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Complete List of Authors:	Afrooz, Nabiul; Stanford University, Civil and Environmental Engineering: Pitol, Ana; Eawag Kitt, Dianna; Stanford University, Civil and Environmental Engineering: Boehm, Alexandria; Stanford University, Civil and Environmental Engineering:

Stormwater represents one of the greatest threats to surface water quality. It contains a myriad of chemical and microbial pollutants. This study examines, at the lab scale, the potential for biochar-amended sand biofilters, a type of green infrastructure, to remove pathogens and pathogen indicators from stormwater and therefore improve receiving water quality.

Role of Microbial Cell Properties on Bacterial Pathogen and Coliphage Removal in Biochar-modified Stormwater Biofilters

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A. R. M. NABIUL AFROOZ[1,2,3], ANA K PITOL[4,5], DIANNA KITT[2,6], ALEXANDRIA B. BOEHM*[1,2]

[1]Department of Civil and Environmental Engineering, 473 Via Ortega, Stanford University, Stanford, California 94305

[2] Engineering Research Center (ERC) for Re-inventing the Nation's Urban Water Infrastructure (ReNUWIt), USA

[3] Southern California Coastal Water Research Project (SCCWRP), Costa Mesa, California 92626

[4] Eawag, Swiss Federal Research Institute of Aquatic Science and Technology, 8600 Dübendorf, Switzerland

[5] Laboratory of Environmental Chemistry, School of Architecture, Civil, and Environmental Engineering (ENAC), École Polytechnique Fédérale de Lausanne (EPFL), CH 1015 Lausanne, Switzerland

[6] Department of Civil and Environmental Engineering, University of Illinois, 205 N. Mathews, Urbana Illinois, 61801

* Corresponding author: Alexandria B. Boehm, Email: aboehm@stanford.edu, Phone: 650 724 9128

Abstract

Stormwater biofilters are distributed stormwater control measures for managing urban runoff. Recent work has shown that adding biochar to biofilters can reduce stormwater contaminant concentrations, including fecal indicator bacteria (FIB). However, the potential of biochar-augmented biofilters to removal human pathogens from stormwater has not been investigated. In this study, we investigated the removal of bacterial pathogens *Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus*, as well as bacterial and viral indicators *Escherichia coli* and MS2 coliphage in laboratory-scale biochar-amended biofilters. Biochar-amended biofilters performed better than sand biofilters in removing the microorganisms from stormwater and removal of pathogenic bacteria was greater than that of FIB. Biochar-augmented biofilters provided up to 3.9,1.9, and 1.8 $\log_{10}$ removal for pathogenic bacteria, *E. coli*, and MS2, respectively. We utilized colloid filtration theory to elucidate potential microbial removal mechanisms. In biochar-amended biofilters, electrostatic interactions between the virus and collector surfaces likely controlled bacteriophage removal whereas the electrostatic interactions likely played a minor role in bacterial removal. Bacterial removal in biochar-augmented biofilters was likely controlled by straining and hydrophobic interactions. The findings of this study inform the design of geomedia-amended biofilters to reduce stormwater-derived microbial contamination in receiving waters.

1. Introduction

Pathogens are important stormwater contaminants and their presence in surface water—inferred by elevated concentration of fecal indicator bacteria (FIB)—is the most frequent cause for impairment of receiving waterbodies in the United States[1]. FIB and pathogen concentrations in stormwater are extremely variable. FIB concentrations can be below the detection limit or higher than observed in sewage[2]. There are few studies of pathogen concentrations in stormwater, but when available, data suggest their levels are also quite variable and can be elevated[2]. Pathogen impairment of surface waters can impact public health[3] and affect local economies that rely on clean surface waters[3,4]. Stormwater control measures, including biofilters, can be used to capture and treat contaminated stormwater before it is discharged to surface waters. However, the conventional biofilter, which typically contains sand and compost, demonstrates inadequate or inconsistent performance in removing microorganisms from stormwater [5, 6]. Stormwater biofilter designs need to be improved to enhance microbial removal.

Addition of geomedia, including biochar, may improve the microbial removal performance of stormwater biofilters [7]. Biochar-amended sand biofilters increase FIB removal from stormwater compared to sand biofilters at the laboratory scale [8, 9]. Recent studies investigated the effects of environmental factors (e.g., weathering, presence of biofilm, antecedent dry period), design parameters (e.g., presence of a saturation zone, biochar particle size), and hydraulic properties (e.g., loading rate) on bacterial removal in biochar-amended biofilters [10-14]. These studies[8-12] primarily used *Escherichia coli* as a process surrogate for microbial contaminants. The number of studies that explored the removal of pathogenic bacteria or viruses in biochar-augmented biofilters is limited.

Two previous studies investigated the removal of pathogenic bacteria in biochar-amended sand or soil under simplified conditions that are not readily extendable to stormwater applications. Both studies were conducted using organisms suspended in deionized water with increasing electrolyte concentrations or pH, in the absence of natural organic matter [15, 16] (which is abundant in stormwater[6]). One of these studies investigated the role of biochar oxidation and pyrolysis temperature on the capacity of biochar to remove pathogenic and non-pathogenic *E. coli* strains [16]. While that study quantitatively demonstrated favorable deposition of bacteria onto the biochar surface compared to the sand surface, the reason for the observed greater removal of the pathogenic *E. coli* strain compared to the non-pathogenic strain was not determined. The second study reported the efficacy of biochar-amended soil for the removal of *E. coli* O157:H7 and *Salmonella typhimurium* [15]. The study suggested that hydrophobic interactions could be a possible mechanism for pathogen removal in biochar-amended soil. However, the underlying reason for the observed greater removal of *E. coli* O157:H7 compared to *Salmonella typhimurium* was not provided [15].

Removal of microbes in porous media has been conceptualized as a two-step process: transport of microbes to the collector grains followed by attachment [17]. The size and shape of microbes dictate the mechanism whereby they come into close proximity to the collector surface. Subsequently, physicochemical properties of the microbes and the collector surface control whether attachment occurs. Attachment is governed by microbe-collector surface interactions. These can be described as Derjaguin-Landau-Verwey-Overbeek (DLVO), hydrophobic, or Lewis acid-base interactions [10, 15, 16, 18]. Physical filtration or straining is another possible mechanism for microbial removal; it is controlled by the geometries of the microbial cells and the collector surface. Understanding the importance of different mechanisms that control the removal of

stormwater microorganisms in biochar-amended biofilters would improve our ability to predict their removal and better inform the design of biofilters.

The present study documented the removal of two bacterial pathogens, and a viral and a bacterial indicator in model biochar-augmented biofilters. Subsequently, we established a link between microbial cell properties and their removal in biofilters. We used two filter media, sand and biochar, and four microbial targets, *E. coli*, *Salmonella enterica* serovar Typhimurium, *Staphylococcus aureus,* and male-specific (MS2) coliphage. *Salmonella enterica* is a Gram-negative, rod-shaped bacterium with a reported concentration of 0.5-420 CFU/100 mL in urban stormwater [19]. *Staphylococcus aureus* is a Gram-positive, spherical bacterium with concentrations ranging from 120-1.8x10^5 CFU/100 mL in stormwater [19]. *E. coli* is a widely used fecal indicator in stormwater with a reported concentration range of 110 to 2x10^6 CFU/100 mL[19]. We used male-specific (MS2) coliphage as a viral indicator, in part, because USEPA may recommend its use in the future as a water quality indicator [20]: reported concentration in stormwater is 0.5-7600 PFU/100 mL[19,20]. The physicochemical properties of the microbial particles and the collectors were characterized for input into a theoretical model to estimate deposition rate constants. Theoretical microbial removal, calculated using colloid filtration theory (CFT), was compared with the observed removal in biochar-amended biofilters.

2. Materials and Methods

2.1 Preparation of the porous media

The porous media for the transport experiments consisted of a 70:30 (by volume) mixture of sand and biochar. Selection of this ratio was motivated by the biofilter soil media (BSM) specification recommended by Los Angeles regional waterboard [21]: the compost/organic fraction

in that recommendation was replaced by biochar. A 7:3 ratio of sand:biochar mix was also described in a previous study as a preferred BSM mix for FIB removal from natural stormwater using biochar[11].

The preparation method for the porous media has been described elsewhere [10]. Briefly, coarse Ottawa sand (20-30 mesh, Fisher Scientific) was soaked overnight in de-ionized (DI) water, acid-washed, and rinsed with DI for pH adjustment to 6.9± 0.3. The sand was then dried (105°C overnight) and autoclaved (121°C, 100 KPa, 15 min), and mixed with the biochar. Biochar particles were procured (Swallow Valley Farm, Valley Ford, CA), crushed, and sieved (to retain particles smaller than 595 μm). The source of the biochar, its preparation method, and physicochemical properties have been described in a previous study[10] and are summarized in the supporting information (SI).

Zeta potentials of the biochar were measured in synthetic stormwater using a Zetasizer (Malvern Instruments Ltd, Worcestershire, UK). Biochar particles passing #200 sieve were suspended in synthetic stormwater and the suspension was placed in contact with the universal dip cell in a standard polystyrene cuvette ensuring no air bubble introduction. Average zeta potential from triplicates was recorded.

2.2 Synthetic stormwater

Based on a protocol described elsewhere [10], we used the following recipe to prepare synthetic stormwater: $CaCl_2$ (0.75 mM), $MgCl_2$ (0.075 mM), $NaHCO_3$ (1 mM), Na_2HPO_4 (0.016 mM), $NaNO_3$ (0.072 mM), Na_2SO_4 (0.33 mM), and NH_4Cl (0.072 mM). 10 mg/L of Suwannee River natural organic matter (International Humic Substances Society, MN, USA) was added to the synthetic stormwater as representative natural organic matter (NOM). This recipe provides

concentrations of inorganic ions and organic matter concentrations within the range of those found in stormwater runoff[19]. The prepared stormwater was autoclaved (15 minutes at 100 KPa) and subsequently, the pH was adjusted to 7.3±0.4 using 0.5 M HCl or 0.5 M NaOH.

2.3 Preparation of bacteria suspensions

A kanamycin resistant strain of *Escherichia coli* (NCM 4236) was used as the indicator bacterium. 15 ml of luria bertani (LB) broth was inoculated by *E. coli* stock culture and incubated for 18 h at 37°C to reach a stationary phase. The growth media was then removed by centrifugation (5000xg for 10 min) and *E. coli* was suspended in synthetic stormwater and stored at 4°C for 16-20 h for acclimatization. The concentration of *E. coli* in the synthetic stormwater was ~10^5 colony forming units (CFU)/mL. Similar procedures were followed to prepare *Staphylococcus aureus* (ATCC 25293) and *Salmonella enterica* servor Typhimurium LT2 (obtained from the Falkow lab at Stanford University) laden synthetic stormwater. Instead of LB broth, brain-heart infusion (BHI) broth and tryptic soy broth (TSB) were used as the growth media for *Staphylococcus* and *Salmonella*, respectively.

The concentrations of *E. coli* and *Staphylococcus* in the synthetic stormwater were within the range of their concentrations found in natural stormwater[19]. However, to avoid non-detects in the effluent samples, we used higher (3 orders of magnitude) *Salmonella* concentration in the synthetic stormwater than has been reported previously for natural stormwater[19].

2.4 MS2 bacteriophage production and purification

We followed a previously published protocol to grow and purify the bacteriophage MS2 (DSMZ 13767) [19]. One liter of Tryptic Soy Broth (Difco) with 0.15 mg/L of streptomycin/ampicillin (Fisher Scientific, NH, USA) and 0.3 mg/L of calcium chloride dehydrate (Sigma Aldrich, MO,

USA) was inoculated with F*amp E. coli* (ATCC #700891). The inoculated suspension was incubated for 4h at 37°C and 220 rpm until reaching logarithmic phase. The media containing log-phase bacteria was inoculated with 100 µl of MS2 at ~10^{10} plaque forming units (PFU)/ml and incubated overnight at 37°C. Subsequently, the *E. coli* cells were lysed by adding 5 ml of chloroform for one hour. After cell lysis, the mixture was centrifuged for 20 min at 4,000g to remove cell debris, and filtrated through a 0.1 µm membrane (EMD Millipore, MA, USA) to recover the MS2 phages. The supernatant was then concentrated using an Amicon Ultra centrifugal filter device (100 kDa; Merck Millipore, MA, USA) to obtain MS2 stocks with ~10^{11} PFU/mL concentrations. The concentration of MS2 in synthetic stormwater was ~10^5 PFU/mL. This is ~4 orders of magnitude higher than those levels reported to occur in natural stormwater[19]. These high levels were necessary to avoid non-detects in the effluent.

2.5 Properties of microbial cells

The sizes of microbial particles were determined using time-resolved dynamic light scattering. A robust light scattering precision instrument (NanoBrook Omni, Brookhaven Instruments Corporation, Holtsville, NY, USA), equipped with a 50 mW solid state laser at 532 nm was used. In brief, a 3 mL volume of microbial suspension (in synthetic stormwater without NOM) was placed in a disposable plastic cuvette and illuminated with the laser to obtain the particle size distribution. The concentrations of bacteria and MS2 were ~10^5 CFU/ml and 10^{11} PFU/ml, respectively. The relatively high MS2 concentration was required to generate an optimum count rate for a reliable estimate of the hydrodynamic diameter. Measurements were done in triplicate.

Zeta potentials of the microbes were measured in deionized (DI) water and synthetic stormwater using a Zetasizer (Malvern Instruments Ltd, Worcestershire, UK). 1 mL of prepared bacteria or MS2 suspension was placed in contact with the universal dip cell in a standard

polystyrene cuvette ensuring no air bubble introduction. Twenty measurements were recorded for each organism.

The qualitative cell surface hydrophobicity (CSH) of the bacterial cells was estimated using microbial adhesion to hydrocarbon (MATH) test [23]. Bacteria laden synthetic stormwater (~10^5 CFU/mL) was mixed with n-dodecane at a 7:2 volume ratio. The mixture was vortexed for 2 min followed by 15 min phase separation. Partitioning of the bacteria into the hydrocarbon was estimated by measuring the attenuation of absorbance (from 300 to 600 nm) with an Uvikon XL UV-vis spectrophotometer (NorthStar Scientific, Leeds, UK). The CSH was calculated using the following formula:

$$\text{CSH (\%)} = \frac{A_c - A_s}{A_s} \text{x} 100 \quad \text{.....(1)}$$

where, A_c is the absorbance of the control culture not subjected to the MATH test and A_s is the aqueous phase absorbance of the cell culture subjected to the MATH test.

2.6 Laboratory-scale biofilters

Polyvinyl chloride (PVC) pipes (2.5x15 cm) were used to hold the filter media. The pipe was glued to end fittings and glass wool was inserted at both ends of the column to prevent porous media from washing out. The details of the column packing have been described elsewhere [10]. Briefly, the biofilters were dry-packed in 3-cm layers and compacted with a wooden rod. The pore volume (PV) of each biofilter was measured gravimetrically by determining the weight difference between a dry and a saturated biofilter. Biofilters were flushed with 30 PV of DI water (arranged in a down-flow configuration) using a peristaltic pump (Masterflex, L/S, Cole-Parmar,IL) to remove any suspended particulates from the pore-water.

2.7 Column experiments

Column experiments were performed in three steps: 1) injecting 4 PV of sterile stormwater (conditioning); 2) feeding 4 PV of microbe-contaminated stormwater; and 3) switching the feed to 4 PV of sterile stormwater. Due to limitations on sample throughput capabilities only the first two steps were performed for MS2 experiments to characterize the rising limb of the breakthrough curve. A Masterflex L/S precision variable-speed peristaltic pump was used for feeding the stormwater into the column in an up-flow configuration. The up-flow columns ensured saturated condition and lowered the possibility of flow short-circuiting [24]. The approach velocity of the water was maintained at 12.3 cm h^{-1} (equivalent to a volumetric flow rate of 1 mL min^{-1}). A total of 24 column experiments were performed: four microbial targets were tested using two types of media in triplicate. The triplicates consisted of three separate columns for each microbe for each of the two media types. All experiments were conducted at room temperature (~25°C).

Column effluent was collected in 0.5 PV (for bacteria) or 1 PV (for MS2) fractions. Bacterial concentrations were measured using spread plate technique with agar plates: LB agar for *E. coli*; BHI agar for *Staphylococcus aureus*; tryptic soy agar (TSA) for *Salmonella enterica* serovar Typhimurium. MS2 detection and quantification was performed using the single agar layer method [25]. The detection limits of the spread plate and single agar layer method were 10 colony forming units (CFU)/mL and 1 plaque forming unit (PFU)/ml of effluent, respectively. Each sample was characterized using 2 decimal dilutions (except for samples with low CFU counts, e.g., samples collected during 0-1 and 7-8 PV) with duplicate technical replicates per dilution. Bacterial and MS2 phage counts on duplicate plates ranging between 10 to 200 CFU and 1 to 200 PFU, respectively, were used to calculate bacteria and MS2 concentrations and

averaged. Concentrations of bacteria or MS2 in the effluent obtained during the conditioning of the column were found to be below the detection limits.

Observed effluent bacterial (CFU/ml) or bacteriophage (PFU/ml) concentrations (C) were normalized by influent concentrations (C_0) and plotted against PV to construct breakthrough curves. The $\log_{10}$ removal of bacteria in a column was calculated by performing numerical integration of the average breakthrough curve (constructed from three column experiments) for a given experimental condition. As we did not collect the data to create full breakthrough curves for MS2 (see previous section), its removal was calculated by averaging the maximum C/C_0 values at the plateau of the breakthrough curve. All the reported values, including $\log_{10}$ removal and maximum C/C_0 (average of the C/C_0 values observed at the plateau of a breakthrough curve) values are reported as average ± standard deviation. Two sample t-tests were performed using R statistical software to test for differences in $\log_{10}$-removal under different experimental conditions. Statistical significance was defined as $p<0.05$. Spearman's ρ was used to test the strength of the association between $\log_{10}$ removal and cell surface hydrophobicity.

2.8 Theoretical prediction for microbial retention in porous media

Theoretical removal of microbes in the biofilters was calculated using colloid filtration theory (CFT). CFT is described in detail by others[26,29,31]. Briefly it conceptualizes the removal of particles (in this case bacteria and viruses) in saturated porous media (here laboratory scale biofilters) as a two-step process. In the first step, particles are transported into close proximity of the porous media grains via Brownian diffusion, gravitational sedimentation, and interception (represented mathematically as a collector efficiency η_0). In the second step, the particle attaches

to the porous media surface (represented mathematically as the attachment efficiency α that varies from 0 to 1). η_0 can be calculated using a correlation equation [26]:

$$\eta_0 = 2.4A_s{}^{0.333}N_R{}^{-0.081}N_{Pe}{}^{-0.715}N_{vdW}{}^{0.052} + 0.55A_sN_R{}^{1.675}N_A{}^{0.125} + 0.22N_R{}^{-0.24}N_G{}^{1.11} N_{vdW}{}^{0.053} \quad \text{.....(2)}$$

where all the variables in the equation are dimensionless. A_s is a porosity-dependent parameter; N_R is the aspect ratio that represents the ratio of particle size and collector size; N_G is the gravity number that describes removal due to sedimentation; N_A is the attraction number that represents the combined effect of van der Waals attraction force and particle interception; N_{Pe} is the Peclet number that represents the relative importance of advective and diffusive transport; and N_{vdW} is the van der Waals number that represents van der Waals attraction forces.

The deposition rate constant with units of 1/time, K_d can be computed using the following equation [26]:

$$K_d = \frac{3(1-f)V\alpha\eta_0}{2fd_c} \quad \text{.....(3)}$$

The deposition rate constant can be used, in turn, to compute theoretical $\log_{10}$ reduction values (LRV)[26,29]:

$$LRV = \frac{fLK_d}{2.303V} \quad \text{.....(4)}$$

where V is the flow velocity, L is the length of the column, d_c is the average filter media diameter, α is the attachment efficiency, and f is the porosity of the packed media. Additional details are described in the SI.

2.9 Interaction energy calculation

One of the factors that can influence α is the electrostatic interaction between the particle and the porous media grains[26-29]. The interaction energy between the microbial and grain surface due to van der Waals and electrostatic double layer forces can be estimated using Derjaguin, Landau, Verwey, and Overbeek (DLVO) theory [27]. We adapted the Wiese and Healy expression for a sphere–flat plate system [28, 29] to calculate the DLVO interaction energy between the microbes and biochar. Details are in the SI.

3. Results

3.1 Physicochemical properties of microbes and porous media

In stormwater, the average hydrodynamic diameters of *Staphylococcus* and *Salmonella* were (average ± standard deviation) 1888±5 nm and 1540±57 nm, respectively (Figure 1a). The hydrodynamic diameter of *E. coli* was 1484±86 nm. The hydrodynamic diameter of MS2 was 71±4 nm.

All bacterial species had negative surface charges (Figure 1b); however, the average zeta potentials (± standard deviations) of *E. coli* (-23.4±0.3 mV) and *Staphylococcus* (-21.2±0.3 mV) in stormwater were more negative than those of *Salmonella* (-11.7±0.4 mV) and MS2 (-13.7±1.6 mV).

At all wavelengths considered, the hydrophobicity values of pathogens *Staphylococcus* and *Salmonella* were greater than that of *E. coli* (Figure 1c). At 420 nm, the most common wavelength at which CSH is reported, the hydrophobicity values (± standard deviation) were 91±3% for *Staphylococcus*; 17±2% for *E. coli*; and 80±5% for *Salmonella*.

Approximately 50% of the biochar particles were smaller than 0.3 mm (Figure 1d). The zeta potential of biochar was measured as -19.6±0.34 mV (see SI). The water contact angle

(WCA) and surface area of sand and biochar particles are reproduced from the previous publication [10]: WCA >100° for biochar, sand was completely wettable; the specific surface areas were 104 and 0.2 m^2/g for biochar and sand, respectively.

3.2 Bacterial removal in biofilters.

The breakthrough curves for bacterial transport in biofilters are presented in the SI (Figure S1). The experimental log_{10} reductions (average ± standard deviations of triplicate experiments) of bacteria in biochar-amended biofilters were 1.9±0.1 (*E. coli*), 3.9±0.2 (*Staphylococcus*), and 3.7±0.2 (*Salmonella*) (Figures 2a and 2b). These values were significantly higher ($p<0.05$) when compared to bacterial removal in biofilters packed with sand. Observed average log_{10} removal of bacteria in sand biofilters was ~0.3. In the biochar-amended biofilters, removal of *Staphylococcus* and *Salmonella* is significantly ($p<0.05$) greater than the removal of *E. coli* by approximately 2 log_{10} units.

3.3 MS2 removal

The breakthrough curves for MS2 transport in biofilters are presented in Figure S2. Log_{10} removal (± standard deviation) of MS2 bacteriophage was significantly greater in biochar-amended biofilters compared to sand biofilters (1.76±0.32 versus 0.39±0.07, $p<0.05$). Removal of MS2 in sand biofilters is similar to the removal of bacteria in sand biofilters. Removal of MS2 in the biochar-augmented biofilters is similar to that observed for *E. coli*, but less than that observed for pathogenic bacteria ($p<0.05$).

3.4 Theoretical maximum depositional rate constants.

The single collector efficiency (η_0) of MS2 bacteriophage was at least one order of magnitude higher than the bacteria (Figure S3). η_0 for bacteria ranged from 0.019 to 0.0036 for *E. coli*,

0.020 to 0.0031 for *Staphylococcus* and 0.019 to 0.0035 for *Salmonella* (reported range is for collector diameters ranging from 0.1 to 0.8 mm). However, in the case of MS2, η_0 ranged from 0.15 to 0.039.

η_0 values were used to compute deposition rate constants for biochar and sand particles (Table 1). Assuming $\alpha = 1.0$ (perfectly favorable conditions for deposition), the theoretical maximum rate constants were one order magnitude higher for biochar collectors ($\sim 10^{-3}$ s^{-1} for bacteria and $\sim 10^{-2}$ s^{-1} for MS2) compared to sand collectors ($\sim 10^{-4}$ s^{-1} for bacteria and 10^{-3} s^{-1} for MS2). Regardless of the type of collector, biochar or sand, the theoretical maximum deposition rate constants for MS2 were one order of magnitude greater than those for bacteria.

3.5 Theoretical maximum log-removal values (LRV) of microorganisms

Using η_0 and the maximum deposition rate constants (which assume $\alpha = 1$), the theoretical maximum LRVs were calculated using equation 4 (Tables S2 and S3). For 15-cm deep sand biofilters, theoretical maximum LRVs were 0.38 for *E. coli*, 0.33 for *Staphylococcus aureus,* 0.38 for *Salmonella,* and 3.8 for MS2 bacteriophage (Table 1). For the biochar-amended biofilters, computed theoretical maximum LRVs were 0.68 for *E. coli*; 0.55 for *Staphylococcus;* 0.69 for *Salmonella;* and 6.67 for MS2 bacteriophage (Table 1).

3.6 DLVO energy barriers

DLVO interaction energy, as a function of separation distance between microbes and biochar, was computed following the adapted Wiese and Healy expression for a sphere–flat plate system (Figure 3). The resultant barrier (the highest resultant energy), relative to thermal energy, KT (where K is the Boltzmann constant and T is temperature) for biochar-*E. coli*, biochar-

Staphylococcus, and biochar-*Salmonella* were 256 KT, 286 KT, and 74 KT respectively. The DLVO energy barrier for biochar-MS2 was 7 KT.

4. Discussion

4.1 Biochar-amendment enhances bacteria and bacteriophage removal

We observed higher retention of pathogenic bacteria (i.e., *Staphylococcus aureus* and *Salmonella enterica)* and *E. coli* in biochar-amended biofilters compared to sand biofilters. The observed $\log_{10}$ reductions for pathogens in biochar-amended biofilters are nine-fold higher than sand biofilters. Biochar also demonstrates potential for virus removal as we observe three-fold increase of bacteriophage removal in the biochar-amended biofilters. However, amending biofilters with biochar offered more advantage in terms of bacterial (nine-fold increase) removal compared to bacteriophage (three-fold increase) removal. Our results corroborate previous studies that report higher microbial removal in biochar-amended porous media compared to un-amended media [10, 15]. The results demonstrate the potential of biochar-amended filter media for enhanced microbial removal in stormwater biofilters, thereby protecting receiving water quality and public health.

Below we explore mechanisms whereby the tested bacteria and virus may be removed in the experimental biofilters. We explore the potential for electrostatic interactions, straining, and hydrophobic interactions to control the removal of microbes by the porous media. We first discuss these mechanisms for bacteria, and then virus.

4.2 Bacterial removal mechanisms

The observed bacterial removal in sand biofilters is comparable to the theoretical maximum LRV estimated using colloid filtration theory (CFT). However, the observed bacterial removal in the biochar-amended biofilters is 2.8 to 7 times higher than the theoretical maximum LRVs, suggesting that CFT is insufficient to explain bacterial removal in the biochar-amended biofilters. Moreover, the energy barrier between bacteria and biochar is too high for electrostatic deposition of bacteria in biochar-amended biofilters[28-31]. This suggests that removal mechanisms not considered in CFT likely contribute to bacterial removal in biochar-amended biofilters. Additional removal mechanisms not included in CFT include straining and attachment via non-DLVO forces such as hydrophobic forces [32, 36, 37].

Straining occurs when the ratio of the bacterial cell diameter to collector diameter is greater than 0.007 [38]. Given the measured bacterial diameters, the ratio is exceeded when the filter media particles are smaller than 250 μm. Therefore, given the large particle size of the sand grains (large than 600 μm), straining of bacterial cells is unlikely to be important in the sand biofilters. The particle size distribution of the biochar used in this study indicates about 40% (by mass) of the biochar particles have diameters smaller than 250 μm suggested that straining may contribute to bacterial removal in biochar-amended biofilters. However, given that the biochar and bacteria in the experiments are of similar sizes, removal due to straining should be similar for all three bacterial species. As we found that removal varied among the bacteria, another removal mechanism that varies among the experimental bacteria may be important.

Removal promoted by hydrophobic interactions between bacteria and collectors may be a possible explanation for the observed differences in $\log_{10}$ removal among three bacterial species. Biochar has high organic carbon content and high hydrophobicity [39]. Strong hydrophobic attraction between bacteria and the biochar surface may help overcome the high DLVO energy

barrier and enable deposition. *Staphylococcus* had the highest hydrophobicity, followed by *Salmonella*, and then followed by *E. coli*. There is a positive correlation between CSH of bacterial targets and their $\log_{10}$ reduction (Spearman's $\rho = 0.79$, $p<0.05$). This is consistent with the conclusion in another study[15] that hydrophobic interactions contribute to bacterial removal in biochar-amended soil. Additional interaction energy computation using directly measured hydrophobic forces and extended DLVO theory could be used to quantify the contribution of hydrophobic forces to the bacterial removal in biochar-amended collectors[40, 41], however such an effort is beyond the scope of the present study.

4.3 Virus removal mechanisms

The observed LRVs for MS2 (both in sand and biochar-amended biofilters) are less than the predicted theoretical maximum LRVs suggesting CFT with $\alpha < 1$ (i.e., $\alpha = 0.1$ and 0.3 in sand- and biochar-amended sand biofilters, respectively) can explain the experimental observations. The low negative surface charge of MS2 combined with its smaller particle size makes the DLVO energy barrier between biochar and MS2 equal to 7 KT. This energy barrier can be overcome by Brownian diffusion or fluid drag force [33] supporting the idea that electrostatic interactions may promote virus removal in our experimental system. Electrostatic deposition of MS2 on a biochar surface was described as completely unfavorable by Sasidharan et al. [18] The deviation of our results from those of Sasidharan et al. [18] can be attributed to differences in solution chemistry (synthetic stormwater with NOM in this study vs. NaCl electrolyte solution in Sasidharan et al.), different measured surface charges on MS2 particles (-27.5 mV in Sasidharan et al vs. -11.7 mV in this study), and different feedstock and physicochemical properties of the biochars used in the studies.

Straining is not expected to be an important removal mechanism for viruses in the biofilters due to their small size[38]. However, hydrophobic interactions could potentially contribute to MS2 removal in porous media containing organic fractions [42, 43] and the high hydrophobicity of biochar particles makes biochar-MS2 hydrophobic attraction a likely mechanism for MS2 removal[44]. Further studies using multiple viruses with a wide range of cell surface hydrophobicity may shed light on the relative importance of hydrophobic interaction compared to electrostatic deposition for virus removal in biochar-amended biofilters.

4.4 Environmental implications

The relative importance of bacterial and viral removal mechanisms identified in this study can inform selection of biochar composition for biofilters designed to improve microbiological quality of urban stormwater. For example, importance of hydrophobic attachment and straining for bacterial removal emphasize the need for selecting fine biochar particles (<0.25 mm) with low volatile matter and polarity (O+N)/C ratio to promote these removal mechanisms. It is important to note, however, that using finer biochar particles in a stormwater biofilter may increase the possibility of filter clogging and thus require more maintenance. So close attention needs to be paid to ensuring that the hydraulic conductivity of biochar-amended biofilters remain sufficiently high.

Our results suggest, virus removal can be facilitated by selecting biochar with relatively low surface potential. Also, chemical modification biochar particles to develop a positive surface charge may significantly enhance virus removal in biochar-amended biofilters.

We used colloid filtration theory (CFT) and calculations of DLVO-forces to explore the mechanisms of microbial removal in sand and biochar-amended sand biofilters. CFT and DLVO force calculations require that simplifying assumptions be made. For example, they require that

particles are spherical and homogenous, and that dispersion is negligible[45]. Despite deviations from some assumptions (i.e., non-spherical particle size of the collectors and heterogenous particle size distributions), this study demonstrates the utility of these models for exploring the relative importance of different microbial removal mechanisms in biofilters. CFT was recently used by Parker et al. [46] to interpret observed FIB removal in stormwater biofilters.

Caution should be taken in extending results observed in this study to field-scale biofilters. The present study was conducted under simplified conditions. We did not explore how unsteady flow conditions, intermittent unsaturated conditions, aqueous chemistry, indigenous microorganisms, plants, and invertebrates, might affect microbial removal. Future investigations of the effect of these factors on microbial removal mechanisms in field-scale biochar-amended biofilters would be useful.

Supporting information

The Supporting Information is available free of charge and includes

Table S1: Physicochemical properties of the collectors; DLVO modeling details; details of collector efficiency and deposition rate constant calculation; and theoretical log removal value (LRV) calculation; Table S2: LRV in 4.5 cm biochar biofilter; and Table S3: LRV in 10.5 cm sand biofilter; Figure S1: Breakthrough curves for bacterial transport experiments; Figure S2: Breakthrough curves for MS2 transport experiments; Figure S3: Theoretical total single collector contact efficiency of the collectors

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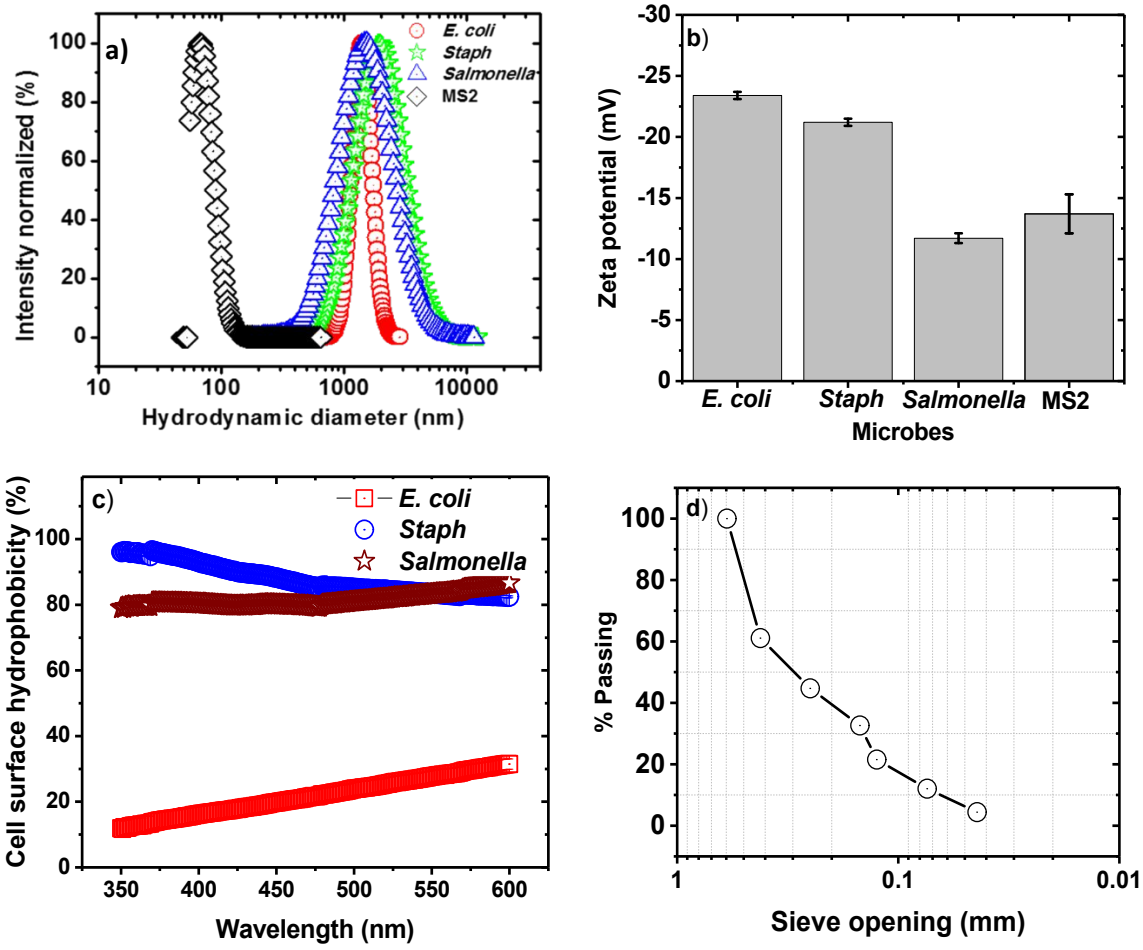


Figure 1: Physicochemical properties of the microbes and biochar: a) hydrodynamic diameter of bacteria and bacteriophage in synthetic stormwater (without NOM) determined by light scattering technique; b) zeta potential (with standard deviations as error bars) of the microbes in synthetic stormwater using electrophoretic mobility; c) hydrophobicity of the bacteria in synthetic stormwater by MATH test; d) particle size distribution of biochar particles by sieve analysis.

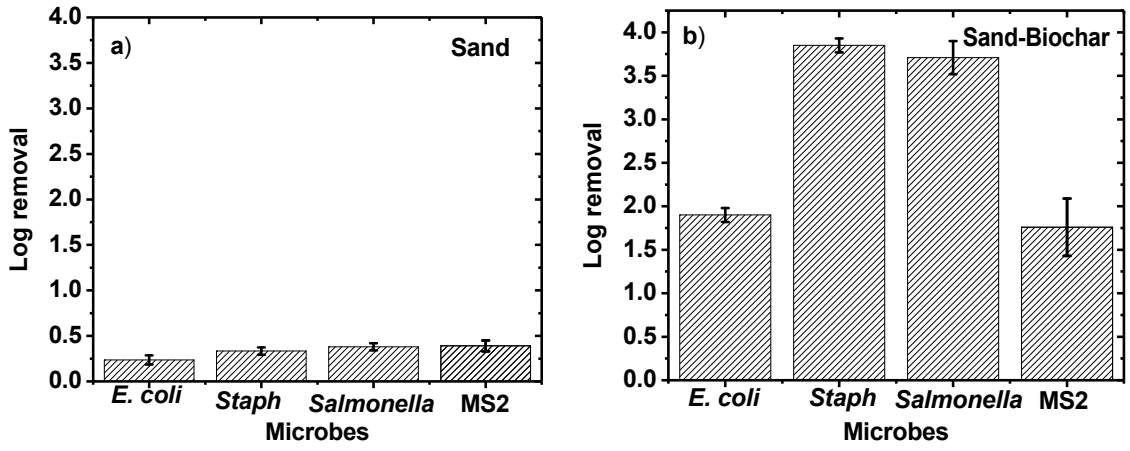


Figure 2: Log_{10} removal of the microbes in a) 100% sand and b) 70% sand, 30% biochar biofilters. Laboratory scale column (2.5 cm x 15 cm) experiments were performed in an up-flow configuration. The error bars indicate standard deviation among triplicate experiments. All experiments were conducted at the room temperature.

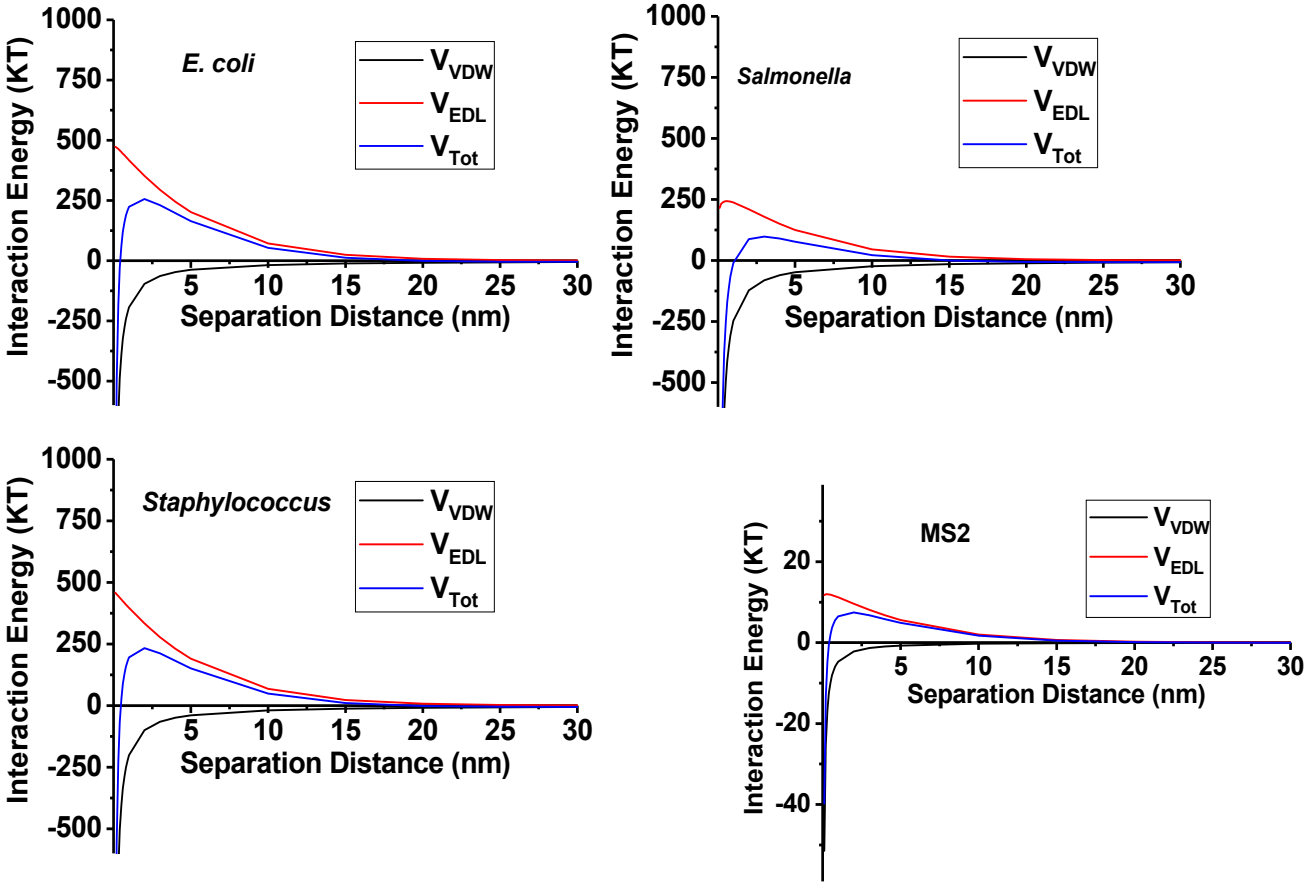
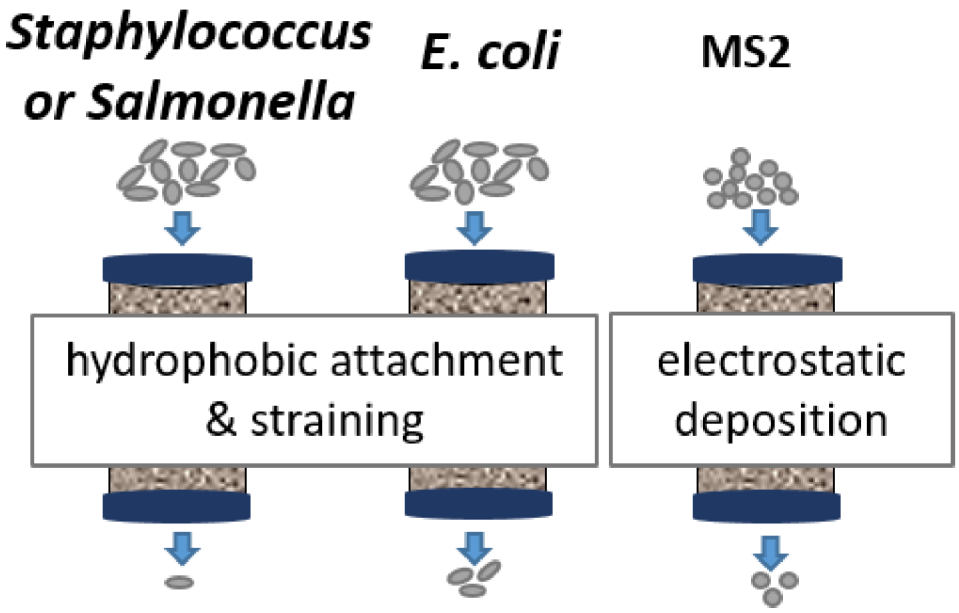


Figure 3: Theoretical DLVO interaction of biochar surface with *E. coli*, *Salmonella enterica* serovor Typhimurium, *Staphylococcus aureus*, and MS2. Interaction energies are expressed in terms of thermal energy, KT (Boltzman constant x temperature). V_{VDW} and V_{EDL} stand for van-der-Waals attractive and electrostatic double layer repulsive energy, respectively. Resultant energy is plotted as $V_{Tot.}$

Table 1: Deposition rate constants times α and maximum theoretical $\log_{10}$ removal values for the studied microbes. Observed removal includes standard deviations of triplicates.

Microbes	**K_d *α for Sand (1/s)**	**Maximum theoretical LRV in sand**	**Average LRV Observed in sand**	**K_d*α For Biochar (1/s)**	**Maximum Theoretical LRV in sand-biochar**	**Average LRV Observed in sand-biochar**
E. coli	5.2×10^{-4}	0.38	0.23±0.03	1.73×10^{-3}	0.68	1.9±0.1
Staphylococcus aureus	4.6×10^{-4}	0.33	0.33±0.02	1.33×10^{-3}	0.55	3.9±0.2
***Salmonella enterica* serovor Typhimurium**	5.3×10^{-4}	0.38	0.37±0.03	1.75×10^{-3}	0.69	3.7±0.2
MS2	5.3×10^{-3}	3.8	0.39±0.1	1.65×10^{-2}	6.67	1.8±0.3



Bacterial pathogens and pathogen indicators suspended in stormwater are removed to a greater extent in biochar-augmented sand biofilters than sand biofilters; the processes governing the removal are distinct.