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**Limited Bacterial Removal in Full Scale Stormwater Biofilters
as Evidenced by Community Sequencing Analysis**

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Abstract

In urban areas, untreated stormwater runoff can pollute downstream surface waters. To intercept and treat runoff, low-impact or “green infrastructure” approaches such as biofilters are adopted. Yet actual biofilter pollutant removal is poorly understood; removal is often studied in laboratory columns, with variable removal of viable and culturable microbial cell numbers including pathogens. Here, to assess bacterial pollutant removal in full-scale planted biofilters, stormwater was applied, unspiked or spiked with untreated sewage, in simulated storm events under transient flow conditions during which biofilter influents versus effluents were compared. Based on microbial biomass, sequences of bacterial community genes encoding 16S rRNA, and gene copies of the human fecal marker HF183 and of the *Enterococci* marker EnterolA, the removal of bacterial pollutants in biofilters was limited. Dominant bacterial taxa were similar for influent versus effluent aqueous samples within each inflow treatment of either spiked or unspiked stormwater. Bacterial pollutants in soil were gradually washed out, albeit incompletely, during simulated storm flushing events. In post-storm biofilter soil cores, retained influent bacteria were concentrated in the top layers (0-10 cm), indicating that the removal of bacterial pollutants was spatially limited to surface soils. To the extent that plant-associated processes are responsible for this spatial pattern, treatment performance might be enhanced by biofilter designs that maximize influent contact with the rhizosphere.

Keywords

44 Stormwater; Biofilters; Microbial community; Transient flow; Source tracking
45

46 **Table of Contents**

47

Introduction

Global urbanization has increased impervious surfaces including roof tops, driveways, parking lots, and streets, resulting in increased stormwater runoff volumes and peak flows. Urban stormwater runoff has been identified as a major pollution source impacting receiving water bodies such as streams, rivers, and coastal waters.¹ Physical, chemical, and microbial pollutants including sediments, nutrients, metals, organic pollutants, and pathogens are transported by stormwater into receiving waters.^{2,3} Mitigation of pollutant loads in stormwater runoff is thus essential for improving receiving water quality. Meanwhile, population growth and climate change are exacerbating short supplies of pristine waters in arid and semi-arid regions. Less desirable water sources such as stormwater runoff have been utilized to augment water supplies.⁴ The challenges of diffuse urban stormwater runoff have historically been addressed by engineered systems. However, increasingly, other low-impact stormwater management techniques are emerging including source control measures such as water sensitive urban designs and green infrastructure approaches.^{5,6}

Biofilters, consisting of a basin or trench filled with planted sand- or soil-based filter media, are widely used green infrastructure stormwater source-control approaches.^{2,3,7,8} In biofilters, stormwater percolates from the surface downwards, retarding runoff volume and peak flow, while retaining or removing pollutants via biotic and abiotic processes. Biofilters can remove suspended sediments and metals from stormwater,⁹⁻¹¹ whereas the attenuation of nutrients and organic pollutants such as pesticides, flame retardants, and chemicals (e.g., benzotriazoles) are highly variable.¹²⁻

¹⁴ The reported removal efficiencies of microorganisms including bacteria, viruses, and protozoa from stormwater passing through biofilters also vary.^{4,8,15} For achieving predictable treatment outcomes, the design and use of biofilters for pathogen removal requires more understanding of pathogen retention and removal, especially for full-scale systems under realistic storm conditions.

Prior studies have monitored fecal indicator bacteria (FIB) as pathogen surrogates in biofilters,^{16,17,8} but FIB can originate from animal feces that are of lower risk to human health than human fecal sources.¹⁵ Therefore, the human fecal marker *Bacteroides* HF183 has been used to trace human waste-associated microbes during stormwater biofiltration.^{15,18} Several pathogens such as *Campylobacter* spp., *Clostridium perfringens*, *Salmonella enterica* and *Staphylococcus aureus*, have also been investigated in stormwater biofilters, but their concentrations did not correlate with either FIB or other pathogens in biofilters.^{16,19,20} Biofilter retention and removal of most bacteria, including putative pathogens, could be assessed through community analysis.²¹ Yet, bacterial community composition has not been used comprehensively to assess bacterial removal from the influent versus effluent over the storm hydrograph, and the role of biofilter media. Combined with statistical analyses such as linear discriminant analysis of effect sizes (LEfSe),²² a Bayesian algorithm SourceTracker,²³ and a modified algorithm fast expectation-maximization microbial source tracking (FEAST),²⁴ bacterial community analysis can allow for understanding the components of the effluents and the fates of overall bacterial communities during infiltration.

In this study, bacterial community composition of stormwater runoff, biofilter soil

media, and biofilter effluent were comparatively analyzed for 4 full scale biofilters of 2 depth regimes undergoing simulated storm events under realistic, transient flow, conditions. Two questions were addressed: 1) how effective are full-scale stormwater biofilters at removing bacteria and retaining them during successive storms? and 2) how does media depth relate to bacterial retention, and its corollary wash-out, through stormwater biofilters? The results contribute to understanding achievable stormwater treatment by full scale biofilters as defined by bacterial, including putative pathogen, removal. Such results are needed to guide future stormwater management including widescale implementation of green infrastructure.

Materials and methods

Biofilters, stormwater filtration, sampling, and sample handling

Four full scale biofilters (C1-C4) at the Orange County Public Works (OCPW) Glassell campus (Orange, CA) were used for challenge experiments with stormwater or sewage-spiked stormwater (mixed influent) as influent. Because human sewage contains copious bacterial human pathogens and most other biotic contaminants common to urban stormwater runoff, this study used raw sewage mixed with stormwater as simulated fecal contaminated stormwater runoff. Each biofilter was 2.4 m long, 1.5 m wide, and 1.8 m deep (Fig. S1 and Table S1). The soil (approximately 0.3 m deep for biofilters C1 and C3, and 0.6 m deep for C2 and C4) was a mixture of fines, sand, and compost to achieve the gradation of 85-88% sand, 8-12% fines (from sandy loam top

soil), and 3-5% organic matter. The biofilters were initially constructed and planted in January 2017, then replanted with *Carex spissa* (San Diego sedge) in February 2019. Besides occasional rain, potable water was used for irrigation.

Transient flow conditions through the biofilters, as would occur during actual storm events, were mimicked following a realistic hydrograph observed in Orange County (Fig. S2). The applied stormwater had been stored in an underground cistern from January to April (2019) and consisted of runoff collected onsite from the adjacent parking lot and a modular treatment wetland. A mixed influent was prepared by combining raw sewage with stored stormwater at a volume ratio of 1:1. Primary influent sewage (750 L) passing the bar screen was collected daily from the Orange County Sanitation District wastewater treatment plant and was then mixed with 750 L stormwater in a 2 m³ tank onsite for each transient flow experiment. The mixing tank was thoroughly cleaned and disinfected (using household bleach) between experiments. Detailed information on the experimental set-up can be found in Parker et al.²⁵

Stormwater or mixed influent were applied to the 4 biofilters under transient flow conditions during 3 experimental phases in May-June, 2019 (Fig. S3). In the first phase, a simulated storm event was conducted whereby each of the 4 biofilters was individually infiltrated with stormwater. Biofilters C1 and C2 served as controls for C3 and C4, and so were sacrificed after phase 1 to collect baseline soil cores. Biofilters C3 and C4 were then infiltrated in a 2nd phase with mixed influent consisting of the 1:1 mix of stormwater and untreated sewage. Soil cores were next collected from biofilter C3, while biofilter C4 was flushed with sewage-free stormwater in a 3rd phase for 4

successive storms. The flushing allowed for assessing retention and release of captured bacterial pollutants before collecting endpoint C4 soil cores.

Initial hydrological experiments using tap water and bromide indicated that the biofilter would outflow for around 2-3 h under transient flow conditions.²⁵ Thus, each transient flow experiment was planned to last approximately 2-3 h, and 6 to 10 flow-weighted composite effluent samples were collected during this period for each experiment, alongside influent stormwater, untreated sewage, or mixed influent samples. The composite stormwater effluent samples were collected using a peristaltic pump drawing from a port located at the discharge of the underdrain inside each biofilter. Water samples were processed in OCPW's water quality laboratory onsite immediately after collection. A maximum 100 mL was vacuum filtered through 0.22 μm filters (MicroFunnel Filter Funnels, PALL Co.), with the volume of water filtered recorded. A filtration blank using sterile Nanopure (Barnstead Thermolyne, Rockland, MA) water was included for each transient flow experiment. Filters were stored on dry ice until transport to the University of California, Santa Barbara (UCSB), and then maintained (-20 °C) until DNA extraction.

Before coring biofilter soils, surface plant material was manually removed by clipping. Clean stainless steel corers (7.6 cm diameter, 45.7 cm long) lined with metal rings (disinfected with 70% ethanol; residual evaporated) were pushed into the ground using a coring rig or, where necessary, a sledge hammer. Six cores were acquired for each biofilter. Once soil cores were obtained, clean metal scrapers were used to cut between metal rings and obtain the desired core intervals. The cored material for each

depth interval was extruded, composited, and sieved (2 mm pore size) fresh, and the six composited soil cores from each depth segment were homogenized into one clean Ziploc bag to obtain one composite sample per depth interval. For biofilters C1 and C3, composite samples at depths of 0-10 cm, 10-20 cm, and 20-30 cm were individually collected; for biofilters C2 and C4, composite samples at depths of 0-10 cm, 10-20 cm, 30-40 cm, and 50-60 cm were individually collected. Soil eluent was freshly generated in the lab onsite for each composite soil sample using a published method,²⁶ and approximately 30 mL of the eluent was filtered through 0.22 μ m filters (MicroFunnel Filter Funnels, PALL Co.) for bacterial recovery, similarly to other aqueous samples. The remaining composited soil samples were stored on dry ice during transport to UCSB, and maintained (-20 °C) until DNA extraction.

DNA extraction, qPCR, and sequencing

DNA extraction from aqueous sample filters was performed using the DNeasy PowerWater kit (Qiagen, Carol Stream, IL). DNA from composited soil samples was extracted using the DNeasy PowerSoil Kit (Qiagen). Duplicate extractions were performed for each soil sample, with the extracted DNA pooled. An extraction blank was included for each filter or soil extraction batch. After DNA concentrations were quantified (Quant-iT dsDNA Broad-Range Assay Kit; Invitrogen, Carlsbad, CA), DNA extracts were archived (-20 °C) until analysis.

Enterococci were quantified using the Entero1A quantitative polymerase chain reaction (qPCR) assay.²⁷ The details of this qPCR assay are described in the SI Methods.

180 All individual qPCR plates had efficiencies of between 96% and 102% with an R^2
181 of >0.998 to 1.000. The human fecal marker HF183 was quantified using the
182 HF183/BacR287 assay²⁸ in simplex format and performed by Source Molecular
183 Corporation (Miami Lakes, FL).

184 Genes encoding 16S rRNA were sequenced on an Illumina MiSeq platform using
185 a MiSeq v3 600 cycle kit (2 by 300 bp) in the California NanoSystems Institute (CNSI),
186 UCSB. The details of DNA amplification, purification, and normalization were as
187 before²¹ (SI Methods). The sequencing data was deposited in NCBI SRA with the
188 BioProject ID PRJNA723423.

189

190 *Bioinformatic and statistical analyses*

191 Illumina sequencing data were processed using the Quantitative Insights Into Microbial
192 Ecology (QIIME v1.9.1) pipeline with default settings,²⁹ with details as before²¹ (SI
193 Methods). After quality filtering, 14,322,411 sequences were obtained for all samples.
194 Sequences were grouped into operational taxonomic units (OTUs) at 97% sequence
195 similarity. A representative sequence for each OTU was picked, and taxonomic data
196 were assigned using the Greengenes 13_8 aligned reference database.

197 Raw sequences for each sample were also processed and aligned through the
198 16SPIP pipeline against a human pathogen 16S rRNA sequence database consisting of
199 29,258 sequences representing 346 bacterial species.³⁰ The BWA-MEM algorithm was
200 utilized for sequence alignment, and the sequences with similarity higher than 99% to
201 reference sequences were identified as taxa of potential human health concern. These

taxa are only potential human pathogens and their pathogenicity is uncertain.

The weighted UniFrac distance matrix generated in QIIME was used for non-metric multidimensional scaling (NMDS) with PRIMER 6.³¹ Bacterial genera significantly associated with stormwater, sewage, and biofilter soil were determined with the LEfSe (the linear discriminant analysis effect size) algorithm²² as well as the DESeq2 method within QIIME. Source proportion analysis was performed using SourceTracker 1.0²³ and FEAST²⁴ with default parameters. For analyzing effluent sources on the basis of bacterial communities, the stormwater, sewage, and biofilter soil sequences were designated as sources, and the biofilter effluent sequences were designated as sinks. For analyzing bacterial communities that were sources to the biofilter soils, the stormwater and sewage sequences were designated as sources, while the soil sequences were sinks. Average source proportions were obtained by individually executing SourceTracker and FEAST in triplicate. Heatmaps were generated using Heatmapper.³² Additional statistical analyses such as Wilcoxon tests (Mann-Whitney for two categories, or Kruskal-Wallis with Steel-Dwass for all pairs comparisons for three or more categories), Spearman rank correlation, and ANOSIM were performed using JMP10 (SAS, Cary, NC) or PRIMER 6.

Results

Stormwater, sewage, and mixed influent bacterial communities

The stormwater was dominated by Betaproteobacteria, Alphaproteobacteria, and

224 Bacteroidetes (Fig. 1). Most bacterial genera specifically associated with stormwater,
225 as identified using LEfSe and the DESeq2 method (Fig. 2), were typical to aquatic,
226 sediments and soil environments, such as *Flavobacterium* spp., *Sediminibacterium* spp.,
227 and *Limnohabitans* spp.. Some stormwater bacterial genera have been associated with
228 specific functions, including *Novosphingobium* that can degrade aromatic compounds
229 such as phenol, aniline, nitrobenzene and phenanthrene,³³ *Methylomonas* and
230 *Methylosinus* that metabolize methane, and *Phenylobacterium* that grow using
231 chloridazon–mineral salts.³⁴ The average relative abundance of potential human
232 pathogens in stormwater was low (0.01%; Table S2) comprised of approximately three
233 species: *Pseudomonas aeruginosa*, *Afipia lausannensis*, and *Acinetobacter baumannii*.
234 Stormwater contained low levels of fecal markers (Table S3), with the human marker
235 HF183 not detectable in most samples, and the EnterolA concentration at less than
236 1.25E+03 copies/100 mL.

237 Raw sewage was enriched with Firmicutes and Epsilonproteobacteria (Fig. 1), and
238 most bacterial genera significantly associated with sewage were human gut
239 microorganisms such as *Streptococcus*, *Blautia*, *Bacteroides*, and *Neisseria* (Fig. 2).
240 Some genera such as *Arcobacter*, *Cloacibacterium*, and *Trichococcus* are typically
241 found in WWTPs.^{35,36} Sewage contained more potential human pathogens, with the
242 relative abundance averaging up to 1.67% and the average number of species up to 36,
243 dominated by *Streptococcus suis*, *Streptococcus lutetiensis*, and *Klebsiella pneumoniae*
244 (Table S2). High HF183 and EnterolA concentrations were quantified in sewage,
245 averaging 3.00E+06 and 1.97E+07 copies/100 mL, respectively (Table S3).

The mixed influent (1:1 stormwater and sewage) bacterial communities were similar to those in sewage (Figs. 1 and 3), likely due to the high biomass in sewage as compared to stormwater (Table S3). Sewage specific genera were thus abundant in the mixed influent (Fig. 2), and the total relative abundance of potential human pathogens in mixed influent was 1.53%, with the number of potential pathogen species averaging 25 (Table S2).

Biofilter soil and eluents

Besides Betaproteobacteria and Alphaproteobacteria, a number of bacterial clades such as Actinobacteria, Acidobacteria, Chloroflexi, Deltaproteobacteria, Planctomycetes, Gemmatimonadetes, and Nitrospirae were also abundant in biofilter soil (Fig. 1). Biofilter soil-specific bacterial genera included the typical soil nitrite-oxidizing bacteria *Nitrospira* (Fig. 2). No potential human pathogens were identified in any biofilter soil samples (Table S2). When comparing to biofilters C1 and C2 that were only dosed with stormwater, bacterial genera specifically associated (as revealed by LEfSe and the DESeq2 methods) with the soils of biofilters C3 and C4 (dosed with stormwater then 1:1 mixed influent) were from sewage, such as *Arcobacter*, *Cloacibacterium*, *Bacteroides*, and *Streptococcus* (Fig. 4). Furthermore, these taxa were relatively abundant at the surface (0-10 cm) of both biofilters C3 and C4 with the total relative abundance of 0.5% and 0.4%, respectively, and present in the depth of 10-20 cm of C3 (0.3%), but generally absent over the depth intervals of 20-30 cm of C3 and 10-60 cm of C4 (all less than 0.2%). These similarities between C3 and C4 soil bacterial

communities were despite that C4 had been flushed with stormwater before coring.

Soil eluent bacterial communities were very similar to those of soil samples (Fig. 1), but they still formed two distinct clusters by NMDS analysis (Fig. 3). Soil eluent harbored potential pathogens (albeit at a low abundance averaging 0.033%), with the highest relative abundance also associated with the top layers (0-10 cm) of biofilters C3 and C4.

Biofilter effluents

When the 4 biofilters initially received only stormwater, biofilter effluent contained many bacterial taxa that were enriched in, or specific to, stormwater (Fig. 1 and 2), and the dominant effluent bacterial genera were also abundant in stormwater (Fig. S4). NMDS analysis confirmed that effluent bacterial communities were more similar to those in stormwater influent than to those in biofilter soils (Fig. 3). The average total relative abundance of potential pathogens in biofilter effluents was 0.022% and the average number of potential pathogen species was approximately six which was comparable to stormwater influent (Table S2). The fecal markers HF183 and EnterolA in biofilter effluents were either not detectable or at similar levels as the stormwater influent (Table S3).

Sewage-enriched bacteria and specific genera were abundant in the effluents of biofilters C3 and C4 when receiving mixed influent (Fig. 1 and 2), and the most abundant genera in the effluents were also abundant in sewage (Fig. S4). As such, the communities in the effluents of biofilters C3 and C4 were highly similar to mixed

influent and sewage (Fig. 3). The average total relative abundance of potential human pathogens approached 1.26% in the effluents (Table S2), and the average number of potential pathogen species was approximately 30. The dominant potential pathogen species were *Streptococcus suis*, *Streptococcus lutetiensis*, *Aeromonas punctata*, and *Klebsiella pneumoniae*, similarly to the mixed influent. Both fecal markers HF183 and EnterolA were at quantifiable levels in the effluents (Table S3).

During the stormwater flushing of C4, the relative abundances of sewage-associated genera gradually decreased in biofilter C4 effluents with each flushing event, and across all 4 rounds (Fig. 1, 2 and S5), while stormwater specific bacteria gradually increased in the effluents during each round (Fig. 2 and S5). Accordingly, the bacterial communities in NMDS analysis exhibited a clear trend, from the first round to the last, of gradually decreasing similarities to sewage and to the mixed influent and associated effluents, while showing increasing similarities to stormwater and to the effluents from earlier rounds of stormwater treatment (Fig. 3). The average total relative abundance of potential human pathogens decreased in effluents, and was 0.69%, 0.37%, 0.27%, and 0.19% over the first to fourth rounds of flushing, respectively; the total relative abundance also decreased within each round of flushing (Table S2). Although the log-reduction values were highly variable between HF183 and EnterolA when comparing their effluent concentrations with those in mixed influent, both markers appeared to decrease in the effluent during the 4 successive rounds of flushing in biofilter C4 (Table S3).

312 *SourceTracker and FEAST analyses*

313 The contributions of stormwater, raw sewage, and biofilter soil to bacterial
314 communities in biofilter effluents were simulated by SourceTracker and FEAST (Fig.
315 S6). The results of these two software approaches were well correlated (Spearman test,
316 all $\rho > 0.890$, all $p < 0.0001$). Stormwater bacterial communities dominated the effluents
317 of all 4 biofilters when stormwater was the influent, with the average percentage being
318 40.0% and 38.7% by SourceTracker and FEAST, respectively. In contrast, the average
319 proportion of the effluent bacterial community sourced from biofilter soil was 11.8%
320 and 17.4% in 4 biofilters by SourceTracker and FEAST, respectively. Such
321 contributions of stormwater or soil to effluents simulated by SourceTracker and FEAST
322 correlated well with the total relative abundances of bacterial genera sourced from
323 stormwater or soil in the effluents of 4 biofilters (Fig. S5) (Spearman test, all $\rho > 0.839$,
324 all $p < 0.0001$). When using mixed influent, sewage became the predominant source of
325 effluent bacterial populations in biofilters C3 and C4, with the average proportion up to
326 58.6% and 41.8% by SourceTracker and FEAST, respectively. In contrast,
327 contributions of stormwater and soil to effluents of biofilters C3 and C4 were
328 comparably trivial, with the average percentages of 4.89% and 4.70% for stormwater,
329 and 2.79% and 4.06% for soil by SourceTracker and FEAST, respectively. The
330 predicted percentages of sewage in effluents of biofilters C3 and C4 when using mixed
331 influent correlated well with the total relative abundance of sewage-sourced bacterial
332 genera in effluents (Fig. S5) (Spearman test, both $\rho > 0.930$, both $p < 0.0001$ for
333 SourceTracker and FEAST). Lastly, during 4 rounds of stormwater flushing in biofilter

C4, contributions of stormwater to effluent bacterial communities increased from 11.0% and 12.6% at the beginning of flushing to 43.3% and 41.2% at the end as predicted by SourceTracker and FEAST, respectively. The percent stormwater contribution also increased within each cycle of flushing, in accordance with the increased total relative abundance of stormwater-sourced bacteria in effluents of biofilter C4 during each cycle of flushing (Fig. S5). The percentage of sewage as a bacterial community source to C4 effluent decreased significantly with each flushing event, from approximately 20% to 3% after 4 rounds of flushing. The contributions of biofilter soils to C4 effluent bacterial communities were similar across all flushing rounds with average percentages of 11.2% and 12.9% by SourceTracker and FEAST, respectively. Overall, the simulated proportions of stormwater, sewage, and soil bacterial population to C4 effluents during stormwater flushing correlated with the total relative abundances of bacterial genera from each source (Fig. S5; Spearman test, $p=0.889$ and 0.918 for stormwater, 0.940 and 0.964 for sewage, and 0.727 and 0.640 for soil, all $p<0.0001$).

The percentages of biofilter soil bacterial communities originating from stormwater and sewage were also simulated using SourceTracker and FEAST (Fig. S7), showing a decreasing trend of stormwater contribution from shallow to deep filtration zones in each biofilter. Similarly, for biofilters C3 and C4 which were treated with mixed influent, there were decreasing percentages of sewage associated bacteria along the biofiltration depth. These results reinforced (Fig. 4) that influent bacterial removal in the biofilters was mainly confined to a shallow surface soil filtration zone.

356

357 **Discussion**

358 Stormwater management using source-control techniques such as biofilters has been
359 implemented worldwide.⁶ Consistent removal of viable and culturable cell numbers of
360 microorganisms including pathogens in biofilters have been summarized previously.¹⁵
361 However, biofilter removal efficiencies appear to vary for various microorganisms,²⁰
362 as reinforced by the disparate decreases of HF183 and EnterolA across biofilters in this
363 study. There is a need to more comprehensively understand bacterial and pathogen fates
364 in stormwater biofilters, such that stormwater management by green infrastructure is
365 performed with realistic expectations. Bacterial 16S rRNA gene sequencing allows for
366 comprehensively understanding bacterial community composition during
367 biofiltration,^{10,14} but until now has not been evaluated under realistic conditions within
368 full scale systems.

369 In this study, with either stormwater or sewage-mixed stormwater as influent, the
370 influent bacteria were major sources of effluent bacterial populations. In contrast,
371 biofilter soil bacterial communities were barely represented in the effluent. This is
372 particularly striking considering the much lower biomass in stormwater compared to
373 biofilter soil (Table S3). These results indicate that indigenous bacteria in biofilter soil
374 media were not eluted during stormwater infiltration, likely owing to soil
375 microorganisms adhering as biofilms firmly to soil particles, particularly when
376 antecedent conditions are desiccating.³⁷ Another possibility is that the large volume of
377 stormwater passing through a biofilter during each storm event (here equal to about 1.4

378 m³, or about 1.6 pore volumes), may effectively dilute to extinction any soil bacterial
379 populations released during infiltration events with influent bacterial populations.
380 Based on total biomass (Table S3) and 16S rRNA gene qPCR results (data not shown),
381 microbial biomasses in the effluents were quantitatively similar to those in the influents
382 of either stormwater or sewage-mixed stormwater. The proportions of potential human
383 pathogens in effluents were also in the same range as the influent, indicating that the
384 removal efficiency of human pathogens in biofilters was limited, at least based on
385 molecular methods used here. This is consistent with the human fecal marker HF183
386 and *Enterococci* marker Entero1A qPCR results, which indicate that log₁₀ reduction
387 values achieved during filtration in biofilters were generally less than 0.5 (Table S3),
388 and thus lower than previous reports.¹⁵ It should be noted that potential human
389 pathogens identified in this study were based on sequence similarities, and thus we
390 cannot infer the viability and pathogenicity of the identified species. While the removal
391 capacity of different stormwater treatment systems including biofilters is variable,
392 previously studied systems achieved 0.5 to 1 log₁₀ reduction for FIB and bacterial
393 pathogens, such as *Campylobacter* spp. (0.78-0.90 log₁₀ reduction) and *Clostridium*
394 *perfringens* (3.20 log₁₀ reduction).¹⁵ The performance of stormwater treatment systems
395 is likely site specific, owing to variations in influential factors including
396 physiochemical characteristics, the selection of plants, incorporation of submerged
397 zones, amendments to the medium, and operations under wet or dry conditions.^{20,38}
398 Also, our experiments were conducted at the field scale under realistic (transient) flow
399 conditions²⁵ and thus may be more representative of treatment efficiencies likely to be

400 achieved in practice. Indeed, removal rates observed in this study were more in line
401 with field-based, rather than laboratory-based, studies in the literature.¹⁵ It should be
402 also noted that molecular methods, such as the qPCR and sequencing methods used in
403 this study, cannot differentiate among DNA from viable microbial cells, DNA from
404 non-viable microbial cells, or cell-free DNA. This caveat might explain the lower
405 removal efficiency of microorganisms, including potential pathogens, observed in this
406 study compared to the consistent removal of microorganisms measured using culture-
407 based methods, as reported by others.¹⁵

408 Still, when considering the final C4 soil bacterial communities and the gradual
409 washing out of sewage-sourced bacteria (including potential human pathogens) during
410 4 rounds of stormwater flushing in biofilter C4, some bacteria were permanently
411 removed during infiltration via retention in the biofilter soil. Bacterial removal
412 decreased with soil filtration depth, with most removal occurring in the top biofilter
413 layer (0-10 cm) even though the biofilter soil depth ranged from 30 cm (C1 and C3) to
414 60 cm (C2 and C4). Since the observed bacterial removal occurred in the rhizosphere,
415 possibly via adsorption or trapping, plants may play an important role, although
416 unplanted systems were not studied here for comparison. The hypothesis that pathogen
417 removal in biofilters is dependent on rhizosphere-associated processes could explain
418 why the selection of plants is a controlling factor for biofilter performance.³⁸ Root
419 exudates including exopolysaccharides change the chemical structure of the
420 rhizosphere compared to bulk soil, promoting the growth of diverse bacterial
421 communities with complex interactions,³⁹ and could affect microbial retention

including pathogens from the influent. Predation and competition among microbes might also contribute to pathogen removal from infiltrating flow.^{40,41} Common soil fauna, such as nematodes and protists preying on bacteria are abundant in the rhizosphere,⁴² and their predation might contribute to the further removal of pathogens after initial sorption or trapping in the rhizosphere.⁴⁰ Here, the 4 rounds of stormwater flushing in biofilter C4 were performed individually during each morning and afternoon of two consecutive days, and remarkable decreases in sewage bacteria overnight in effluents between the second and third round of flushing were observed (Fig. S6 and Fig. S5c). Such decreases might be caused by inactivation and predation of sewage bacteria, but mechanistic studies would be needed to clarify the role of predation by soil fauna on pathogen removal.

Bacterial community analysis data were complementary and confirmatory of qPCR results, suggesting the value of both approaches to understanding biofilter pathogen removal. High throughput sequencing-based bacterial community characterization has been applied to distinguish environmental sources of microbial inputs, such as drinking water, river water, stormwater runoff, groundwater, and sediments.⁴³⁻⁴⁶ SourceTracker has allowed for discerning source contributions to bacterial communities in ecological patches with high accuracy, sensitivity, and specificity.^{23,47} The more recently-established FEAST software estimates proportions of source contributions using an expectation-maximization algorithm with much higher computational efficiency.²⁴ In this study, the fates of overall bacterial communities during infiltration were similarly simulated by SourceTracker and FEAST (Fig. S6), and results correlated well with the

444 results of specific microbial genera revealed by LEfSe and DESeq2 algorithms (Fig. 2)
445 and with dominant genera in the effluents (Fig. S4). Such correlations among diverse
446 statistical methods owe to the distinctly different bacterial communities among source
447 samples of stormwater, sewage, and biofilter soil (Fig. 1 and 3). There was a strong
448 correlation between the total relative abundance of potential pathogens identified in all
449 effluents and the estimated relative abundance of potential pathogens by summing the
450 multiplied values of the average relative abundance of potential pathogens in
451 stormwater (0.010%) and sewage (1.67%) and their individual proportions in each
452 effluent sample predicted by SourceTracker or FEAST (Spearman test, both $\rho > 0.930$,
453 both $p < 0.0001$). The simulated sewage proportions further strongly correlated with
454 proportions of HF183 and EnterolA in biofilter effluent versus sewage when using
455 mixed influent or during stormwater flushing (Table S3) (Spearman test, $\rho = 0.934$ and
456 0.943 for HF183, and 0.896 and 0.905 for EnterolA, all $p = 0$), indicating that
457 community sequencing and qPCR results were consistent with each other.

458 Besides microbial pollutants, community analysis can reveal bacterial taxa
459 associated with specific functions in water and biofilter soil. In a prior biofilter column
460 study, salt-enriched artificial stormwater was altered by soil bacterial communities,
461 with effects on the effluent concentrations of nitrate, phosphate, and metals.¹⁰ In another
462 study, nitrogen cycling and organic pollutant metabolizing bacteria were enriched in
463 biochar-amended stormwater biofilters.¹⁴ In this study, stormwater specific bacteria
464 were mainly microbial clades common to oligotrophic conditions, with some taxa as
465 known biodegraders. Nitrite-oxidizing *Nitrospira* spp. bacteria in the biofilter soil had

a measurable average relative abundance of 0.96%. Furthermore, ammonia-oxidizing bacteria *Nitrosomonas* were also present in some soil samples of this study (data not shown), indicating their potential to confer nitrogen transformation in stormwater during biofiltration. Additional research would be needed to understand how indigenous soil bacteria in the biofilters studied here were involved in chemical transformations, including potentially the inoculated and retained taxa. It should be noted that the number of 16S rRNA gene operons per cell can vary significantly among bacterial groups,⁴⁸ thus the community sequencing results of this study only provide a rough estimation of the relative abundances of the bacterial taxa including potential human pathogens.

In summary, four full-scale biofilters conveying the flow of several realistic storms, both with and without sewage contamination, appeared to be mostly pass-through systems for bacterial communities including potential pathogens based on qPCR and sequencing methods. This determination was made by holistic quantitative examination of microbial communities entering, exiting, and persisting in the biofilters. Because a subset of the entering sewage-associated microbial contaminants were potential pathogen taxa, the dynamics of these biofilters with regards to actual bacterial filtration might not bode well for biofilters achieving stated goals of pathogen removal from stormwater. More research, such as using viability PCR with propidium monoazide (PMA) to allow preferential detection of membrane intact bacteria, is needed.⁴⁹ The removal that did occur was limited to a narrow surface soil lens, consistent with the predictions of clean bed filtration theory¹⁷ and perhaps demonstrative of how plant root

zones control pathogen removal. Considering the water volume reduction provided by biofilters, bacterial load reductions may be more significant than concentration reductions.⁵⁰ More research on pathogen removal in biofilters is needed given that pathogens are the top cause of waterbody impairments nationally.⁵⁰ The results based on qPCR and sequencing herein cast a critical light on conventional stormwater biofilters for achieving significant pathogen removal goals, yet the results may also motivate biofilter design innovations that expand zones of rhizosphere influence.

Supplementary Information

Additional information regarding biofilter set-up, stormwater biofiltration study design, qPCR assays, 16S rRNA sequencing procedures and data analyses, the most abundant 20 bacterial genera, SourceTracker and FEAST prediction results, potential human pathogens identified, and qPCR quantification results of HF183/Bac287 and EnterolA.

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Fig. 1 Relative abundances of major bacterial phyla and super classes in all stormwater (SW), raw sewage (RS), mixed influent (MI), effluents associated with stormwater influent in biofilters C1, C3, C2, and C4 (Effluent-SW), effluents resulting from mixed influent in biofilters C3 and C4 (Effluent-MI), effluents during 4 rounds of stormwater flushing in biofilter C4 (Effluent-flush), soil from biofilters C1, C3, C2, and C4 (Soil), and soil eluent from biofilters C1, C3, C2, and C4 (Eluent). The results of stormwater, sewage, mixed influent, and effluents from each biofilter across all challenge experiments are in the order, from left to right, of sampling time. The soil and soil eluent results are presented (left to right) in the order of depth from shallow (0-10 cm) to deep (20-30 cm or 50-60 cm) zone across the biofilters. For simplicity, the several simulated storm events and coring events are not marked in the figure.

Fig. 2 Heat map of relative abundances of bacterial genera individually significantly associated with stormwater (SW), raw sewage (RS), and soil in all samples of this study. For each category of samples, the top 10 bacterial genera with the highest average relative abundance were selected and are shown in order from top to bottom. In total, 30 bacterial genera are shown. From left to right: stormwater (SW), raw sewage (RS), soil of biofilters C1, C3, C2, and C4, mixed influent (MI), effluents associated with stormwater influent in biofilters C1, C3, C2, and C4 (Effluent-SW), effluents resulting from mixed influent to biofilters C3 and C4 (Effluent-MI), effluents during 4 cycles of stormwater flushing in biofilter C4 (Effluent-flush), and soil eluent from biofilters C1, C3, C2, and C4. Results for stormwater, sewage, mixed influent, and effluents from each biofilter are presented in order (left to right) of sampling time. The soil and soil eluent results are presented in order of depth from shallow (0-10 cm) to deep (20-30 cm or 50-60 cm) zone across the biofilters.

Fig. 3 Non-metric multidimensional scaling (NMDS) analysis of bacterial community composition in all samples of this study including raw sewage, mixed influent, stormwater, effluents associated with stormwater influent from biofilters C1-C4 (Effluent-SW-C1 through -C4), effluents with mixed influent from biofilters C3 and C4 (Effluent-MI-C3 and C4), effluents during 4 cycles of stormwater flushing in biofilter C4 (Effluent-Flush-1 through -4), soil, and soil eluent (ANOSIM test global $R = 0.866$, $p = 0.001$).

Fig. 4 Heat map of relative abundances of bacterial genera specifically associated with soils of biofilters C3 and C4 receiving mixed influent (MI), compared to biofilters C1 and C2 receiving only stormwater (SW). The top 10 bacterial genera with the highest average relative abundance in soil samples of biofilters C3 and C4 were selected and are shown, ordered from top to bottom. The relative abundances of the bacterial genera are displayed for: a) soil samples of biofilters C1 to C4, and b) stormwater (SW), raw sewage (RS), mixed influent (MI), effluents resulting from stormwater influent to biofilters C1, C3, C2, and C4 (Effluent-SW), effluents resulting from mixed influent applied to biofilters C3 and C4 (Effluent-MI), effluents during 4 cycles of stormwater flushing applied to biofilter C4 (Effluent-flush), and soil eluent from biofilters C1, C3, C2, and C4. Results from samples of stormwater, sewage, mixed influent, and effluents from each biofilter are, from left to right, in the order of sampling time. Results from the soil and soil eluent samples are, from left to right, in the order of depth (from shallow to deep zones) for each biofilter.