant level of pvalue < 0.1 (0.7005997, t = 2.1955, df = 5, p-value = 0.07955). Overall alpha diversity before the growth and removal study was significantly anti-correlated to the amount of *E. coli* present in the effluent after initial removal and quantified biomass was significantly correlated to subsequent remobilization (**Figure 4.6**). Both increased diversity and biofilm presence were significantly correlated with improved removal. Diversity is most significant for initial release, while biofilm presence is most significant for subsequent remobilization. All correlation values and significance values are presented in SI 4.4 and SI 4.5.

4.4 Discussion

Pathogen removal from stormwater with engineered infiltration systems varies greatly from system to system, but it is unclear how much biofilms growing within the filtration media contribute to this variability. Because of the high degree of variability observed in stormwater microbial communities over space and time (Chapter 2), we wanted to investigate how much variation in microbial community inocula contributes to variability of pathogen removal. We inoculated sand columns with stormwater from different locations that had substantially different microbial community structures (i.e. dominated by different taxa). The microbial communities formed on the sand columns resulting from different inocula varied significantly in the amount of biomass present. Despite these differences, all of the stormwater-inoculated columns had a similar performance with respect to model pathogen (*E. coli*) removal and remobilization. Interestingly, less diverse columns performed worse for initial removal. Although diversity had significant correlation with removal, the amount of biomass was more significant for

subsequent remobilization. This suggests that more diverse communities have a higher potential of containing organisms that can colonize and influence removal ability for *E. coli* and possibly any other contaminant of interest. However, more work is needed to better determine the impact of microbial community diversity on pathogen removal.

We assume that columns inoculated with substantially different community composition sustain these differences over the course of the experiment. However, this may not be the case, as we did not verify how the community developed during the week of biofilm growth. Community composition was shown to change after being exposed to conditions in the lab [42], so we do not expect the community composition on the column to be the same as in the inocula. We also know from previous experiments that replicates can change in a similar way [6]. As we started with three communities that were initially different, it is possible they converged to be more similar under similar physical and chemical conditions [25] resulting in a similar transport phenotype. While this may be true, it is unlikely given the differences in biomass quantification via ATP or 16S rRNA gene copy numbers. Since we observed large disparities in the physical number of organisms present, we expect that the communities are still substantially different (**Table 4.1**).

Biomass has been suggested as significant factor associated with *E. coli* removal [22], [30] though other reports show more nuance [15]. Single isolates have been widely used to characterize the removal of *E. coli* by biofilms [15], [22], [30]. However our results show that single isolates may not be

representative for initial removal of complex community biofilms. Here *Pseudomonas aeruginosa* served as a very useful positive control, however even with introducing antibiotic that *Pseudomonas* is resistant to, there may be possible contamination and a complex, but likely much less diverse community could have developed over the experimental period. Though it was unlikely that a pure *P. aeruginosa* culture formed given the numerous OTUs detected, there was likely exponentially less diversity compared to the number of OTUs detected in the stormwater communities. Despite the significant differences in initial removal between the single isolate positive control and stormwater communities, remobilization results were not significantly different between them.

Diverse communities have been shown to be important to *E. coli* and other contaminant removal under various conditions but have not been extensively investigated [21], [43], [44]. The presence of eukaryotic microorganisms (carrying the 18S rRNA gene), such as protozoa, have been shown to consume *E. coli* and contribute to removal within filters [21]. This was not explored in this study but extracted DNA is available for exploration into the diversity and potential of eukaryotic organisms to interact with *E. coli* in a future study.

In long-term sustainability studies over an 18-month period, infiltration systems in the field are believed to stabilize in removal efficiency. Conventional bed media initially achieved a mean of 72% removal efficiency for *E. coli* O157:H7 strain B6914 [23]. The removal efficiency improved over time, achieving 97% or higher efficiency after six months. The trapped B6914 cells died off rapidly between runoff application events. The improved removal efficiency was

believed to be due to mechanistic straining over time as the pores clogged as well as an observed increase in indigenous protozoa from the stormwater. Our results were collected over the initial growth period and tracer test results do not detect significant differences in pore volume change from mechanical pore volumetric test and tracer test (**SI 4.3**).

Results of this study suggest the disproportionate influence of a small number of similar taxa between inocula on initial removal, as the most abundant organisms varied between inocula. Alternatively, there may be redundant functions present between different dominant taxa resulting in similar function from different communities. The communities that remained on the column were not evaluated for this study and can be investigated to interpret the insignificant difference in *E. coli* removal associated with inocula taken from different stormwater locations.

4.5 Conclusions

Here, we investigated whether differences microbial community composition in the inocula could contribute to variable or inconsistent pathogen removal in sand filters. Three environmental communities (from rooftop and roadway run-off) dominated by substantially different taxa were used to inoculate sand filters. Columns from three environmental inocula performed better than clean sand and single isolate columns for initial removal and had similar remobilization rates. Taken together, diverse stormwater community biofilms retained more pathogens than a single isolate or clean sand. Differences in inocula based on location did not result in significantly different removal

efficiency. Biomass had the highest absolute correlation with subsequent remobilization, and Shannon diversity had the highest absolute correlation with initial removal results. More work is needed to determine whether the biofilm communities in this study at the time of *E. coli* challenge were significantly different and, if so, to repeat the findings with a larger diversity of inocula to determine if diverse microbial communities biofilms have a consistently better performance than the individual isolate biofilms.

Maintenance and engineering practitioners have struggled to control biofilms for optimal filtration removal, dealing with biofouling and clogging, scraping and even bleaching the schmutzdecke. The influence of biofilm diversity and the potential to improve removal could be expanded on to optimize filter function.

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Chapter 5. Conclusions

The overall objective of this research was to assess the influence of stormwater microbial ecology on sand filtration systems and contaminant removal. Microbial communities colonizing the filtration media are known to influence colloid removal, though it is currently challenging to control biofilms for improved contaminant removal. To investigate characteristics of bacterial biofilms within filtration systems that influence pathogen and nutrient removal, three objectives, summarized below, were developed. The overall hypothesis of this thesis is that stormwater microbial communities found across potential stormwater treatment locations (roadways, rooftops, outfalls, etc.), contribute to the variability in contaminant removal efficiency, specifically for pathogens. After assessing the spatial and temporal dynamics of microbial community composition across urban waterways, samples representing the breadth of variability in community composition were used to inoculate experimental filters to determine how community composition impacts pathogen removal. Overall, we found that increased community diversity was correlated with improved initial removal and biomass was associated with reduced remobilization of a model pathogen.

5.1 Community composition, diversity, and pathogens within urban stormwater

In Chapter 2 we investigated the diversity of microorganisms found in urban waters and compared them to previous findings. We found that taxa known to be stormwater-associated were not specific to stormwater types, but found more broadly across urban waterways under both wet and dry environmental conditions. The biggest variation in community composition of environmental samples could be explained by sampling conditions (i.e. wet or dry weather), but only when using diversity metrics without relative abundances. Samples tended to cluster by source type (roof, road, outfall and stream) better when not considering relative abundance metrics. Additionally, road and roof run-off samples tended to look more similar to each other compared to outfall and stream samples. Potential pathogens and nutrient remediation functions, inferred from amplicon data, were found to be highly variable between samples, even of the same type. Hydrocarbon degradation and dark iron oxidation was found at higher relative abundance in wet than dry samples.

The results of this work provided insight into the sources of pathogens and changes in bacterial community composition across the urban waterways. While a number of studies have shown that fecal indicators tend to increase during wet weather, few studies have looked at how wet and dry conditions impact community composition and pathogen diversity of baseflow from an outfall. We found all locations contained some degree of pathogen pollution. The dynamic changes in the bacterial community structure that might serve as an inoculum for a stormwater filter, could influence filter performance and receiving water health.

Increased understanding of taxonomic and functional changes in urban water sources improves our understanding of the factors that need to be considered when implementing infrastructure management practices specific to bioremediation.

5.2 Stormwater biofilm community growth in experimental sand filtration columns

In Chapter 3, we investigated the growth dynamics of natural bacterial communities within experimental sand filter systems. We determined that stormwater inoculated columns did not resemble the initial inoculum, suggesting substantial growth within the column, or the non-inoculated columns, demonstrating that laboratory contamination was not a substantial driver of community composition. Environmental columns remained distinct from negative columns despite some contamination. 16S rRNA and *nosZ* genes were quantified in the columns over time to estimate trends associated with overall biomass and specific denitrifier activity. Potentially pathogenic and denitrifying organisms decrease in relative abundance from their respective peaks, but increased in total abundance over the month-long incubation period. One of many genes in the denitrification pathway, nitrous-oxide reductase (*nosZ*) increased over time along with the total abundance of potential denitrifiers. Increases in total pathogens as well as denitrifiers suggests that removal efficiency of pathogens and nitrogen could be impacted by community composition. Results inform the design of mixed community biofilm filtration

studies, which are uncommon but necessary to better understand the function of filtration systems deployed in the field.

5.3 Influence of stormwater biofilm inocula on *E. coli* removal from experimental columns

In Chapter 4, experimental columns inoculated with environmental communities from different stormwater runoff locations outperformed clean sand and other controls at initial *E. coli* removal, but were more similar with respect to remobilization. All stormwater community biofilms retained more pathogens on the column than biofilm forming isolates. Rooftop and roadway stormwater runoff communities have similar pathogen removal and remobilization performance, despite having significantly different community composition when originally collected. We found that Shannon diversity had the highest absolute correlation with initial removal, which was statistically significant, and total biomass as measured with quantitative PCR had the highest absolute correlation with subsequent remobilization, though barely statistically significant. These results suggest that the diversity of the filter inoculum may influence filter performance for *E. coli* and other pathogen removal, although it is unclear whether this trend would hold over long operational periods or other sources of inocula.

It is important to consider, these results were obtained under controlled environmental conditions that are not meant to completely reproduce environmental conditions that would be naturally occurring in the field. There are a wide range of microbial and biogeochemical conditions relevant to field conditions that would need to be investigated before the results of this work can be broadly applied or implemented in the field. Continuously adding

microorganisms over time to the column or the re-addition or colonization of microorganisms in a subsequent rain event or from continual inoculation were not investigated. Sequencing the 16S rRNA gene is only one method for estimating the taxonomic composition of the microbial community and does not directly represent the activity or function of organisms present. The eukaryotic community including macroorganisms such as invertebrates, worms, and plants also influence stormwater systems, but were not investigated here.

5.4 Practical Significance

From a fundamental standpoint, this research expands upon model biofilm removal studies conducted with single isolates, suggesting that changes based on single community members may be masked by properties of diverse communities. Engineering practitioners are beginning to incorporate biological methods, such as antimicrobial media and disinfectants [1], [2], into field installments, though the practice is not widespread. With increased regulation of biological contaminants, more attention is needed from practitioners as to what is most effective. Biofilms are largely regarded as a nuisance associated with biofouling. Also known as “gutter slime”, scraping the top layer and even bleaching filters have been used, but these methods could be refined as more is learned about the potential influence of microorganisms in biological filter performance metrics.

By improving the biological function and overall engineering of stormwater filters, public health outcomes can be improved. Pathogens and contaminated runoff have caused significant damage to water quality globally. With improved

and more efficient and effective techniques, widespread application of these technologies might be achieved. These initial result assessing biological function of these filters can be built on for removal of additional pathogens, plants that are connected to microbial communities and can improve filter function, the potential for water reuse, and considering the connections that stormwater runoff and treatment have to gentrification [3], [4], abuse [5], war and water conflict [6].

5.5 Future work

This work can be continued and taken in a number of different directions. The most relevant next objective to pursue would be to develop a biofouling framework that results in additional control to improve biofilm management, by connecting and working with designers and practitioners. With connections to practitioners, depth profiles of *in situ* filters can be determined to monitor biological function throughout the filter, with specific attention to biofoilung maintenance such as scraping and bleaching. Additionally, schmutzdecke function could be noted and analysis of filters in the field could be conducted under relevant environmental conditions. Evaluating filters and biofilms in different environments, with different pathogens and filtration media, would be necessary to optimize performance across locations. Citizen science stormwater monitoring and installments are gaining popularity, in which ordinary citizens assist in sample collection, analysis, and publication of results, as well as tailoring research questions to a community driven public health approach. In this way, important stakeholders are engaged and involved in the project from

assessing the community needs and problem definition, to study or installment design and implementation, as well as evaluation, redesign and maintenance. It is crucial that we improve management and water quality of stormwater as an important resource for reuse and take advantage of this opportunity to advance public health and reduce the environmental and health burdens of mismanagement and pollution.

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APPENDIX A: Chapter 2 Supplemental Information

Supplemental methods

Replicates, negative and positive control analysis

Sterile water or reagents were processed along side environmental samples to identify contaminants generated during sample collection, DNA extraction or library preparation (negative controls). A total of 14 negative laboratory controls were extracted and sequenced, 7 of which had fewer that 5,000 reads associated with it, while the other 7 had from 5,000-3,188,436 reads. The microbial community composition of the negatives that were above the sequence read cut-off of 100,000 reads used in the analysis cluster distinctly from environmental samples with Bray Curtis, Jaccard and Unweighted Unifrac distances metrics, but form a broad cluster using the Weighted Unifrac metrics in principle coordinates analysis (Fig. S1).

A mock community sample comprised of DNA extracts of 16S rRNA genes cloned into E. coli and mixed together in various proportions as previously described (dbOTU paper) was also sequenced. The observed read count in the mock community was correlated to that expected from the input concentration of each mock community sequence variant identified in the final OTU table (Pearson's product moment correlation coefficient = 0.75) for templates without primer binding site mismatches. Considering all templates, the correlation coefficient was 0.61. This suggests that the protocol is working as expected, and amplifies templates in proportion to their concentration in the DNA extract, barring known GC content and primer binding site mismatches.

We also evaluated whether the results could be explained by differences between the three sequencing runs used during analysis. Taxonomic and functional analysis with ANCOM analyses on Wet/Dry categories were repeated using Sequence Run category and any result that are identical were not considered further.

We also sequenced technical replicates for many biological samples. The average distance between technical replicates was substantially smaller for technical replicate than biological replicates taken on different days or at different locations for the same sample type. This demonstrates that the variability introduced by the sample preparation was small compared to the variation produced by the biological variability of the samples.

Supplemental Figures and Tables

Table S2.1. Spatial and temporal sampling scheme

	Location	Sample dates	Sample number	Wet/dry	Extraction date	Sequence run
Rooftop	N 39°19′35″, W 76°36′37″; N 39°19′06″, W 76°37′04″	6/30/16 8/16/16 10/24/17	12	wet	6/30/16 8/16/16 7/6/17	1,3
Roadway	N 39°19′36″, W 76°36′36″; N 39°19′33″, W 76°36′51″; N 39°19′34″, W 76°37′26″; N 39°19′33″, W 76°37′29″	10/24/17	12	wet	7/6/17	3
Outfall	N 39°19′36.172″, W 76°37′32.355″	6/30/16 8/16/16	6	wet	6/30/16 8/16/16	1
Outfall	N 39°19′36.172″, W 76°37′32.355″	6/2/16 6/9/16 6/15/16 9/29/16	12	dry	6/2/16 6/30/16 9/29/16	1, 2
Stream	N 39°19′36.172″, W 76°37′32.355″	8/16/16	3	wet	8/16/16	1
Stream	N 39°19′36.172″, W 76°37′32.355″	6/9/16 6/15/16	6	dry	6/2/16 6/30/16	1

Table S2.2. Dominant taxonomic group at each taxonomic level across sampling categories

Sample type	**Kingdom**	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
Rooftop (wet; n=4)	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	*Enterobacteriaceae*	*Enterobacteriaceae*	unknown
Roadway (wet; n=4)	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	*Oxalobacteraceae*	*Massilia*	unknown
Outfall (wet; n=1)	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	*Oxalobacteraceae*	*Massilia*	unknown
Baseflow (dry; n=4)	Bacteria	Proteobacteria	Clostridia	Clostridales	*Lachnospiraceae*	*Bacteroides*	*Faecalibacterium prausnitzii* (Firmicutes)
Stream (wet; n=1)	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	*Comamonadaceae*	unknown	unknown
Stream (dry; n=2)	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	*Comamonadaceae*	*Comamonadaceae*	unknown

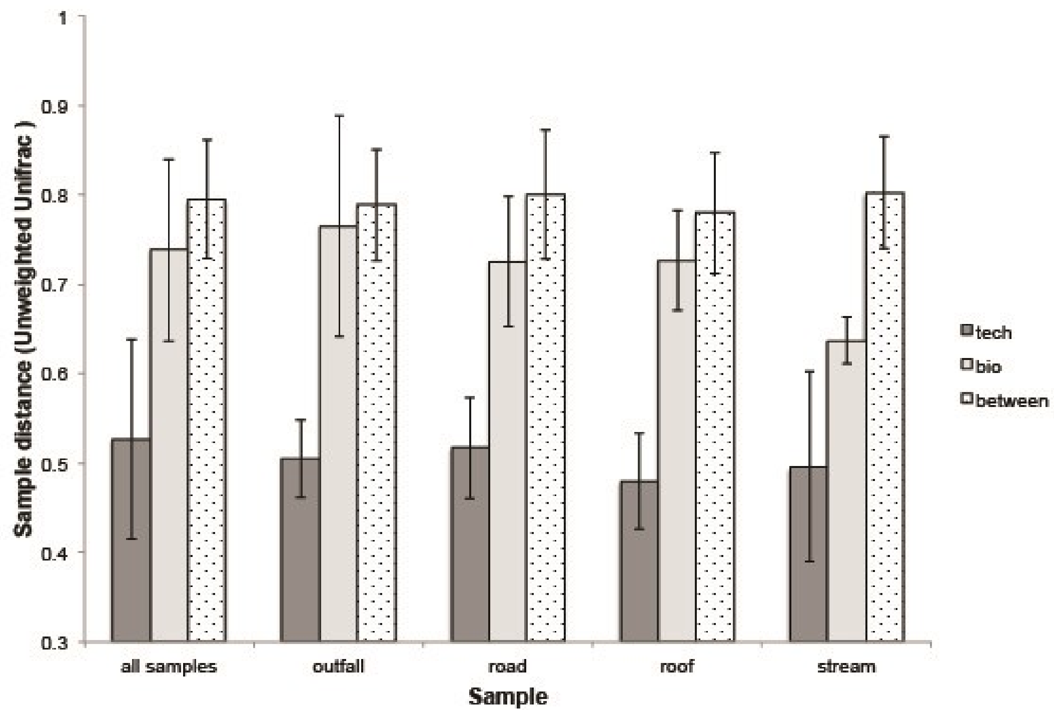

Figure S2.1. Average distance (Unweighted Unifrac) between technical and biological replicates compared to the average distance between samples types. Technical (dark gray bars) replicates were samples that were divided into 2-4 replicates after sample collection. Biological (light gray bars) replicates are samples collected from the same sample type, but potentially at different locations or times. The average distance between sample types (white dotted bars) is a comparison between samples of different types. The averaged value represents the average of all possible pairwise comparisons within each category, and error bars represent the standard deviation. The average distance between biological replicates is lowest for the stream and then rooftop samples. The average distance between biological replicates of outfall and roadway samples is not significantly different from the average distance between sample types, demonstrating that the difference in community composition between biological replicates of these samples is as large as the difference between different sample types.

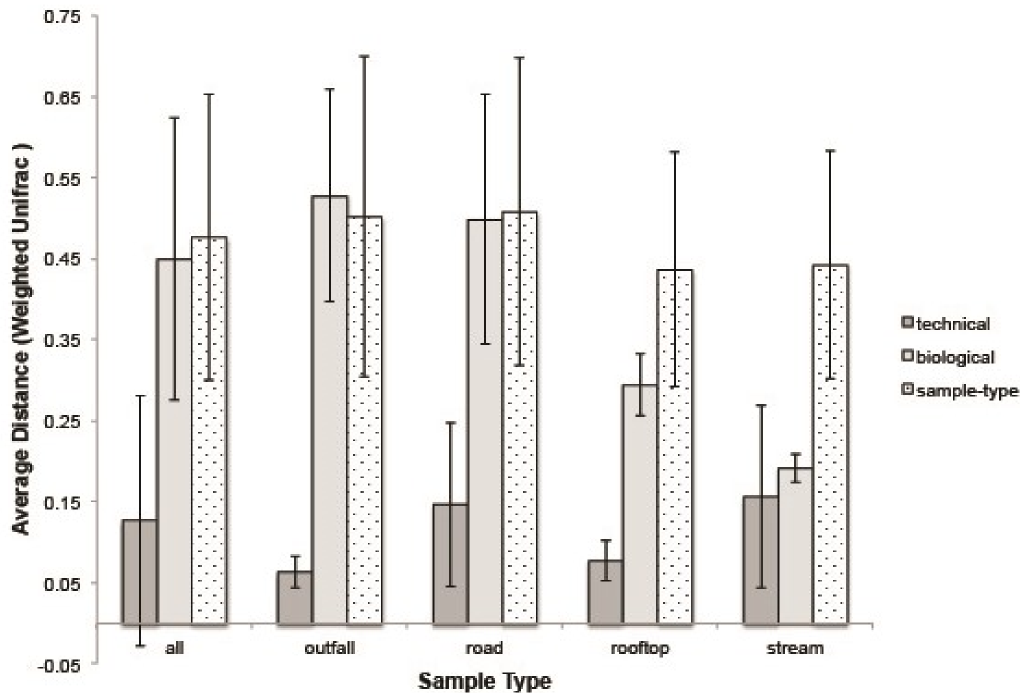

Figure S2.2. Average distance (Weighted Unifrac) between technical and biological replicates compared to the average distance between samples types. Technical (dark gray bars) replicates were samples that were divided into 2-4 replicates after sample collection. Biological (light gray bars) replicates are samples collected from the same sample type, but potentially at different locations or times. The average distance between sample types (white dotted bars) is a comparison between samples of different types. The averaged value represents the average of all possible pairwise comparisons within each category, and error bars represent the standard deviation. The average distance between biological replicates is lowest for the stream and then rooftop samples. The average distance between biological replicates of outfall and roadway samples is not significantly different from the average distance between sample types, demonstrating that the difference in community composition between biological replicates of these samples is as large as the difference between different sample types.

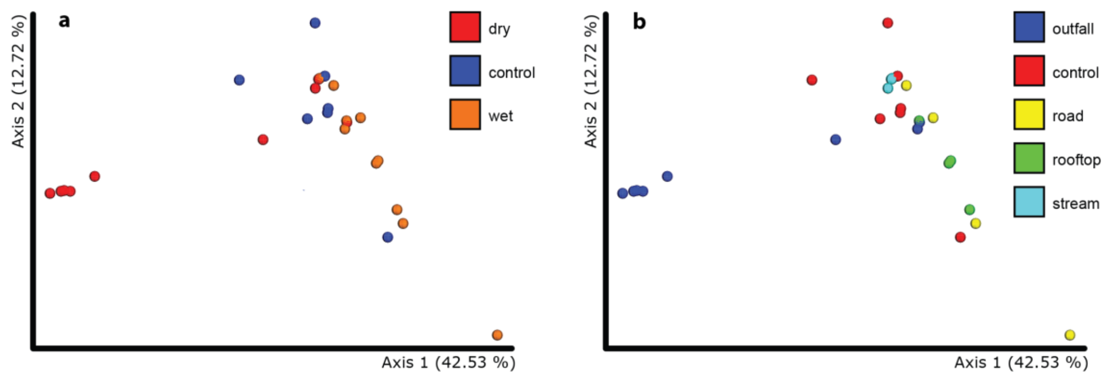

Figure S2.3. Principal coordinates analysis of microbial community composition using Weighted Unifrac distance metric, colored by a.) collection category (wet/dry/control) and b.) sample type. Ordination is identical between (a) and (b), but samples are colored according to categories. The first two axes of ordination explain 55.25% of the variation, but samples of the same type do not cluster based on either weather events or sample type.

APPENDIX B: Chapter 3 Supplemental Information

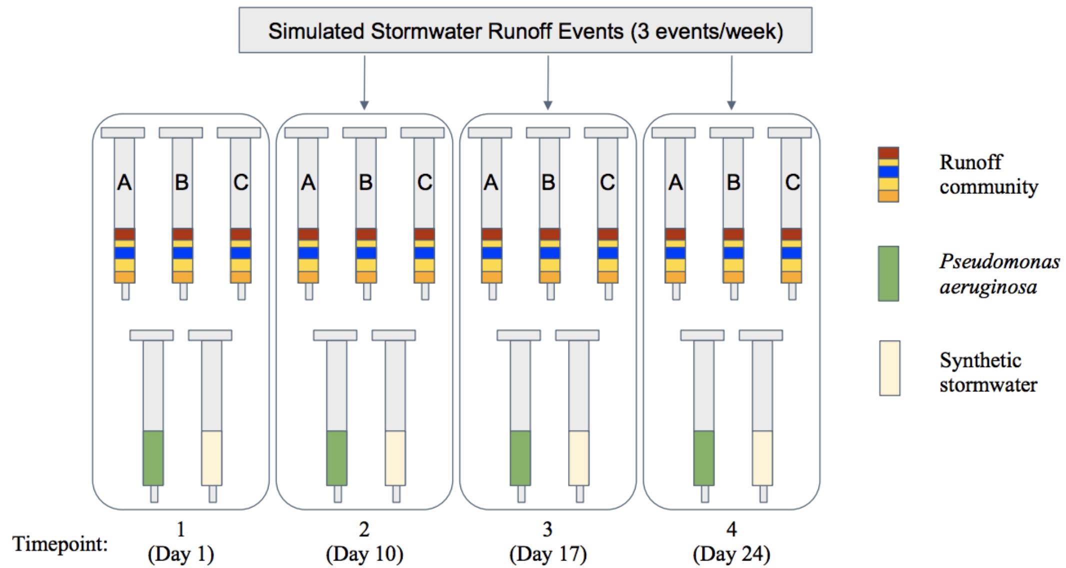

Figure S3.1. Diagram of experimental plan. A total of 20 columns were analyzed over a 24- day period. On day one, twelve columns were inoculated with stormwater run-off (multi-colored columns), four were inoculated with Pseudomonas (green) and four with sterile synthetic stormwater media (cream). After inoculation, day 1 columns were analyzed. Synthetic sterile stormwater was added to the remaining columns 3 times per week to simulate storm events. The columns were analyzed on day 10, 17 and 24 for microbial community analysis and gene quantification.

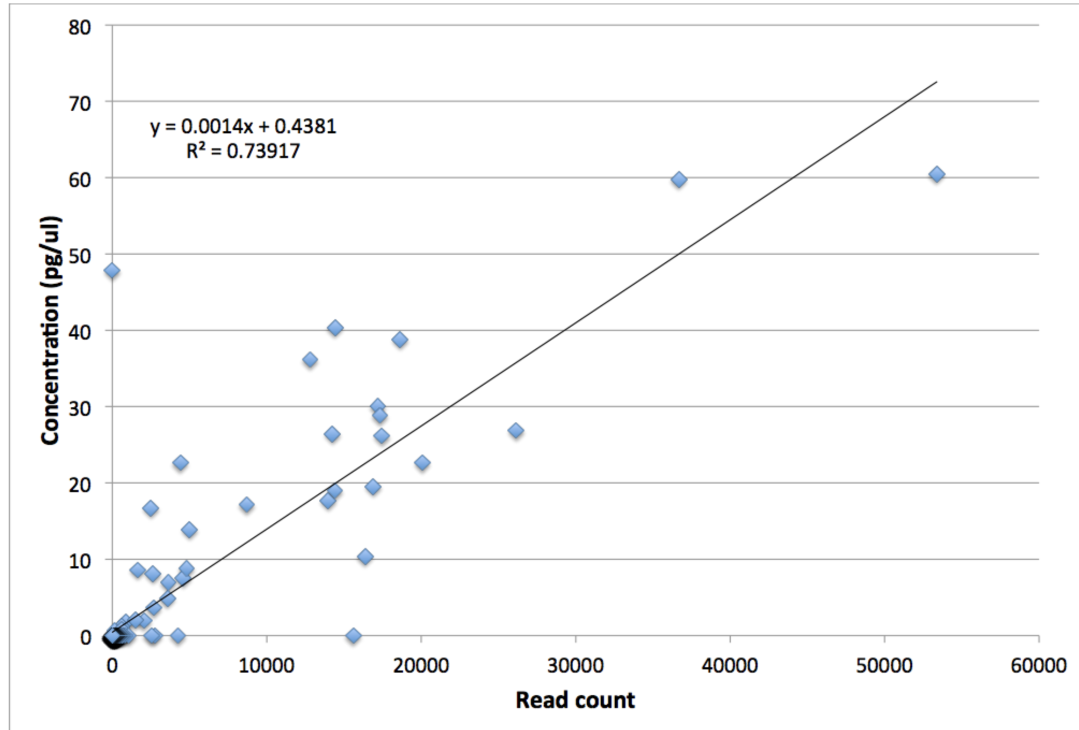

Figure S3.2. Relationship between input concentration and resulting read count for mock community sequences. Mock community templates were quantified and added together. The resulting total number of reads in the sample correlated to the input concentration for most templates. One template was not observed in the final dataset because it was flagged as a chimera and removed. Another sequence was not a 16S rRNA gene sequence and was removed during subsequent analysis. By analyzing the relationship between the input concentration and resulting read count, we adjusted the analysis parameters to improve correlation (R2 18 =0.88).

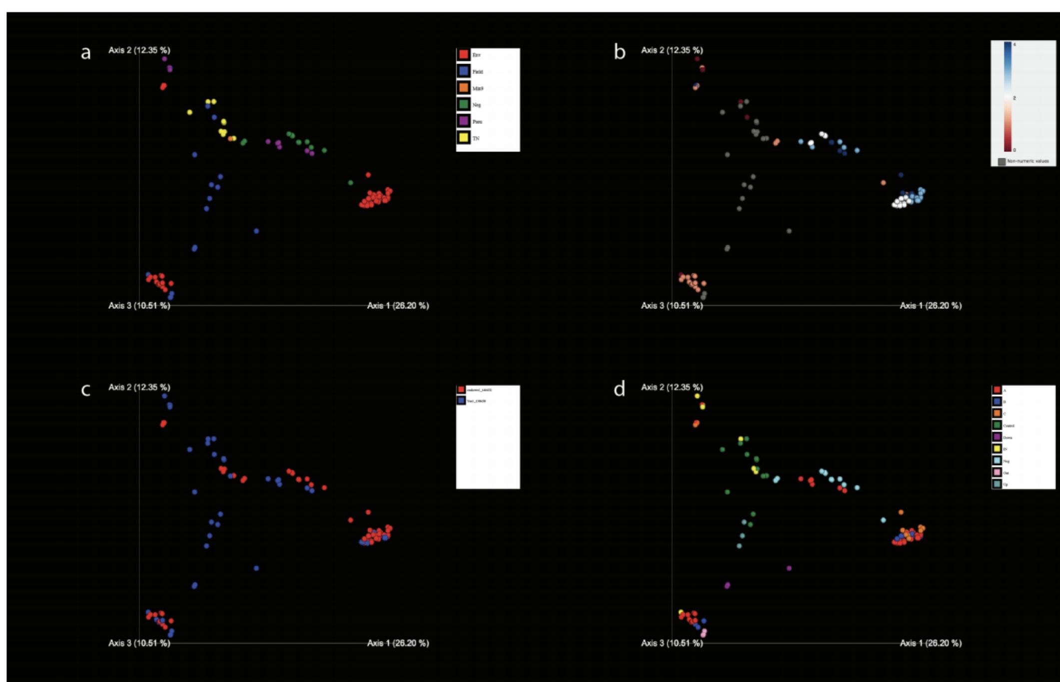

Figure S3.3. Principle coordinates analysis of all samples colored according to various categories. a.) Colored according to sample type, including column samples (Env, red; Pseu, purple; Neg, Green), environmental samples (Field, blue) and controls (TN, yellow). b.) Colored according to time after inoculation. Input samples in dark red (inoculum), column day 1 samples in pink (week 1), after 10 days (week 2), 17 days (week 3) and 24 days (week 4). Field samples and negative controls not added to the column are in gray. c.) Samples colored according to the two different batches of samples processed and sequenced together. The environmental samples cluster into distinct groups that cannot be explained by batch effects. d.) Samples colored according to replicate description (A, B or C) or field type (Up, Down, Out). Replicate columns were arbitrarily assigned A (red), B (blue) or C (orange), although this designation only describe true biological replicates within week and column type. Control (green) includes positive and negative controls. In (yellow) indicates input inoculum for the environmental, negative and Pseudomonas columns. Up (light green) and Down (purple) indicate field samples taken around the outfall and are distinct from the outfall samples (Out, pink) used on the columns.

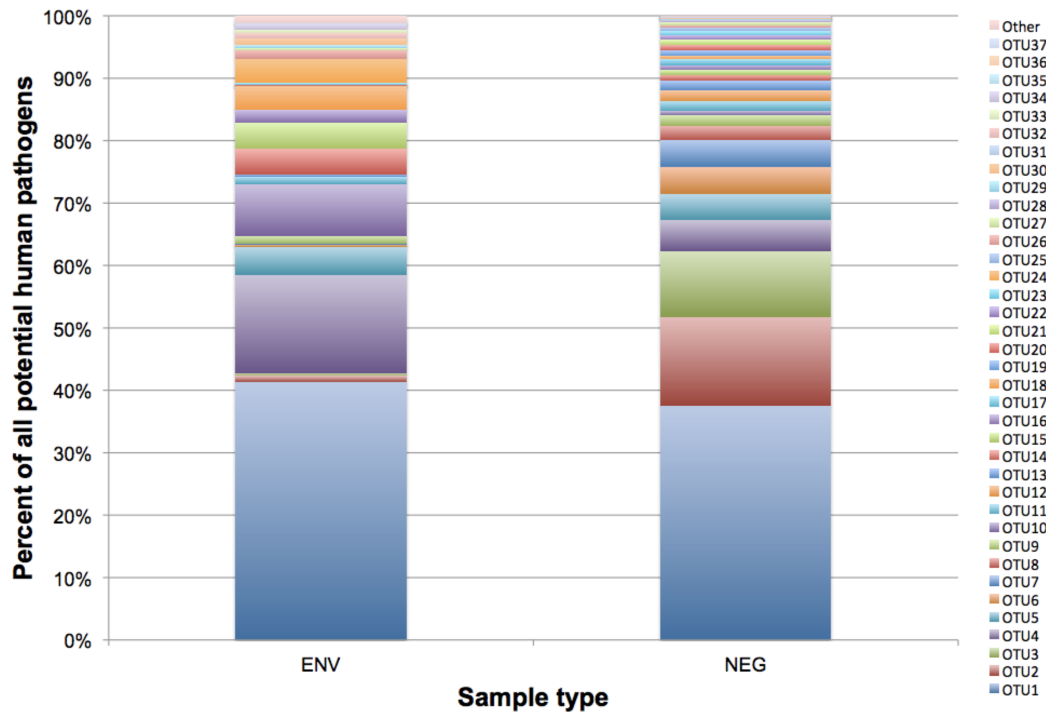

Figure S3.4. Potentially pathogenic OTUs in stormwater inoculated columns (ENV, left) and non-inoculated columns (Neg) across all time periods. The relative abundance of all OTUs with taxonomic classifications identified as human pathogens by FAPROTAX across all time periods are displayed. OTU composition is slightly different between inoculum types, although the groups share many OTUs. The most abundant OTU (OTU1) is similar to Stenotrophomonas acidaminiphila, suggesting that it may have come from the laboratory environment, rather than directly from stormwater. Other OTUs more common in the stormwater columns than the non-inoculated columns (e.g. OTU4, OTU10, OTU14) are classified as Acinetobacter and could have originated from the stormwater community.

APPENDIX C: Chapter 4 Supplemental Information

Table S4.1. ATP top mid bottom of test columns before homogenization. Total ATP by Column Location (pg ATP/g sand)

	Roadway 1		Roadway 2	
	average	stdev	average	stdev
top	1.04E+05	2.19E+04	4.81E+04	5.72E+03
mid	1.06E+05	5.33E+04	7.51E+04	1.08E+04
bottom	1.21E+05	7.15E+04	8.60E+04	1.42E+04

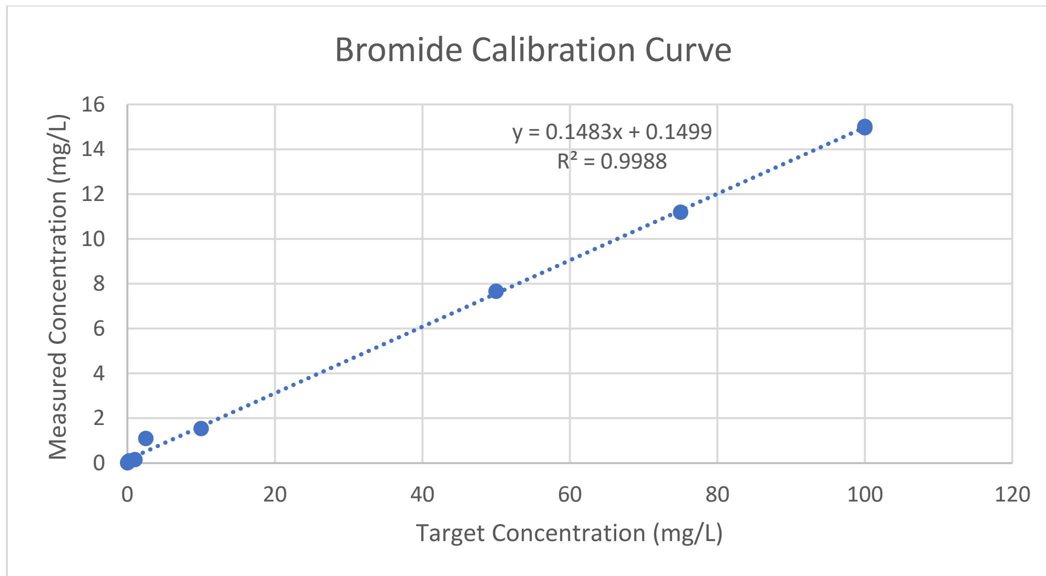

Figure S4.2. Tracer test calibration curve and results. Tracer samples were analyzed on a Thermo Fisher Dionex ICS-2100 system equipped with a conductivity cell detector, KOH eluent generator, ASRS suppressor, 25 µL injection loop, and 0.5 mL Polyvial® autosampler (Dionex AS-40) was utilized.

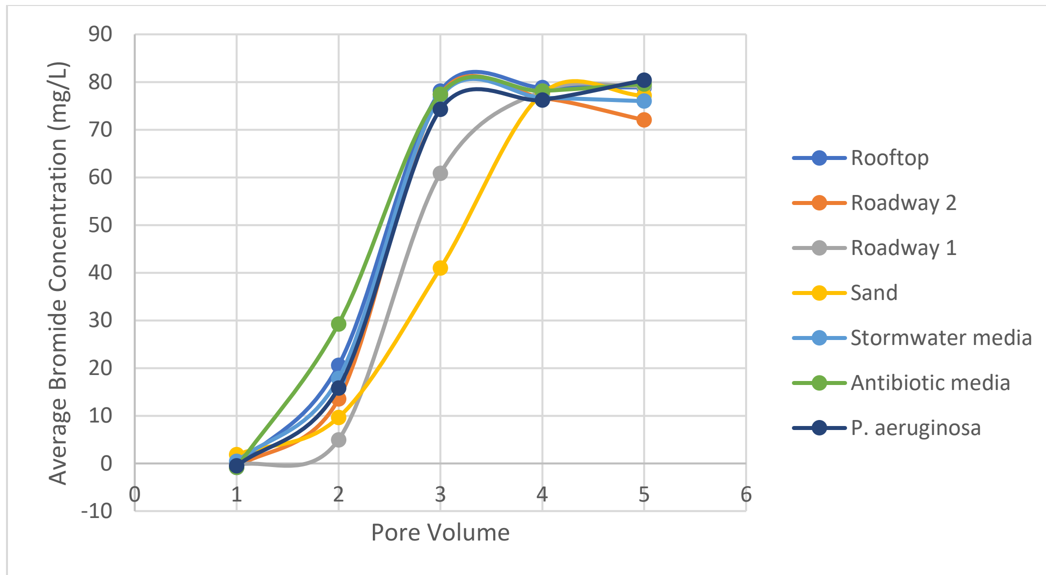

Figure S4.3. Conservative tracer NaBr was added as a step tracer starting at 0mg/L tracer and stepping to 100 mg/L tracer. Breakthrough was sampled every half pore volume (2.5mL). We assume t=0 as 0

Figure S4.4. Pearson correlation values for diversity and biomass presence

qpcr	1			
ATP	-0.5	1		
shannon_diversity	-0.13	0.43	1	
observed_OTU	-0.57	0.55	0.89	1

Table 4.2 Pearson's product-moment correlation results summary

	Pearson's product-moment correlation value	t	df	p-value	95 percent confidence interval	
qpcr and Subsequent_remobilization	0.7005997	2.1955	5	0.07955	-0.1110448	0.9516006
shannon and Subsequent_remobilization	-0.5535206	-1.4861	5	0.1974	-0.9221825	0.342162
qpcr and Initial_release	0.3210861	0.75811	5	0.4826	-0.5697309	0.8649922
shannon and Initial_release	-0.8292872	-3.3183	5	0.02105	-0.9740498	-0.2030077

Table S4.3. DNA concentrations of sequenced samples measured by qubit assay and qPCR.

Sample	DNA concentration	1:5 Dilution	Sample	Cq	Average	Stdev	Sample	Cq	Average	Stdev	Sample	Cq[1]	Average
s-dep	Too Low (TL)	No	3	14.40	14.49	0.115325626	X-96	33.47	33.10891	0.46912322	3C	20.19	20.26767
s-3-48	TL	No	3	14.62			X-96	32.58			3C	20.29	
s-5-96	TL	No	3	14.45			X-96	33.28			3C	20.32	
s-1-rv	0.533	No	2	14.97	15.02	0.113578167	K-9-RV	25.83	25.81659	0.043034197	4A	22.86	22.98464
s-4-rv	0.692	No	2	15.15			K-9-RV	25.85			4A	23.08	
s-8-rv	0.464	No	2	14.94			K-9-RV	25.77			4A	23.01	
s-7-rb	0.488	No	2-7-RB	16.09	16.09	0.035118846	S-4-RV	16.60	16.58051	0.085547115	4B	23.82	23.86884
s-10-rb	0.100	No	2-7-RB	16.12			S-4-RV	16.66			4B	23.88	
s-2-rb	0.198	No	2-7-RB	16.05			S-4-RV	16.49			4B	23.91	
p-dep	TL	No	K-7-96	32.34	32.39	0.072341781	P-8-96	21.25	21.25378	0.087832179	S	26.57	26.55166
3-dep	TL	No	K-7-96	32.47			P-8-96	21.34			S	26.58	
3-3-96	0.163	No	K-7-96	32.35			P-8-96	21.17			S	26.50	
3-6-48	0.112	No	4-5-RV	17.45	17.82	0.609289203	3-8-RV	18.98	18.96388	0.087097892	4C	23.25	23.30771
2-dep	0.0620	No	4-5-RV	18.52			3-8-RV	19.04			4C	23.38	
2-1-48	0.419	No	4-5-RV	17.48			3-8-RV	18.87			4C	23.30	
2-9-96	0.377	No	4-10-RV	16.26	16.27	0.015275252	K-dep	31.10	31.07810	0.211919862	2C	18.24	18.53448
2-10-rv	1.06	No	4-10-RV	16.29			K-dep	31.28			2C	18.35	
2-6-rv	0.933	No	4-10-RV	16.27			K-dep	30.85			2C	19.01	
2-5-rv	0.313	No	2-4-RB	16.82	16.81	0.030550505	5-1-RV	16.50	16.53638	0.030044708	3B	19.13	19.11048
2-8-rb	0.463	No	2-4-RB	16.78			5-1-RV	16.54			3B	19.09	
2-4-rb	0.492	No	2-4-RB	16.84			5-1-RV	16.56			3B	19.11	
2-7-rb	0.809	No	P	18.07	18.02	0.06244998	3-1-RV	16.06	15.98076	0.07039396	2-dep	20.00	19.97434
p-8-96	0.148	No	P	18.04			3-1-RV	15.93			2-dep	20.00	
3-1-rv	0.678	No	P	17.95			3-1-RV	15.96			2-dep	19.92	
3-8-rv	0.150	No	X-1-RV	25.92	25.97	0.05033223	3-4-RV	19.05	19.07186	0.033824492	2B	17.18	17.20440
3-4-rv	0.171	No	X-1-RV	26.02			3-4-RV	19.06			2B	17.27	
4-2-rb	0.909	No	X-1-RV	25.98			3-4-RV	19.11			2B	17.16	
4-dep	TL	No	K-5-RV	27.57	27.57	0.01	3-dep	21.76	21.76624	0.073044788	K-10-RV	24.70	24.74502
4-8-48	TL	No	K-5-RV	27.58			3-dep	21.70			K-10-RV	24.71	
4-7-96	TL	No	K-5-RV	27.56			3-dep	21.84			K-10-RV	24.82	
4-10-rv	1.19	No	S-7-RB	16.42	16.40	0.034641016	2-9-96	17.63	17.74473	0.129780884	Mixa		#DIV/0!
4-9-rv	0.950	No	S-7-RB	16.42			2-9-96	17.88			Mixa		
4-5-rv	0.746	No	S-7-RB	16.36			2-9-96	17.72			Mixa		
4-3-rb	0.668	No	K-3-RB	23.09	23.08	0.015275252	3-2-RB	17.67	17.63358	0.102262903	4	25.13	25.04101
4-1-rb	0.837	No	K-3-RB	23.08			3-2-RB	17.71			4	25.05	
3-9-rb	0.187	No	K-3-RB	23.06			3-2-RB	17.52			4	24.95	
3-7-rb	0.0850	No	4-1-RB	16.97	16.98	0.055677644	4-8-48	31.00	31.20729	0.20113927	3A	18.93	18.99111
3-2-rb	0.255	No	4-1-RB	17.04			4-8-48	31.23			3A	19.05	
p-1-rb	0.212	No	4-1-RB	16.93			4-8-48	31.40			3A	18.99	

k-dep	TL	No	3-3-96	20.96	20.97	0.15011107	X-3-RV	28.86	29.06783	0.201553898	K-2-RB	26.11	26.17345
k-4-48	TL	No	3-3-96	20.82			X-3-RV	29.08			K-2-RB	26.13	
k-7-96	TL	No	3-3-96	21.12			X-3-RV	29.26			K-2-RB	26.28	
k-5-rv	TL	No	P-6-RB	20.25	20.34	0.080829038	2-1-48	16.00	16.00448	0.019101309	2A	17.02	17.08671
k-9-rv	TL	No	P-6-RB	20.35			2-1-48	16.02			2A	17.07	
k-10-rv	TL	No	P-6-RB	20.41			2-1-48	15.99			2A	17.17	
k-2-rb	TL	No	S-3-48	23.54	23.69	0.132287566	s-dep	32.25	31.93237	0.301612228	B	32.04	31.95644
k-3-rb	0.0800	No	S-3-48	23.79			s-dep	31.91			B	31.71	
x-5-rb	TL	No	S-3-48	23.74			s-dep	31.64			B	32.12	
k-1-rb	TL	No	P-10-48	24.14	24.14	0.01	5-2-RB	18.26	18.27725	0.029752002			
p-10-48	TL	No	P-10-48	24.15			5-2-RB	18.31					
p-5-rv	0.205	No	P-10-48	24.13			5-2-RB	18.26					
p-6-rb	0.232	No	S-8-RV	17.24	17.29	0.078102497	X-6-RB	28.27	28.24966	0.029197866			
x-dep	TL	No	S-8-RV	17.25			X-6-RB	28.22					
x-48	TL	No	S-8-RV	17.38			X-6-RB	28.27					
x-96	TL	No	4-dep	31.54	31.58	0.109696551	P-9-RB	20.09	20.12603	0.034240701			
x-2-rv	TL	No	4-dep	31.70			P-9-RB	20.15					
x-3-rv	TL	No	4-dep	31.49			P-9-RB	20.14					
x-1-rv	TL	No	X-5-RB	26.95	27.11	0.136503968	K	26.12	26.05888	0.052059162			
x-4-rb	TL	No	X-5-RB	27.20			K	26.02					
x-6-rb	TL	No	X-5-RB	27.17			K	26.03					
p-4-rv	0.156	No	2-6-RV	16.20	16.25	0.055075705	5-10-RB	21.08	21.17417	0.090987007			
p-2-rv	0.135	No	2-6-RV	16.31			5-10-RB	21.26					
s	TL	No	2-6-RV	16.25			5-10-RB	21.18					
k	TL	No	4-2-RB	16.86	16.94	0.072111026	P-4-RV	20.12	20.12528	0.019775145			
p	0.431	No	4-2-RB	17.00			P-4-RV	20.11					
b	TL	No	4-2-RB	16.96			P-4-RV	20.15					
4	TL	No	4-9-RV	16.93	17.01	0.085440037	3-9-RB	18.80	18.83734	0.053836506			
3	3.99	Yes	4-9-RV	17.00			3-9-RB	18.81					
2	5.56	Yes	4-9-RV	17.10			3-9-RB	18.90					
p-9-rb	0.294	No	3-7-RB	20.51	20.54	0.03	X-dep	32.30	32.65215	0.38006362			
			3-7-RB	20.57			X-dep	32.60					
			3-7-RB	20.54			X-dep	33.06					
			P-1-RB	19.74	19.82	0.086216781	P-dep	29.01	28.93399	0.252100059			
			P-1-RB	19.80			P-dep	29.14					
			P-1-RB	19.91			P-dep	28.65					
			4-7-96	28.95	29.06	0.115036226	X-4-RB	27.19	27.12895	0.07812663			
			4-7-96	29.06			X-4-RB	27.15					
			4-7-96	29.18			X-4-RB	27.04					
			2-8-RB	19.06	19.05	0.070237692	X-48	32.02	32.09035	0.063575222			
			2-8-RB	18.98			X-48	32.10					
			2-8-RB	19.12			X-48	32.15					
			K-4-48	31.57	31.84	0.240208243	X-2-RV	26.31	26.25723	0.133847899			

			K-4-48	32.03			X-2-RV	26.36					
			K-4-48	31.92			X-2-RV	26.11					
			2-5-RV	18.05	18.06	0.02081666	2-10-RV	15.49	15.52480	0.066644306			
			2-5-RV	18.08			2-10-RV	15.60					
			2-5-RV	18.04			2-10-RV	15.49					
			4-3-RB	17.59	17.65	0.077674535	P-2-RV	21.02	21.04741	0.032771029			
			4-3-RB	17.74			P-2-RV	21.08					
			4-3-RB	17.63			P-2-RV	21.03					
			3-6-48	18.38	18.41	0.036055513	S-5-96	23.41	23.39827	0.042073538			
			3-6-48	18.45			S-5-96	23.35					
			3-6-48	18.40			S-5-96	23.43					
			K-1-RB	26.08	26.09	0.011547005	P-5-RV	20.82	20.80096	0.0201055			
			K-1-RB	26.10			P-5-RV	20.80					
			K-1-RB	26.08			P-5-RV	20.78					

[1] Cq value represents quantification of the 16s rRNA gene using quantitative PCR (qpcr). A higher Cq value represents lower concentration of the gene present. These values can be used to standardize relative abundance data to total abundance. It is assumed that there is 1.75 doubling efficiency, therefore the fold difference between each Cq value can be calculated to standardize the amount of DNA being amplified and PCR cycle number for amplification. Replicate samples are analyzed for qPCR are averaged for the final concentration used in amplification.

APPENDIX D: The Theoretical Principles of Particle Removal and Flow Through Porous Media

1. Introduction

Stormwater runoff is a reusable water resource. Many stormwater filtration systems can remove pathogens and other contaminants when they are employed as stand-alone units or in a treatment train to treat runoff. Biofilms that form on porous media and stormwater filters should theoretically alter the performance of the filters. However, this has been investigated experimentally for pathogen removal under limited scenarios and modeled under even fewer [1]. Sand is a widely-used media specifically in stormwater treatment. Therefore, pathogen removal in sand filters must be understood to optimize use in treatment trains. Known physical and chemical forces must be adequately described to investigate unknown biological components in the biofilm and how it relates to removal in water treatment.

This appendix describes the theoretical principals of particle removal and flow through porous media, as they relate to the design of biofilm growth studies on sand filters to assess the removal of fecal indicator bacteria from stormwater. First, I will detail the historical context of water filtration and applications to stormwater management. Then, we will explore basic filter principles, the characterization of flow through porous media, and establish ways to predict or experimentally measure particle removal parameters. The current state of mathematical modeling is also discussed. After each section, an understanding of the stated principles will then be applied to design my filtration experiments.

2. Historical Context on Water Filtration and Application to Stormwater Management

Sand filters used to treat stormwater have evolved from a long history of civic water treatment systems. Filters have been used to clarify water for thousands of years dating back to at least 2000 BC in India [2]. The use of filters even dates back to early records of monkeys observed digging for water beside a river, using the bank to filter the source stream. The first modern slow sand filter was designed and implemented by Chelsea Water Works Company, London, 1829 [2]. Wastewater treatment began implementing sand filters as tertiary treatment in the 1990s [3]. Most water treatment facilities have moved away from slow sand filters for rapid, and ultra rapid filters. However, similar slow sand filtration processes are still employed for drinking water [2],

[4], [5]. The differences in slow sand filtration and its successors are shown in **TABLE D1**.

Process Characteristic	Slow Sand Filtration	Rapid Filtration	Precoat Filtration
Filtration rate	0.05-0.2 m/h	5-15 m/h	1.3-5 m/h
Media diameter	0.3-0.45 mm	0.5-1.2 mm	4-30 μm
Bed depth	0.9-1.5 m	0.6-1.8 m	2-5 mm
Required head	0.9-1.5 m	0.6-1.8 m	2-5 mm
Run length	1-6 months	1-4 days	6h – 30 days
Ripening period	Several days	15 min – 2 h	None
Pretreatment	None required	Coagulation	None required
Dominant filtration mechanism	Straining, biological activity	Depth filtration	Straining
Regeneration method	Scraping	Backwashing	Bed replacement
Maximum raw-water turbidity	10 – 50 NTU	Unlimited with proper pretreatment	10 NTU

TABLE D1 *common filter types for water treatment and their respective parameters. Slow sand filters largely resemble stormwater sand filters based on the major parameters listed. Table recreated from* [2]

As described by Montgomery Watson Harza Firm (MWH), slow sand filters were largely superseded by rapid filtration in other water treatment processes; however, some designs resemble the stormwater filters employed today. They consist of gravity filtration in a submerged bed with finer particles than rapid filters. The schmutzdecke or bioactive layer forms in the first centimeters, which can filter particles and biologically degrade organics. Thus, destabilization of particles is not necessary through added coagulants. Head is allowed to build up before it is regenerated by scraping off the schmutzdecke layer. The major benefit for use is the ease of operation. There are differences between traditional slow sand filters and stormwater management systems, including but not limited to, the fact that stormwater facilities experience subsequent dry periods and many designs are not always submerged [2]. Schematic designs of a slow sand filter and stormwater sand filter are shown in **FIGURE D1** for comparison.

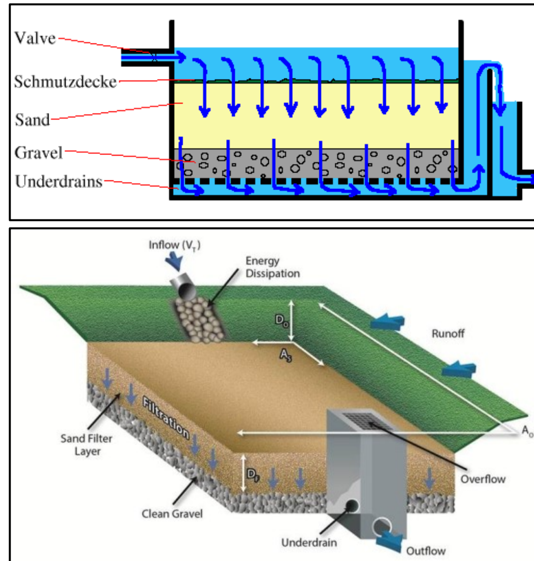

FIGURE D1 *Top image shows a traditional slow sand filter for water treatment commonly found in drinking and wastewater plants* [6]*. Bottom image shows a stormwater sand filter located at a stormwater outfall. Both have sand, gravel, underdrains, the ability to develop a schmutzdecke layer* [7].

The topic of pathogen removal in stormwater sand filtration also touches the studies of groundwater recharge, potable reuse of wastewater, and water treatment for a range of conditions, environments, and needs [1], [8], [9]. Ultimately, when it comes to stormwater, we as engineers are understanding that runoff is a resource. Instead of treating and releasing stormwater runoff, we can give it a job for reuse and infiltration, and to address issues of water scarcity [10], [11].

Experimental Design

Within stormwater management, sand filters can be used to treat many source water types - rooftop runoff, overland flow, roadway sources, residential and industrial sites upstream of a stormwater collection system, or at the outfall of a collection system. I am precisely interested in the heterogeneity of bacterial biofilms that form on these filters and how the biofilm influences pathogen removal from stormwater sources. To evaluate and predict important bacterial community parameters, physical and chemical influences must be controlled for and accurately described in filtration.

The experimental setup I have employed relates most closely to a slow sand filter with constant pressure head on initial installation- in other words, an idealized case before the first scraping to clear the schmutzdecke. This setup is appropriate to simulate a reuse scenario using valves or pumps to maintain the pressure head, or an idealized sand filter in the field that operates at a single approach velocity. With these

specifications, I can describe the applicable theoretical principles of flow through porous media, particle removal through depth filtration, and the applications of mathematical modelling to my results.

3. Basic filter principles

a. Hydraulics of flow: clogging, Detachment, breakthrough

As described by MWH, the filtration rate is equal to the flow rate through the filter bed divided by the cross-sectional area of the bed and is measured in units of volumetric flux. This is known as the superficial velocity because the average velocity in the bed is higher due to the volume taken up by the filter media. The two major parameters of concern when running a filter are the head loss and removal efficiency (i.e., effluent concentration) over time [1], [2], [12]. These relationships over the time of a filter run are shown in **FIGURE D2**.

MWH also describes how the effluent concentration follows a characteristic pattern, as shown in **FIGURE D2**. Effluent turbidity rises as the clean filter ripens to a point where additional particles have been attached and improve removal rate, in the first segment of the graph. This results in a peak. The additional particles increase the collector surface available for removal. This relates to theory of how biofilms augment filtration media. Over time, the head loss increases in the filter as it clogs and ultimately there is a breakthrough of contaminant in the effluent. Breakthrough of contaminants or increase of head loss trigger maintenance of the filter i.e., scraping of the schmutzdecke to regenerate it [13]–[15].

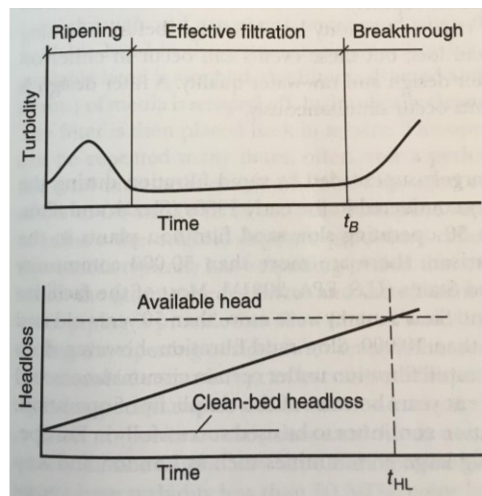

FIGURE D2 *Trends of head loss and effluent turbidity over the life of a slow-sand filter before scraping to remove the schmutzdecke* [2]

Important considerations that must be balanced during a filter run are detailed by Benjamin and Lawler [12]:

> *" (1) Effluent should be of sufficient quality in the early part of a run that little or no water needs to be wasted or recycled before meeting the effluent guidelines.*
> *(2) Effluent should meet water quality guidelines over a long time period (or, more precisely, until a high normalized production is achieved).*
> *(3) Head loss should increase slowly enough that the filter can operate until a high normalized production is achieved.*
> *(4) Requirements for backwashing flow and volume must be reasonable."*

Balancing the effluent concentration with head loss is a challenging task. For example, increasing the size of filter media or the depth of the filter bed may help achieve better effluent quality; however, this will increase head loss and the backwashing requirements. These decisions are not trivial, specifically with drinking water, therefore increasing prioritizing the quality of effluent over the length of the filter run can vary situationally. For example, a drinking water plant would prioritize effluent quality over length of filter run more than a wastewater plant because of the direct destination of the effluent and different sacrifices each plant can make in effluent quality without damaging the health of the public.

From this information, stormwater management falls closer under wastewater concerns but could trend towards drinking water concerns as reuse increases. Specific to pathogens in the biofilm, regulations vary on required removal, therefore methods of optimizing these concerns may vary circumstantially. These descriptions are used to describe general particles, however physiology, biofilm phylogeny, function and biological characteristics of pathogens in stormwater are not well incorporated into filtration theory.

Experimental Design

The biofilm growth represents a ripening period shown in **FIGURE D2**. The columns will then be tested for the breakthrough of fecal indicator bacteria to generate breakthrough curves that can be compared between different bacterial community assemblies. Theoretically, different communities will augment the columns to generate significant differences in removal rate under the same initial physical and chemical controls. In the field, head would be allowed to build over the filter to a certain extent

depending on the filter design; however, head is controlled using a peristaltic pump to set the same approach velocity for each column.

b. Filter media

As described by MWH, properties of filtration media include grain size, size distribution, density, shape, bed porosity, and specific surface area, to name a few. Grain shape can be characterized by their sphericity or shape factor which are dimensionless characteristics. Unfortunately, these definitions do not have much practical application because they are difficult to measure directly and most media is assumed spherical for modelling purposes. Density is most important to consider when designing multimedia filters, specifically ones that are disrupted from backwashing. The material hardness is also important to consider as the media may degrade over time. Filter bed porosity has a crucial impact on head loss and removal efficiency of the filter [2]. It is the fraction of free space in the bed and can be calculated using EQUATION 1 below, as well as measured observationally. Filter media is widely varied in the literature when studying pathogen removal from stormwater and ranges from sand, biochar, and compost and soil, to highly engineered media [16]–[18]. Completely sand filters are commonly used in the field across the United States [7], [19], [20] because they are relatively easy to fund, design and maintain.

$$\varepsilon = \frac{V_V}{V_T} = \frac{V_T - V_M}{V_T}$$

EQUATION 1 where ε = porosity (dimensionless), V_V = void volume in filter bed (L^3), V_T = total volume of filter bed (L^3), V_M = volume of filter media (L^3) using MLT units. [2]

Experimental Design

Completely sand filters are commonly used in the field across the United States because they are relatively easy to fund, design and maintain. In other designs, sand may be a layer in an engineered stormwater infiltration system. Sand used in my stormwater column study is 50-70 mesh or 0.25mm effective average grain size (Sigma cas # 14808-60-7) which falls within the range of grain sizes used in the field [9], [21]–[23]. To prepare filter media, 32g (about 2.5cm depth) of sand is added then compacted 25 times with the a standardized weigth tamper dropped from the 2.5cm above the sand surface. This is done for the entire length of the 45cm column (Ace glass cat #5820-34, 5837-56). This depth is representative of filters employed in the field. The density of

sand added has been standardized to 0.475 ± 0.011 g/mL column resulting in a standardized porosity of 92.3 ± 3 mL by volumetric measure. This is important to standardize and control the physical properties of the filter, as well as replicate how they are compacted in the field to minimize settling.

c. Reynolds number and flow regimes

An important hydraulic characteristic for the design of filtration systems is the flow regime which can be described by the flow around spheres using the Reynolds number. The flow regime for a given filtration technology can be defined using **FIGURE D3** below based on the design filtration rate and effective media size [2]. As described in MWH, slow sand filters control head loss by operating at a low enough filtration rate that biodegradation removes accumulated organic particles. The net available head loss in a slow sand filter is considered consistent as the clean bed head loss is insignificant. Therefore, additional applications of Darcy's flow regime, Forchheimer flow regime, and calculations of clean bed head loss applies to rapid filtration, and not our experimental scenario.

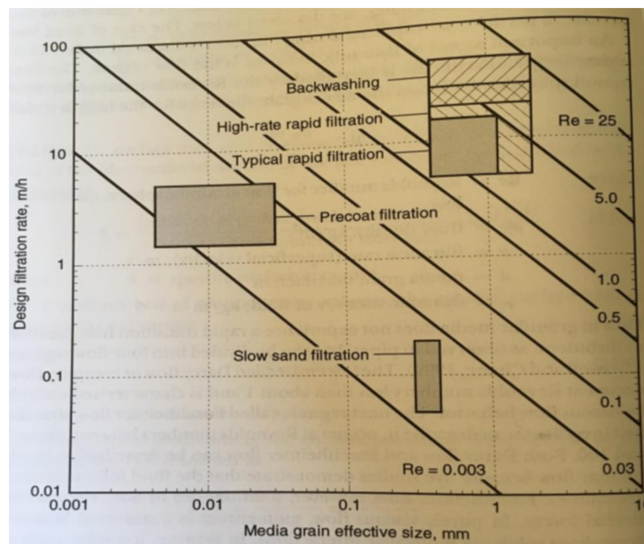

***FIGURE D3** The flow regimes of filtration technology can be classified by comparing the range of design filtration rate and effective media grain size* [2]

<u>Experimental Design</u>

The experimental filters I employ have a design filtration rate <0.15 m/h with 0.25 mm effective average grain size. These experiments operate right at the Darcy flow regime for slow sand filtration between the 0.003 and 0.03 Reynolds number range. Flow through the column and porosity will be measured using NaBr, a conservative tracer [24].

4. **Particle removal**

a. Basic mechanisms

The removal of particles, particularly fecal indicator bacteria has been described as mechanical filtration, straining, physicochemical filtration, and transformation (growth and death of bacteria) [1]. Mechanical filtration occurs at the filter cake and removes particles larger than the filter media pores. Straining happens in the narrow pores between the filter media and has been measured as particles >0.18 particle to median grain size ratio and as wedging with ratios greater >0.005 [1]. There is debate as to how significantly different this is from classic physicochemical filtration. Under classical physicochemical filtration theory, the particle is transported to the surface by either Brownian diffusion, interception by a particle, or gravity sedimentation. Under ideal or favorable conditions, all particles that are transported to the collector would attach. Under unfavorable conditions, solution chemistry largely controls the removal rate by establishing energy conditions for irreversible removal at the primary energy minimum or reversible removal at the secondary energy minimum [1], [25], [26]. This is shown in **FIGURE D4**. In general, a particle will or will not attach based on the forces it undergoes as it approaches the collector surface.

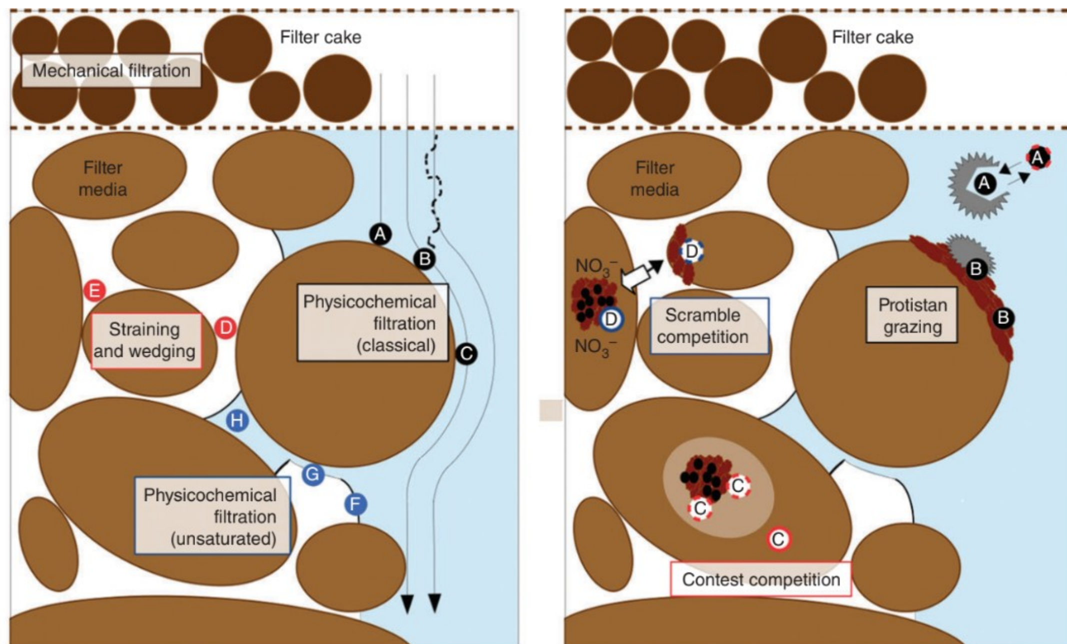

FIGURE D4 *Factors under saturated and unsaturated conditions impacting particle removal and bacterial removal are shown on the left and right, respectively. Specific mechanisms in the graphic are described in the original article* [1].

There are many physiochemical forces that influence the transport and removal of

bacteria in sand filters. Derjaguin–Landau–Verwey–Overbeek (DLVO) theory describes the net force a particle experiences as it approaches a surface [27], [28]. They simplify the forces to a Van der waals attractive force and an either attractive or repulsive electric double layer forces which allows the identification of a primary and secondary energy minimum. This model is described in FIGURE 5. Born repulsion, hydration effects, steric repulsion, polymer chains on particle surfaces, hydrophobic interactions, and polymer bridging are all important as well [1], [25], [29]–[32]. Bacteria are considered to be negatively charged particles when modeling filtration at typical environmental pH [1]. This is true for both gram-positive and gram-negative cells [33]. The bacterial cell surface contains carboxyl and amino groups. These groups become protonated and unprotonated at low and high pH, respectively [12]. Clean sand grains in a filter bed are considered negatively charged as well, creating a repulsive force between the particle and collector [34].

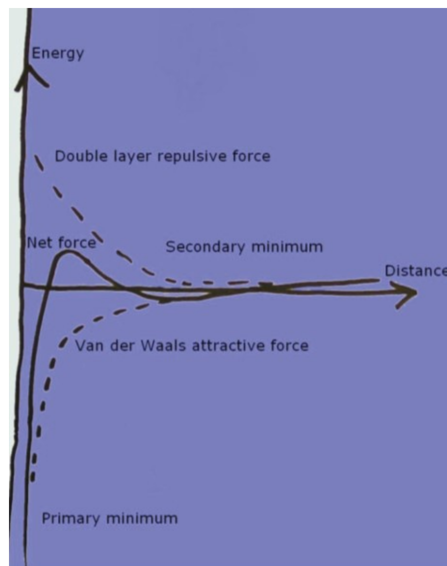

FIGURE D5 *By simplifying the forces impacting the energy a particle has over a distance away from a collector to the double layer force and Van der Waals attractive force, the location of important energy minimums can be identified* [8]

b. Iwasaki's general mathematical model of depth filtration and the filtration coefficient

Iwasaki's model uses classic filtration theory to describe particle removal in a filter [12], [35], [36]. The model assumptions are that the bed is uniform in shape and media, and that particles do not aggregate, meaning interactions between suspended particles are negligible. The probability of particle capture per unit length or time can be

described by the filter coefficient, λ. If we describe an incremental filter depth as ΔL as in **FIGURE D6**, the probability of particle capture in unit length $\Delta L = \lambda^* \Delta L$. Therefore, the probability of particle passage through $\Delta L = 1 - \lambda^* \Delta L$. If N_L and $N_{L+\Delta L}$ represent the concentration of particles at location L and ΔL, then $N_{L+\Delta L} = (1 - \lambda^* \Delta L) N_L$ given **FIGURE D6**. This can be rearranged so that $N_{L+\Delta L} - N_L / \Delta L = \Delta N / \Delta L = -\lambda^* N_L$. As ΔL -> 0, $dN/dL = -\lambda^* N_L$. This is described as EQUATION 2.

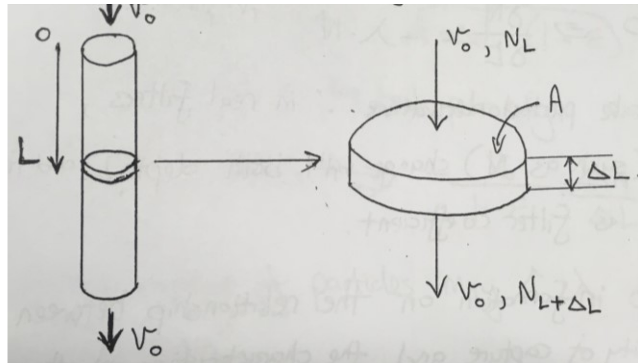

***FIGURE D6** Iwasaki's model diagram where L= the column length (L), A= cross sectional area (L^2), N=concentration of particles (M/L^3), v_0= approach velocity (L/T) using MLT units where v is assumed to be constant.*

This removal can be described by the filter rate coefficient and has been experimentally described by a first-order rate equation (EQUATION 2). The filter coefficient can also be described as a function of time instead of length.

$$\frac{\partial C}{\partial z} = -\lambda C$$

EQUATION 2 Where λ = filtration coefficient (L^{-1}), C = mass or number concentration of particles (M/L or L^{-1}), z = depth in filter (L) using MLT units

We can further quantitatively compare the columns based on the rate of deposition of particles or bacteria using EQUATION 3.

$$k_d = -\frac{U}{fL} \ln\left(\frac{C}{C_0}\right)$$

EQUATION 3 The deposition rate can be described for each column where U= is the approach velocity (L/T), f= porosity (L^3), L= length of the filter bed (L), C= outflow concentration (M/L^3), C0= inflow concentration (M/L^3) in MLT units [24], [37]

As the filter bed ripens, the ability to remove contaminants is impacted by the addition of collector surfaces. Iwasaki described this as a linear relationship between filter coefficient and deposition rate in EQUATION 4. Though originally described by Iwasaki in units of particle numbers, the filter coefficient has been expressed in many other dimensions[12].

$$\lambda = \lambda_0 + \alpha\sigma$$

EQUATION 4 Where λ=filter coefficient after ripening λ_0= initial filter coefficient, α= ripening coefficient, σ= the deposition rate [2]

c. Fundamental filtration theory

As described in MWH, fundamental filtration theory measures the relative importance of factors and predictors relating to particle contact in filtration media. It is useful for modeling, however not representative for long term performance or full scale filtration. Filter media and particles are idealized to be spherical, variable hydrodynamics of media angularity are not addressed, models predict one value for the filter coefficient when realistically the value varies by depth and time, and lastly, many models don't account for porosity changes as accumulation occurs within the bed. These models are considered clean bed filtration models and despite shortcomings are useful for determining the relative importance of different filtration mechanisms [2].

Described above generally as physicochemical filtration, clean bed filtration theory is widely used to design packed beds for water filtration and particle removal. The basic model was described by Yao et. al (1971) and termed colloid filtration theory (CFT) [26]. Removal is predicted by two steps, collection and attachment. Respectively these can be described by the single collector contact efficiency, defining the transport of particles to the sand grain, and the attachment efficiency, defining the extent of attachment to the sand grain surface Equation 5 and 6 below.

$$\eta = \frac{particles\ contacting\ collector}{particles\ approacing\ collector}$$

$$\alpha = \frac{particles\ adhering\ to\ collector}{particles\ contacting\ collector}$$

EQUATIONS 5 AND 6 (respectively) Where η = transport or collector efficiency (dimensionless), α = attachment efficiency (dimensionless)

The accumulation on a single collector is the product of eta and alpha which represents the rate at which particles enter the region of influence of the collector multiplied by a transport (eta) and efficiency (alpha) factor. To model particle removal, eta is usually predicted using equations and alpha calculated through experimentation. For example, once the removal rate for a single collector can be calculated, the number of collectors at incremental layer, ΔL, can be calculated using the incremental volume. These equations are combined, differential elements are simplified to infinitesimal dimensions, and integrated across the filter length, L with influent and effluent concentrations. If we assume heterogeneity throughout the bed, EQUATION 7 can be

derived to relate the single collector removal to the inflow and outflow particle concentrations. These are considered the fundamental equations when considering particle removal with a single spherical collector model.

$$\ln\frac{N_{\mathrm{out}}}{N_{\mathrm{in}}} = -\frac{3}{2}\frac{(1-\varepsilon)\alpha\eta}{d_{\mathrm{c}}}Z$$

$$N_{\mathrm{out}} = N_{\mathrm{in}}\exp\left[-\frac{3}{2}\frac{(1-\varepsilon)\alpha\eta}{d_{\mathrm{c}}}Z\right]$$

EQUATION 7 can be represented in either form where N_{out} = number of particles exiting, N_{in} = number of particles entering, Z = column length, d_c= collector diameter of the filter media, and all other terms previously defined [12]

The removal of the total filter bed can also be expressed as (eta filter), EQUATION 8, where eta represents the removal of one single collector. Again, eta is usually predicted using equations and alpha is calculated by experiment.

$$\eta_{\mathrm{filter}} = 1 - \frac{N_{\mathrm{out}}}{N_{\mathrm{in}}}$$

EQUATION 8 where η_{filter} = the approximated collector efficiency of the entire filter and other terms are previously defined. [12]

The Rajagopalan and Tien (RT) Model and other Phenomenological Models are important for expanding further on shortcomings of the Yao model describing collector and attachment efficiency, however the underlying principles are still largely the basis of modelling [2], [12].

Experimental Design

The underlying attractive and repulsive forces that are altered by the colonizing biofilm will not be investigated in this study but could be further probed using atomic force microscopy.

Iwasaki's general model establishes the basis of the column design, however the experiment focus is not on how the filter coefficient or filtration rate changes over the depth of each filter. Straining is more of the mechanism for removal in sand filters over depth filtration as described in **TABLE D1**. The deposition rate, EQUATION 3, will be used as one way to compare the columns as it is appropriate for comparison over time. As described in previous sections, the overall removal rate will also be compared.

EQUATION 8 can be used to estimate Eta filter or the overall removal rate of each filter.

Alpha can be calculated by comparing the removal in an idealized, positively charged column to the columns colonized by different biofilms [24]. However, in order to compare the relative removal rates of each community, these values will not need to be calculated or compared.

5. State of modelling

As described by Molnar et al. [34] in a review for the American Geophysical Union, the earliest applications of colloid transport research began before the 1990s for the removal of pathogens in drinking water. Since then, models have been developed from pore to field scales. As they are used to delineate source zone protection for drinking water and remediation applications, it is important that these models can assess both favorable and unfavorable conditions.

Since Yao et al. introduced CFT, significant research has been developed to model, control and predict colloid retention in porous media. The importance of DLVO forces has been expanded on significantly through the use of column studies to fit rate parameters to investigate the influence of short range forces under favorable and unfavorable conditions [29], [38]–[42]. Continuum-scale models are used to describe transport at a macroscopic level where mass balances are solved using a representative elementary volume (REV) [34], [43]–[46]. Colloid filtration theory has been largely investigated under favorable conditions with emphasis now placed on unfavorable conditions as well, to increase applicability to environmental scenarios. Most CFT models have two major components to predict eta (η), a mechanistic force model that describes the particle transport and attachment, and correlation equations used to predict the retention of colloids [34]. Modelling efforts seek to better adapt equations to environmental conditions for example, by attempting to predict the influence of the secondary energy minimum under favorable and unfavorable conditions, the importance of immobilization, heterogeneity of the macropores, and other mechanistic simulations [38], [47]–[50]. In general, physical and chemical heterogeneities are widely investigated to understand environmental implications [34]. Biological heterogeneity is less investigated but the body of research is growing [1], [30], [34], [51].

Specific to pathogen removal from stormwater, previous studies have associated pathogen removal rate to filter media type, the presence of protozoa, biomass presence, ionic strength, and varied single isolates [13], [16], [22], [24], [52]. Modelling efforts have been applied to previously conducted column studies to identify the important predictors

for removal [51]. Mohanty et al. designed a column study investigating the influence of plant species and turbidity on the removal of fecal indicator was conducted to show that both variables are correlated to bacterial removal rate [30]. Parker et al. later followed up to predict the extent that clean bed filtration theory played a role in the removal. They retroactively calculated alpha in additional experiments and predicted eta using a combination of single collector efficiencies. Using multiple linear regression modelling to associate filtration rate as largely dominated by the clean bed filtration theory rate constant with some attribution to antecedent dry period, study design, column age, and the presence of plants in the filter. This supports the hypothesis that clean bed filtration theory can predict the removal of fecal indicator bacteria, but only with the inclusion of other environmental, ecological, and biological design parameters.

Experimental Design

Column studies investigating biofilm associated removal in porous media have not investigated the influence of varied community assemble. Pathogen removal rates of filters in the field have been assessed and correlated to design parameters. However, the importance of biofilm community heterogeneity, diversity, phylogeny and function have not been established as correlated. Microbial ecological theory offers significant insight on stochastic and deterministic properties of bacterial community assembly, though they have not been applied to understand pathogen removal in stormwater and filters. Predictors such as, the presence of the 16s gene, presence of enteric organisms or keystone species in the biofilm, marker gene presence, species richness and abundance, Simpson's diversity index, and beta-diversity metrics are indicators of biofilm community diversity and function when compared between columns. Not only will the significance in variability of removal between columns be tested, but these metrics will be compared to the deposition rate to determine significance. This parameters can then be analyzed in future modelling studies as removal predictors similar to the Parker et al. study.

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About the Author

EDUCATION

Johns Hopkins University, Baltimore, MD
PhD student, Department of Environmental Health Engineering, Fall 2015-2020
Advisor: Sarah Preheim
Dissertation title: "Assessing Bacterial Community Assembly and Function for Improved Biological Removal of Pathogens and Contaminants in Stormwater Filtration Systems"

Johns Hopkins University, Baltimore, MD
Master of Science in Environmental Health and Engineering, Whiting School of Engineering, Spring 2017

The Honors College of George Mason University, Fairfax, VA
Bachelor of Science in Civil, Environmental, and Infrastructure Engineering, December 2014
Advisor: Liza Durant and Denise Akob

PROFESSIONAL EXPERIENCE

Bloomberg School of Public Health- Baltimore, MD

- Independent Policy Research with Vice Dean for Public Practice and Community Engagement, Dr. Joshua Sharfstein on the Safe Drinking Water Act and Lead and Copper rule. Developed white paper on amendments after the Flint Water lead poisoning and *legionaries* outbreak.
- Developed client intake and outcomes tool for Intersection of Change organization for community practicum with Martha's Place housing program for women and continued the relationship outside of class until the project was complete.
- Intro to Public Health Teaching Assistant: lead weekly lessons, discussions, exams on public and environmental health.

Urban League of Essex County- Newark, NJ
Textile Recycling Program Development and ThriftWorks Intern- March 2015-August 2015

- Participated in nonprofit community effort to reduce clothing and textile waste and create jobs and wealth for our community through a secondhand thrift store enterprise.
- Managed online retail operation and customer service
- Tasks included personally interacting with community members, engaging others in the importance of reuse and recycle of materials, donation solicitation, expanding the nonprofit's skillset with my engineering

expertise, understanding the ways that my work in engineering can be applied improve the lives of low income people

U.S. Geological Survey, Reston Microbiology Lab- Reston, VA
Student Contractor- May 2014-February 2015
Projects related to the biodegradation of produced water from hydraulic fracturing as well as implementing microbiology techniques on various ecosystems
Tasks and skills include:

- Field sampling for microbial analysis
- Building microcosms
- Measuring and analyzing headspace gas using GC/MS
- Hungate anaerobic technique
- PCR/qPCR
- Microbial cultivation
- Analyzing data
- Research presentations

Louis Stokes Alliance for Minority Participation- Fairfax, VA
Mentor- Fall 2012-Summer 2014

- Hold weekly tutoring hours during school year
- Mentor incoming freshman and grade math assignments for summer program

George Mason Department of Civil, Environmental and Infrastructure Engineering- Fairfax, VA
Student Grader- Spring 2014

- Assisted professor by grading weekly homework assignments
- Held weekly office hours with students

Balfour Beatty Construction- Fairfax, VA
Jobsite Intern- Summer 2012

- Worked for a Construction Management firm at a jobsite in Washington D.C.
- Set up a digital system for viewing and posting questions and corrections to the Construction Drawings to be used by site supervisors and subcontractors
- Monitored building elevations and jobsite safety during the construction process
- Interacted with permitting process and subcontractor, manager, and owner relationships

Ironbound Community Corporation, Environmental Justice Team - Newark, NJ
AmeriCorps Volunteer - Summer 2011

- Completed Quarter Term of service

- Assisted in planning and maintaining community garden
- Worked to combat food deserts in the inner city by publicizing organizations Farmers Market
- Assisted in addressing environmental issues in the area through education and involvement in the community

City of Newark, Housing and Economic Development Division- Newark, NJ
Intern - January 2011

- Created site plan reports
- Worked with AutoCAD, Adobe Photoshop and Adobe Illustrator programs for the Newark Master Plan project

MZM Construction & Management- Newark, NJ
Intern - Summer 2010

- Assisted with business plan development and visited job sites
- Observed small business structure and function

SYSTRA USA- Little Falls, NJ
Intern - Summer 2009

Assembled blueprints and design plan books for a civil engineer working on a major rail transit project in the metropolitan New York City area

RESEARCH INTERESTS

urban microbiomes, public health, community driven development, urban infrastructure, environmental health, civil engineering, science communication, crisis and risk communication, infrastructure and sociology, urban design, low impact development, storm water management, bioremediation, experimental methods, culture independent microbiology techniques

RESEARCH EXPERIENCE

Johns Hopkins University, Department of Environmental Health and Engineering (2015-2020)

- Green infrastructure and community development around storm and rainwater catchment
- Examining engineered infiltration systems as a viable bioremediation component in stormwater management
- Pathogen fate and transport in municipal separate storm sewer systems
- Methods in microbial community analysis
- Flint water crisis white paper to amend the Safe Drinking Water Act

George Mason University Undergraduate Research Scholars Program (Summer - Winter 2014)

- Understanding the impacts of produced water from hydraulic fracturing operations on streambed microbial communities and the potential for bioremediation of a spill

- Research conducted with the Reston Microbiology Lab of the U.S. Geological Survey
- Wrote grant and was selected for funding through competitive application process
- Skills Employed: Creating microcosms, measuring headspace gas concentrations using gas chromatography, qPCR, microbial analysis, evaluating degradation of organic compounds, theoretical calculations, analyzing data, technical presentations, PRISM graphing software

RESEARCH PRESENTATIONS AND PUBLICATIONS

Fraser, A.N.; Zhang, Y.; Sakowski, E.G.; Preheim, S.P. Dynamics and Functional Potential of Stormwater Microorganisms Colonizing Sand Filters. *Water* **2018**, *10*, 1065.

Fraser, A. 2016 "Hydraulic Fracturing and Natural Gas Extraction Associated Environmental Health Concerns and Bioremediation Potential" Johns Hopkins University IGERT. Talk.

Fraser, A. 2015 "Examining the United States Infrastructure Crisis through Communication Models" Johns Hopkins University. Talk.

Fraser, A. 2014 "Degradation of Hydraulic Fracturing Fluid Additives by Stream Microbial Communities" U.S. Geological Survey. Talk

Fraser, A. 2014 "Message Efficacy around the US Infrastructure Crisis: Crisis Communication Models Applied to the American Society of Civil Engineers' Infrastructure Report Card" Crisis Communication, George Mason University. Talk.

Fraser, A. 2014 "Engineering Bacteria to Form a Biofilm and Induce Clumping in Caenorhabditis elegans: Analysis and Applications" Environmental Engineering Microbiology, George Mason University. Talk

Fraser, A., Butt, M., Khan, D., **2012** "Transportation and Air Pollution Mitigation in Johannesburg, South Africa" Applied Ecology, George Mason University. Poster.

CERTIFICATIONS AND TECHNICAL SKILLS

- Community Based Public Health Certificate, Bloomberg School of Public Health, Johns Hopkins University, 2017
- FE/EIT Certification
- Science communication, crisis communication, persuasive communication, logistics communication, public briefings, public health communication
- Python, MATLAB, Linux/Unix, Command Line
- R for correlation, regression and statistical modeling
- VBA for Excel, PRISM graphing software

- Shell script programming and batch job scheduling (SLURM), github
- QIIME2 bioinformatics pipeline, FAPROTAX database for phenotype prediction
- PRISM Graphing Software
- AutoCAD: Civil 3D, Google Sketchup
- Bluebeam Revu
- EPANet, HY-8 Culvert Analysis, Bentley Flowmaster
- Microbiology Techniques
- Permaculture Design Certification

AWARDS AND ACCOMPLISHMENTS

- Middle East Mutual Aid Partnership founder with Abell and Charles Village Mutual Aid collective, Baltimore Redevelopment Action Coalition for Empowerment, and Reclaiming Our Community (2020)
- NSF Integrative Graduate Education and Research Traineeship program: Water, Climate and Health (2016-2018)
- Intersection of Change and Martha's Place Community Development Practicum participant to develop health program evaluation and community, health, and engineering partnership to meet technical program needs (2017)
- Maryland Science Olympiad volunteer (2016)
- Science outreach and education at John Ruhrah Elementary and Henderson Hopkins Elementary School (2016)
- Walter L. Robb Fellow- Department of Geography and Environmental Engineering (2015-2016)
- Hydraulic Fracturing Water Cycle briefing with nonprofit Food and Water Watch canvassing unit (October 2015)
- Best Presenter Award- Civil Engineering Undergraduate Senior Design Presentation (December 2014)
- International Scholar Laureate Program: Student Delegate on Engineering Studies in China (Summer 2013)
- Office of Diversity: Vision Award recognition for Leadership and Academic Excellence (2012-2014)
- George Mason Civil Engineering Undergraduate Advisory Council Member (2010-2011)
- AmeriCorps Service on Environmental Justice Projects (Summer 2011)
- Louis Stokes Alliance for Minority Participation Program, Member and Peer Advisor (2010-2014)