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Dynamics and Functional Potential of Stormwater Microorganisms Colonizing Sand Filters

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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 up 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.

Keywords: 16S rRNA gene; microbial community; diversity; potential pathogen; denitrification

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*).

2. Materials and Methods

2.1. 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.

2.2. 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 S1). 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.

2.3. 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.

2.4. 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.

2.5. 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 S1). 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 S2a). Clustering was not driven by batch effects, as replicates from the same samples processed in different batches clustered together (Figure S2c,d).

2.6. 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].

2.7. 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. Results

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

3.1.1. 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 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.

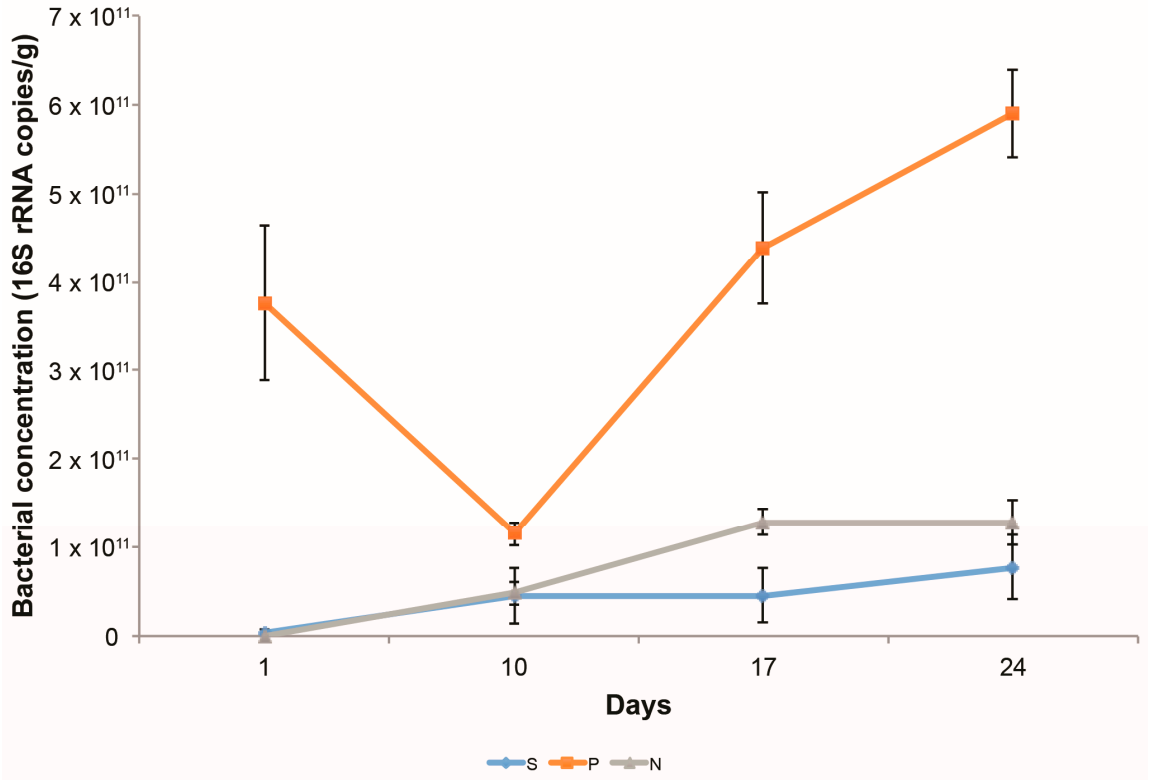

Figure 1. Change in bacterial cell concentration within environmental and control columns over time, as estimated by 16S rRNA gene copy number/µL. Abbreviations: S (blue), stormwater-inoculated columns; P (orange), *Pseudomonas aeruginosa* -inoculated columns; N (gray), non-inoculated columns. Significantly 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.

3.1.2. Bacterial Community Composition on Columns

Columns were colonized by a diverse range of microbial taxa (Figure 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*.

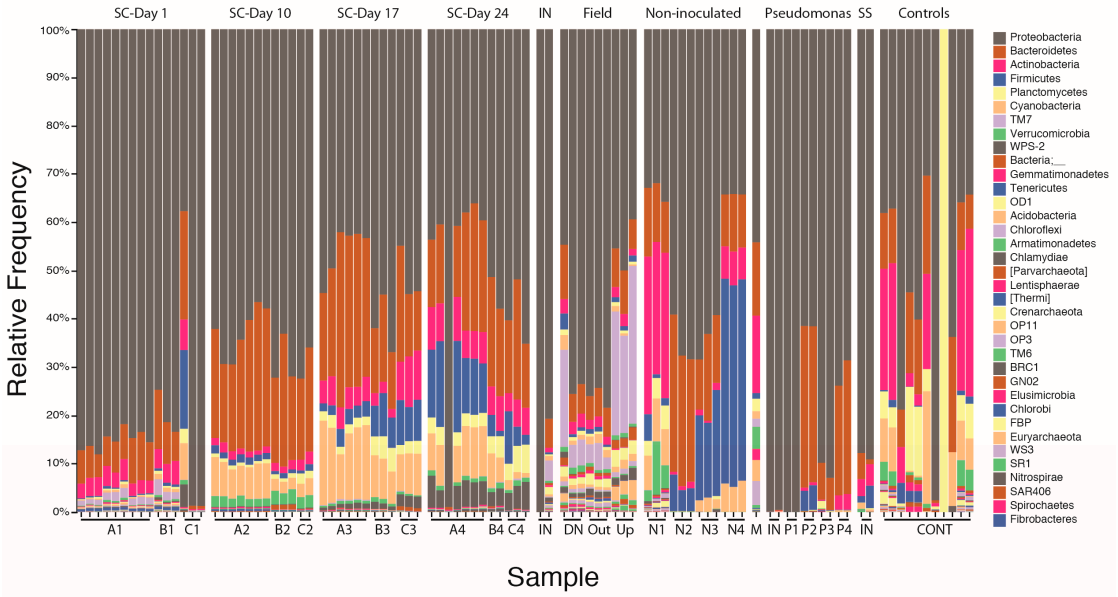

Figure 2. Taxa plots (phylum level) of microbial communities across replicate stormwater columns, *Pseudomonas* columns, non-inoculated columns, field samples, and controls. Legend provides phylum level classification although phyla comprising less than 1% of the samples overall are not listed. Sample 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). 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 2) or specific taxa.

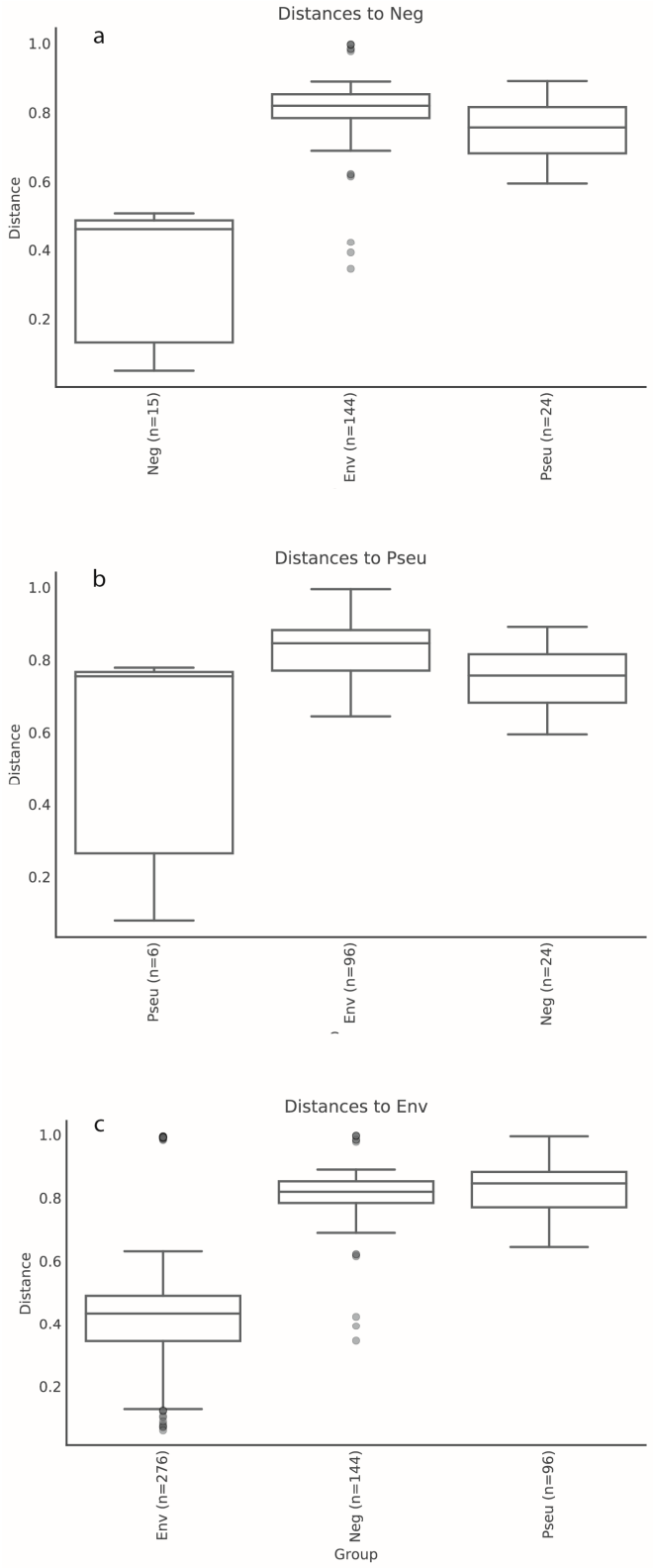

Figure 3. Bray-Curtis distance between different inoculum types from the last two time-points combined. Median, interquartile range and outliers distances between all column samples and (**a**) non-inoculated (Neg) column distance (**b**) *Pseudomonas aeruginosa* (Pseu), and (**c**) Stormwater inoculated 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).

3.2. Microbial Community Succession on Stormwater Columns

3.2.1. 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 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 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.

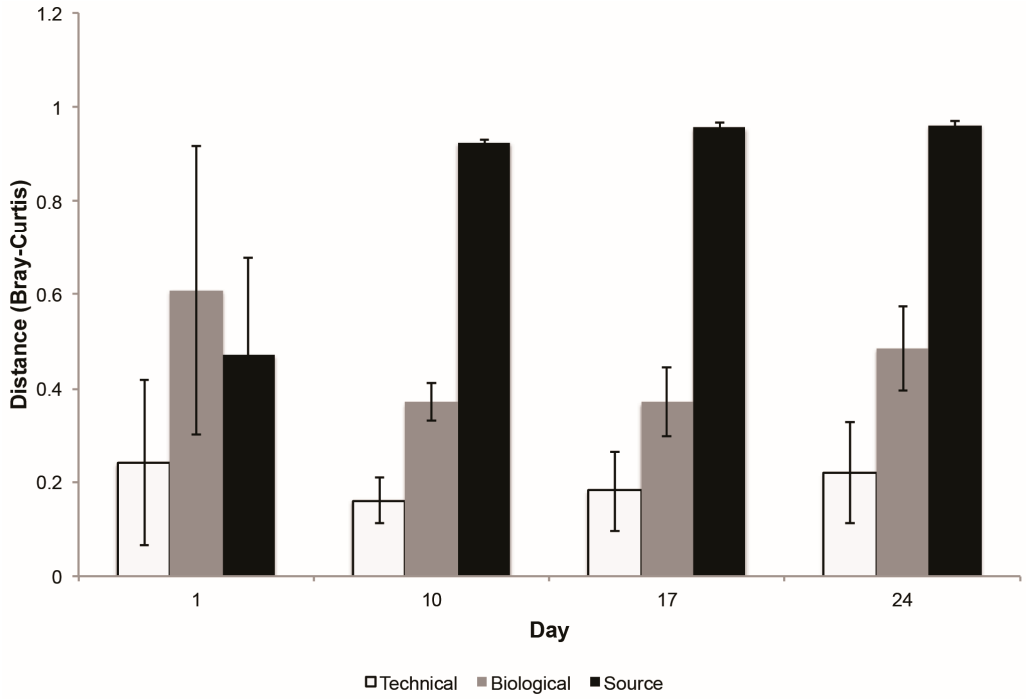

Figure 4. The average Bray-Curtis distance between technical replicates (T), biological replicates (B) and the distance it diverged from the starting inoculum community (S) each day. Numbers following single letter comparison group designations indicate the day of the experiment (e.g., S24 is the distance 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.

3.2.2. 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 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) demonstrates dispersal of the lab environment to the columns is not high enough to cause the observed the similarity between replicate columns.

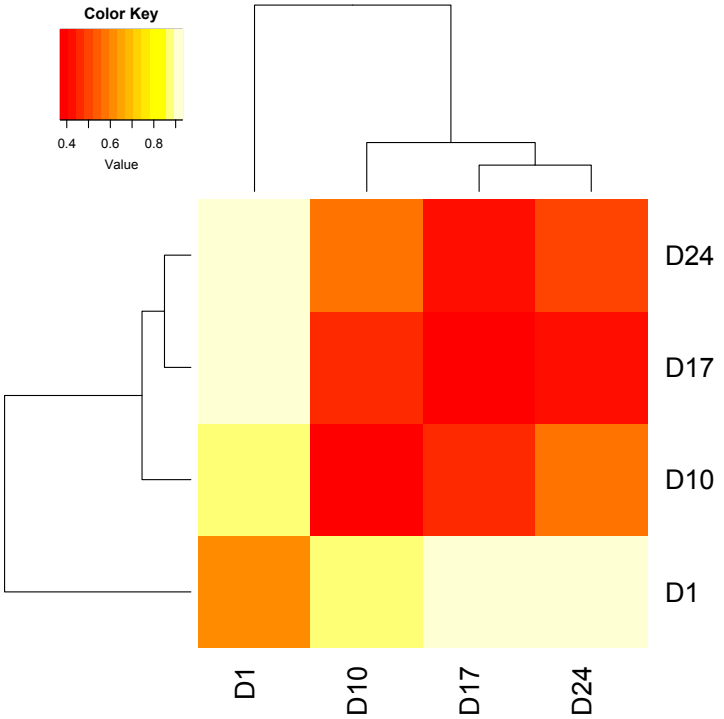

Figure 5. Heatmap of mean Bray-Curtis distance between time-points from stormwater inoculated columns. Colors indicate mean distances between biological replicates (diagonal) or all sample comparisons between different time-points, with red indicating more similar and yellow indicating more different. 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.

3.2.3. 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). 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 6). The relative abundance of potential pathogens on stormwater columns was high on day 1 and was maintained through day 10 (Figure 6c). 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 6a). 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 6a). 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.

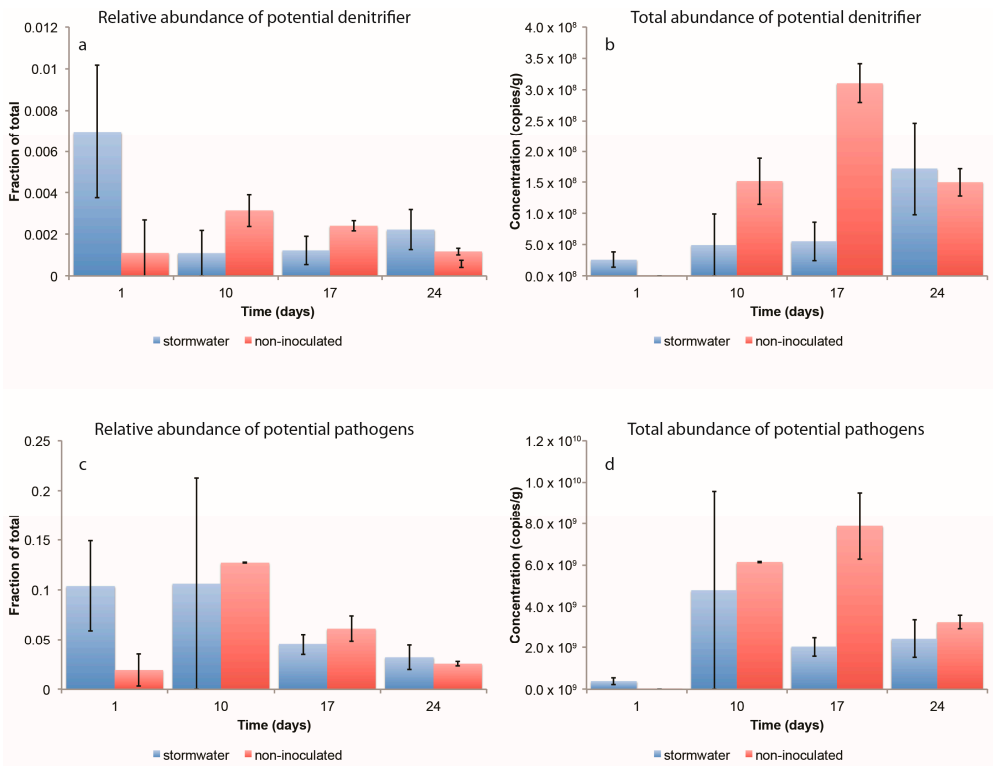

Figure 6. Relative (**a**,**c**) and total (**b**,**d**) abundance of potential denitrifying (**a**,**b**) and pathogenic (**c**,**d**) microbial taxa over time within non-inoculated, (red) and stormwater inoculated (blue) columns. (**a**) The relative abundance of potential denitrifying taxa within 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 6c), the overall bacterial cell concentration was lower than at later time-points (Figure 6d). However, both potential pathogens and denitrifying taxa expanded within the column on day 10, reaching a maximum between day 10 and 17 (Figure 6b,d). This initial increase was not maintained, and both types decreased from their peak by day 24.

3.2.4. 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 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.

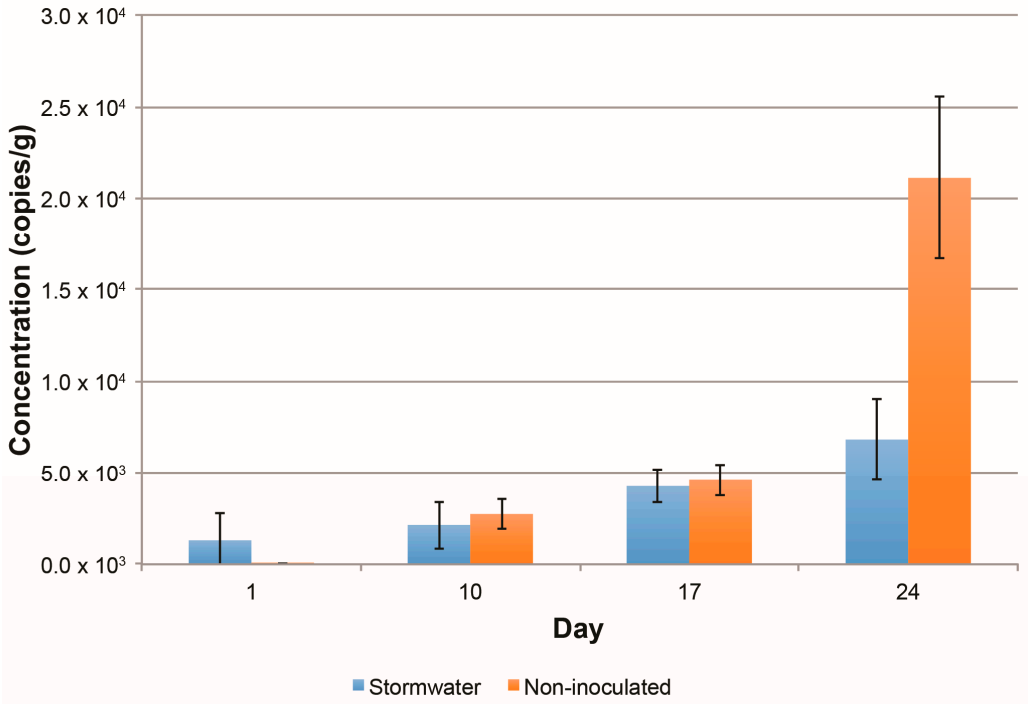

Figure 7. Total abundance of *nosZ* over time within non-inoculated (Neg) and environmental columns. Env-stormwater inoculated columns; Neg-Non-inoculated columns.

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.

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.

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