Engineering *E. coli* Removal from Stormwater

Claire Wayner, Baltimore Polytechnic Institute, Baltimore, MD

Objective

The purpose of this research is to identify a diverse range of bacteria isolates that are capable of removing *Escherichia coli* from polluted urban stormwater. Stormwater pollution is an enormous problem for urban areas, and solutions such as engineered infiltration systems (EIS) have been proposed to remediate stormwater pollution. Previous research has been performed on the effectiveness of these systems at removing inorganic pollutants, but very little is known about the impact of EIS on pathogenic bacteria pollutants (e.g., fecal coliform, *E. coli*). The bacteria naturally present in stormwater and on these systems in particular are expected to impact pathogen pollutant transport in EIS. Thus, it is crucial to understand how the bacteria already present on EIS in the environment interact with pathogen indicators like *E. coli* (the focal species of this research) to help inform better engineering design of EIS and potential manipulation of environmental factors to promote reduction of pathogen pollution.

Abstract

Stormwater runoff often becomes contaminated with biological pathogens, such as *E. coli*, that can have negative impacts on urban aquatic ecosystems and the health of human communities associated with those water systems. In order to mitigate stormwater pollution, engineered infiltration systems (EIS, see Figure 1) have become a popular solution, but little is known about the specific characteristics such as growth inhibitors of bacterial biofilms naturally present on EIS that aid in the removal of biological contaminants like *E. coli* from urban stormwater. In this investigation, a lab EIS was constructed and the bacterial community present on the system after four weeks was sequenced and compared to a larger dataset of bacteria present in urban stormwater to provide a better understanding of the microbial diversity selected for by EIS growth conditions. Results suggest that EIS is fairly selective and that only a small portion of the diversity present in urban stormwater can grow on EIS. Bacteria isolates grown from EIS were then used in a growth inhibition assay to investigate whether these isolates have the potential to antagonistically react to *E. coli*. Assay results suggest that only a limited subsection of isolates present on EIS have the potential to kill *E. coli*. This study has the potential to inform the modification of EIS design to best remove *E. coli* from stormwater and thus reduce stream contamination.

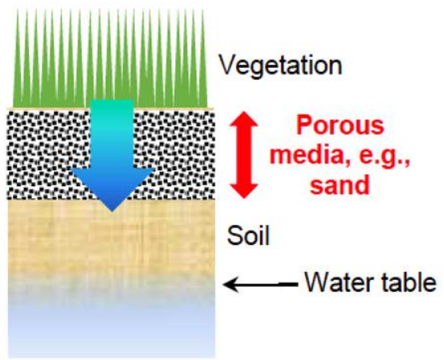

Figure 1. A schematic of a sample EIS.

Materials/Methods A

The first component of this research involved identifying the bacteria already present in urban stormwater and which portion of these bacteria are able to grow on a column simulation EIS constructed in the lab.

Stormwater samples were collected from an outfall pipe discharging into a Baltimore urban stream, Stony Run. 50 mL of each water sample was then filtered using a peristaltic pump through an in-line Swinnex filter holder onto sterile 0.22 µm filters (Millipore). A 16S ribosomal library of bacteria species present in water samples was then constructed through DNA extraction and Illumina library setup and sequencing.

The column simulation EIS was constructed out of Econo-Pac 14 cm polypropylene chromatography columns (Bio-Rad) packed with 6 cm of white quartz sand (SiO_2, 50-70 mesh particle size). Sand and columns were autoclaved before usage a total of three times. Columns were connected with sterilized feed tubing to a peristaltic pump (Cole-Parmer). For inoculation, 1 L per column of collected stormwater runoff was cycled through three columns for three hours at the rate of 10 cm/hr. Columns were then gravity drained and incubated at room temperature for the next 19 days and were fed 1 L of synthetic stormwater solution (SSS, see end of methods section for contents) on days 5, 12, and 19. SSS was filter sterilized before usage.

On day 19 of the column experiment, effluent exiting the columns during the first 10 minutes of feeding was collected and grown on plates in 200 µL, 100 µL, and 10 µL amounts (diluted with SSS to be 200 µL total volume when necessary) containing 50% purified agar and 50% SSS. Plates were incubated at 22°C for approximately 120 hours before isolated bacteria were restreaked on additional plates. 54 bacteria were isolated in total. 11 isolated bacteria were sequenced to limit repetition and to provide a representative sampling of what bacteria were expected to grow on EIS.

Materials/Methods A (con't.)

After bacteria were purified through several rounds of restreaking, colonies were picked and resuspended in 200 µL of polymerase chain reaction (PCR)-grade H_2O. First- and second-step PCR on the 16S ribosomal region was then performed on extracted DNA with the following conditions: 98°C for 10 minutes; 30 cycles of (1) 98°C for 30 seconds, (2) 52°C for 30 seconds, and (3) 72°C for 60 seconds; and 72°C for 10 minutes. Forward sequencing on all isolates was performed at the Johns Hopkins School of Medicine Genetic Resources Core Facility (GRCF) on an Illumina MiSeq with 600 base pairs through the usage of the 27F primer. Sequenced isolates were then preserved in glycerol cultures at -80°C until the growth inhibition assay could be performed.

All sequences were aligned using mothur against the Silva reference alignment. The tree was then generated using ClustalX and visualized using the Interactive Tree of Life (iTOL) for tree visualization.

SSS Contents: 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 mM NH_4Cl, and 0.02 mM Na_2SO_4. To increase carbon content, 0.0015% (by weight) peptone, 0.0011% meat extract, 0.0003% urea (16 mg/L BOD), and 3 g/L of yeast extract were added. 3 mg/L hexanes was added to mimic presence of petroleum products naturally found in polluted stormwater.

Results and Interpretation A

By characterizing the bacterial community on EIS compared to the community found in stormwater, the research aims to identify how EIS is selective. In total, 11 bacteria isolates from the simulation EIS effluent were sequenced, but a total of 54 unique isolates were produced. Multiple bacteria of similar physiology were seen throughout isolation, and to avoid repetition, specific isolates were selected as representatives of their physiological groups to be sequenced. Although the limited sequencing did not provide the most complete picture of the diversity present within the 54 total isolates, the sequencing results that were obtained are notable, however, when compared to the overall diversity of the stormwater. Of the 38 phyla and 237 families present in the larger community runoff data, four phyla and eight families were observed in the EIS isolates. This could be due to two main factors, one being the expected selective nature of the lab EIS. The constant temperature of the laboratory environment, coupled with the specific growth media used, selected for particular organisms best adapted to this simulation EIS. In addition to the selectivity of the EIS, the plate method used for sequencing, which involved the streaking and restreaking of isolates on agar plates, was also selective; only certain isolates could grow on an agar media with the given growth media. If sequencing had been done on the effluent samples collected from the columns instead of on the isolates grown from the effluent on plates, a more complete and less biased picture might have been presented. However, the plate isolation technique was necessary to this experiment to save viable isolate cultures for the growth inhibition assay and future EIS research with these isolates.

While comparing the 11 isolate sequences to the larger data taxonomically provides some superficial analysis, a better way to visualize the data and identify evolutionary patterns is through a phylogenetic tree, displayed in Figure 2. The tree was generated using the larger data set from the runoff samples and 11 sequenced isolates. All 11 sequences pertaining to isolates were highlighted in red, and the areas of isolates are boxed in red, with the number to the side of the box indicating the number of isolates found within that region. The tree is meant to provide a larger visual picture for broader analysis of potential patterns present in bacteria found on EIS. It is not meant to show specific isolate names or categories.

Overall, the diversity of isolates present on EIS were found to be clustered toward one end of the phylogenetic tree, particularly one node, which contained six unique isolates. This clustering of the isolates toward one part of the tree is not entirely surprising, given that these isolates are required to have evolved particular beneficial characteristics that can allow them to survive on EIS. In addition, all but one isolate found toward the bottom of the tree were found to be Gram positive bacteria, a category suggesting that these bacteria are primarily soil bacteria. Toward the top of the tree, all but one were Gram negative bacteria. It is at first surprising that Gram negative bacteria can grow on the tree at all; Gram negative bacteria are typically not found in sediment. The presence of both types of Gram and stratification of isolates by Gram is an interesting factor that should be investigated further.

Granted that the plate sequencing method used does select for particular organisms, which could be one of the causes behind the grouping of diversity, the division of isolates along the tree could very well be an indicator that EIS primarily selects for a particular phylogenetic division of bacteria.

One potential result of the limited diversity on EIS could be that all of these bacteria that have evolved for EIS have also evolved to inhibit *E. coli* growth to curtail competition. Another possibility could be that *E. coli* have evolved to recognize these bacteria typically present on EIS and could have adapted to either benefit from or at least not be harmed by these bacteria. Further research into the interactions between isolates and *E. coli* on a simulation EIS would be necessary to answer these hypotheses.

Results and Interpretation A (con't.)

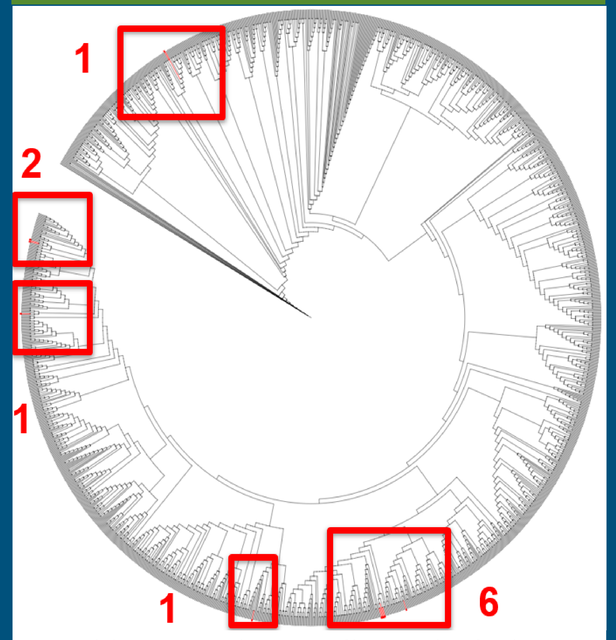

Figure 2. Phylogenetic tree with sequences of bacteria isolates found on EIS overlaid on the diverse range of bacteria found in urban runoff. Bacteria isolates are highlighted in red. For better visualization, the areas of isolates are boxed in red, with the number to the side of the box indicating the number of isolates found within that region.

Materials/Methods B

The second component of the research is to characterize relationships between *E. coli* cells and isolates through growth inhibition assays performed on SSS and agar plates. The purpose of a growth inhibition assay is to identify hostile relationships between two bacteria species by placing an isolated culture of one onto a thin lawn culture of another, grown up at the same time. Any hostile or parasitic reactions are clearly defined by a zone of clearing, indicating that the one bacteria species is able to outcompete the second species.

The growth inhibition assay was carried out between all 54 isolates and a lawn of *E. coli*. Saved glycerol cultures of sequenced isolates were restreaked on plates containing 50% agar and 50% SSS and incubated at 22°C for 96 hours. Colonies of each isolate were then resuspended in 1 mL of SSS to create liquid cultures, which are required to perform the growth inhibition assay. After 200 µL of *E. coli* culture was spread onto SSS plates as a lawn, 5 µL of liquid culture of isolates was placed on *E. coli* cultured plates in defined areas and allowed to dry for 20 minutes onto the lawn of *E. coli* to ensure that the droplets remained within their areas. After 48 hours of incubation, isolates were investigated to see if zones of clearing had formed, indicating an aggressive response toward *E. coli*. The radius of clearing, if present, was measured in millimeters around specific isolates from the edge of the isolate colony to the edge of the clearing zone. Photographs of zones of clearing were also obtained using a standard phone camera; see Figure 3.

Results and Interpretation B

Radii of clearing in the growth inhibition assays were observed in only 3 out of 54 isolates. Figure 3 shows a typical radius of clearing. The radii were measured at three different points on the plate and averaged, with the average value across the three isolates being 2.8 mm. Note that sequencing results are available for only one of these isolates, identified as a member of genus *Pseudomonas*. Based on physiological similarities, it is suspected that the other two isolates are also members of genus *Pseudomonas* but not known for certain.

Although there is no sequencing data available for the bulk of the isolates tested, the breakdown between those bacteria showing an adverse reaction and those showing none at all still establishes that very few of the bacteria present on EIS have the capabilities to behave aggressively toward *E. coli*. These low numbers may be impacted by the fact that relationships were tested on agar plates and not in EIS, where realistically, *E. coli* and these species would be interacting.

Results and Interpretation B

Yet the plates still suggest that only a small fraction of bacteria present on EIS hold potential for reducing *E. coli* contamination in stormwater. In most cases, bacteria isolates grew without reacting to *E. coli*, suggesting that these species have adapted to *E. coli*'s presence and could even have developed beneficial symbiotic relationships. If this ratio holds true on EIS, then EIS could prove to be a host site for *E. coli*, encouraging its growth and the continual reintroduction of pathogenic cells into stormwater, putting into question the effectiveness of EIS at remediating stormwater quality. These initial results could thus be used to modify the design of EIS to make its stormwater remediation more effective. Engineers could manipulate the conditions of EIS to be more favorable to those organisms that antagonize *E. coli*, thus reducing *E. coli* concentration in stormwater. More research in a column simulation EIS, however, is necessary to see if these results obtained from an assay conducted on agar plates are transferrable to the actual environment.

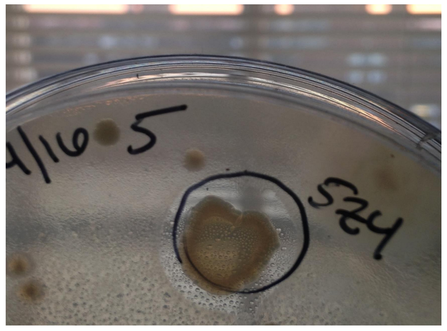

Figure 3. Isolate 5z4 (unsequenced) shown with radius of clearing. Note transparent zone around isolate.

Conclusions

To better understand the microbial communities present in urban stormwater and those bacteria able to grow on EIS, a column simulation EIS study was conducted and results sequenced and compared to sequencing results from stormwater samples. Sequencing revealed that only a select portion of the bacterial diversity present in urban runoff are able to grow on EIS. An additional growth inhibition assay study indicated that an even smaller subset of the bacterial community present on EIS have the potential to kill or inhibit the growth of *E. coli*. This suggests that the bacterial diversity on EIS, while being incredibly limited, does not include many species that can reduce the concentration of *E. coli* in stormwater. In continuing research, biofilms of these individual isolates that display an ability to kill *E. coli* and those that do not will be grown on a lab simulation EIS to better imitate the conditions experienced in the field. The removal and remobilization rates of *E. coli* culture through these biofilms grown on EIS will be investigated through breakthrough curves. Results from these future studies will be used to draw broader conclusions about the roles of underlying physical characteristics of biofilms, such as the Gram of the bacterium, on the removal of *E. coli* in EIS and how the design of EIS could be modified to remove the most *E. coli* from stormwater, reducing the pollution levels of stormwater and urban aquatic ecosystems.

Relevant Applications to Biotechnology

This research identifies varieties ("isolates") of bacteria that could be used on EIS in the field to promote *E. coli* and other pathogen removal. By identifying the beneficial bacteria, engineers and biologists can design EIS to contain these bacteria or to promote the natural growth of these bacteria. When these EIS are then installed in the environment, there would be enhanced removal of pathogenic bacteria, reducing pollution in urban stormwater. Biotechnology could be used to construct EIS with designs to promote the growth of beneficial bacteria. The beneficial bacteria could also be engineered to grow biofilms on installed EIS. Bacteria could also be engineered to inhibit *E. coli* to an enhanced degree, removing the most pathogens from polluted stormwater.

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