Title

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

Journal

Environmental Science and Technology, 55(13)

ISSN

0013-936X

Authors

Li, Dong
Van De Werfhorst, Laurie C
Rugh, Megyn B
et al.

Publication Date

2021-07-06

DOI

10.1021/acs.est.1c00510

Peer reviewed

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

Journal:	*Environmental Science & Technology*
Manuscript ID	es-2021-005108.R1
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Li, Dong; University of California Santa Barbara, Bren School of Environmental Science & Management Van De Werfhorst, Laurie; University of California Santa Barbara David Bren School of Environmental Science and Management, Donald Bren School of Environmental Science & Management Rugh, Megyn; University of California Los Angeles, Department of Civil and Environmental Engineering Feraud, Marina; University of California Santa Barbara David Bren School of Environmental Science and Management, Bren School Hung, Wei-Cheng; University of California Los Angeles, Department of Civil and Environmental Engineering Jay, Jennifer ; University of California Los Angeles, Department of Civil and Environmental Engineering Cao, Yiping; Source Molecular, Technology Parker, Emily ; Virginia Tech, Department of Civil and Environmental Engineering Grant, Stanley; University of California Irvine, Civil and Environmental Engineering Holden, Patricia; University of California Santa Barbara, Bren School

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

Dong Li[1], Laurie C. Van De Werfhorst[1], Megyn B. Rugh[2], Marina Feraud[1], Wei-Cheng Hung[2], Jennifer Jay[2], Yiping Cao[3,4], Emily A. Parker[5], Stanley B. Grant[5,6], Patricia A. Holden[1]*

[1]Bren School of Environmental Science & Management, University of California, Santa Barbara

[2]Department of Civil and Environmental Engineering, 420 Westwood Plaza, 5731 Boelter Hall, UCLA Los Angeles, 90095

[3]Source Molecular Corporation, 15280 NW 79th Court, St 107, Miami Lakes FL 33016

[4]Santa Ana Regional Water Quality Control Board, 3737 Main Street, St 500, Riverside CA 92501

[5]Occoquan Watershed Monitoring Laboratory, Department of Civil and Environmental Engineering, Virginia Tech, 9408 Prince William Street, Manassas VA 20110, USA

[6]Center for Coastal Studies, Virginia Tech, 1068A Derring Hall (0420), Blacksburg, VA 24061, USA

*Corresponding author:

P A. Holden

E-mail: holden@bren.ucsb.edu

Abstract

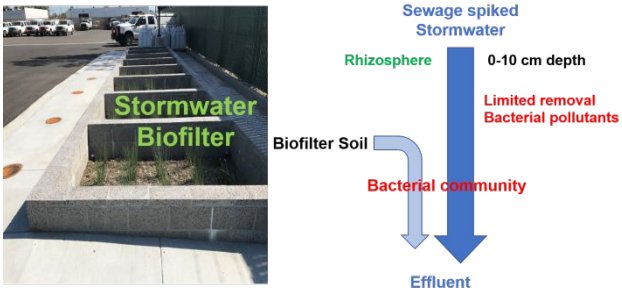
In urban areas, untreated stormwater runoff can pollute downstream surface waters. To intercept and treat runoff, low-impact or "green infrastructure" approaches such as biofilters are adopted. Yet actual biofilter pollutant removal is poorly understood; removal is often studied in laboratory columns, 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 Entero1A, 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

Table of Contents



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

92 media, and biofilter effluent were comparatively analyzed for 4 full scale biofilters of
93 2 depth regimes undergoing simulated storm events under realistic, transient flow,
94 conditions. Two questions were addressed: 1) how effective are full-scale stormwater
95 biofilters at removing bacteria and retaining them during successive storms? and 2) how
96 does media depth relate to bacterial retention, and its corollary wash-out, through
97 stormwater biofilters? The results contribute to understanding achievable stormwater
98 treatment by full scale biofilters as defined by bacterial, including putative pathogen,
99 removal. Such results are needed to guide future stormwater management including
100 widescale implementation of green infrastructure.
101
102

103 # Materials and methods

104 *Biofilters, stormwater filtration, sampling, and sample handling*

105 Four full scale biofilters (C1-C4) at the Orange County Public Works (OCPW) Glassell
106 campus (Orange, CA) were used for challenge experiments with stormwater or sewage-
107 spiked stormwater (mixed influent) as influent. Because human sewage contains
108 copious bacterial human pathogens and most other biotic contaminants common to
109 urban stormwater runoff, this study used raw sewage mixed with stormwater as
110 simulated fecal contaminated stormwater runoff. Each biofilter was 2.4 m long, 1.5 m
111 wide, and 1.8 m deep (Fig. S1 and Table S1). The soil (approximately 0.3 m deep for
112 biofilters C1 and C3, and 0.6 m deep for C2 and C4) was a mixture of fines, sand, and
113 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 m^3 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

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

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

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

158 depth interval was extruded, composited, and sieved (2 mm pore size) fresh, and the six
159 composited soil cores from each depth segment were homogenized into one clean
160 Ziploc bag to obtain one composite sample per depth interval. For biofilters C1 and C3,
161 composite samples at depths of 0-10 cm, 10-20 cm, and 20-30 cm were individually
162 collected; for biofilters C2 and C4, composite samples at depths of 0-10 cm, 10-20 cm,
163 30-40 cm, and 50-60 cm were individually collected. Soil eluent was freshly generated
164 in the lab onsite for each composite soil sample using a published method,[26] and
165 approximately 30 mL of the eluent was filtered through 0.22 μm filters (MicroFunnel
166 Filter Funnels, PALL Co.) for bacterial recovery, similarly to other aqueous samples.
167 The remaining composited soil samples were stored on dry ice during transport to
168 UCSB, and maintained (-20 ºC) until DNA extraction.
169
170 *DNA extraction, qPCR, and sequencing*
171 DNA extraction from aqueous sample filters was performed using the DNeasy
172 PowerWater kit (Qiagen, Carol Stream, IL). DNA from composited soil samples was
173 extracted using the DNeasy PowerSoil Kit (Qiagen). Duplicate extractions were
174 performed for each soil sample, with the extracted DNA pooled. An extraction blank
175 was included for each filter or soil extraction batch. After DNA concentrations were
176 quantified (Quant-iT dsDNA Broad-Range Assay Kit; Invitrogen, Carlsbad, CA), DNA
177 extracts were archived (-20 °C) until analysis.
178 *Enterococci* were quantified using the Entero1A quantitative polymerase chain
179 reaction (qPCR) assay.[27] The details of this qPCR assay are described in the SI Methods.

All individual qPCR plates had efficiencies of between 96% and 102% with an R^2 of >0.998 to 1.000. The human fecal marker HF183 was quantified using the HF183/BacR287 assay[28] in simplex format and performed by Source Molecular Corporation (Miami Lakes, FL).

Genes encoding 16S rRNA were sequenced on an Illumina MiSeq platform using a MiSeq v3 600 cycle kit (2 by 300 bp) in the California NanoSystems Institute (CNSI), UCSB. The details of DNA amplification, purification, and normalization were as before[21] (SI Methods). The sequencing data was deposited in NCBI SRA with the BioProject ID PRJNA723423.

Bioinformatic and statistical analyses

Illumina sequencing data were processed using the Quantitative Insights Into Microbial Ecology (QIIME v1.9.1) pipeline with default settings,[29] with details as before[21] (SI Methods). After quality filtering, 14,322,411 sequences were obtained for all samples. Sequences were grouped into operational taxonomic units (OTUs) at 97% sequence similarity. A representative sequence for each OTU was picked, and taxonomic data were assigned using the Greengenes 13_8 aligned reference database.

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

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

203 The weighted UniFrac distance matrix generated in QIIME was used for non-
204 metric multidimensional scaling (NMDS) with PRIMER 6.[31] Bacterial genera
205 significantly associated with stormwater, sewage, and biofilter soil were determined
206 with the LEfSe (the linear discriminant analysis effect size) algorithm[22] as well as the
207 DESeq2 method within QIIME. Source proportion analysis was performed using
208 SourceTracker 1.0[23] and FEAST[24] with default parameters. For analyzing effluent
209 sources on the basis of bacterial communities, the stormwater, sewage, and biofilter soil
210 sequences were designated as sources, and the biofilter effluent sequences were
211 designated as sinks. For analyzing bacterial communities that were sources to the
212 biofilter soils, the stormwater and sewage sequences were designated as sources, while
213 the soil sequences were sinks. Average source proportions were obtained by
214 individually executing SourceTracker and FEAST in triplicate. Heatmaps were
215 generated using Heatmapper.[32] Additional statistical analyses such as Wilcoxon tests
216 (Mann-Whitney for two categories, or Kruskal-Wallis with Steel-Dwass for all pairs
217 comparisons for three or more categories), Spearman rank correlation, and ANOSIM
218 were performed using JMP10 (SAS, Cary, NC) or PRIMER 6.

219

220

221 # Results

222 *Stormwater, sewage, and mixed influent bacterial communities*

223 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 Entero1A 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 Entero1A concentrations were quantified in sewage,
245 averaging 3.00E+06 and 1.97E+07 copies/100 mL, respectively (Table S3).

246 The mixed influent (1:1 stormwater and sewage) bacterial communities were
247 similar to those in sewage (Figs. 1 and 3), likely due to the high biomass in sewage as
248 compared to stormwater (Table S3). Sewage specific genera were thus abundant in the
249 mixed influent (Fig. 2), and the total relative abundance of potential human pathogens
250 in mixed influent was 1.53%, with the number of potential pathogen species averaging
251 25 (Table S2).
252
253 *Biofilter soil and eluents*
254 Besides Betaproteobacteria and Alphaproteobacteria, a number of bacterial clades such
255 as Actinobacteria, Acidobacteria, Chloroflexi, Deltaproteobacteria, Planctomycetes,
256 Gemmatimonadetes, and Nitrospirae were also abundant in biofilter soil (Fig. 1).
257 Biofilter soil-specific bacterial genera included the typical soil nitrite-oxidizing bacteria
258 *Nitrospira* (Fig. 2). No potential human pathogens were identified in any biofilter soil
259 samples (Table S2). When comparing to biofilters C1 and C2 that were only dosed with
260 stormwater, bacterial genera specifically associated (as revealed by LEfSe and the
261 DESeq2 methods) with the soils of biofilters C3 and C4 (dosed with stormwater then
262 1:1 mixed influent) were from sewage, such as *Arcobacter*, *Cloacibacterium*,
263 *Bacteroides*, and *Streptococcus* (Fig. 4). Furthermore, these taxa were relatively
264 abundant at the surface (0-10 cm) of both biofilters C3 and C4 with the total relative
265 abundance of 0.5% and 0.4%, respectively, and present in the depth of 10-20 cm of C3
266 (0.3%), but generally absent over the depth intervals of 20-30 cm of C3 and 10-60 cm
267 of C4 (all less than 0.2%). These similarities between C3 and C4 soil bacterial

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

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

274

275 *Biofilter effluents*

276 When the 4 biofilters initially received only stormwater, biofilter effluent contained
277 many bacterial taxa that were enriched in, or specific to, stormwater (Fig. 1 and 2), and
278 the dominant effluent bacterial genera were also abundant in stormwater (Fig. S4).
279 NMDS analysis confirmed that effluent bacterial communities were more similar to
280 those in stormwater influent than to those in biofilter soils (Fig. 3). The average total
281 relative abundance of potential pathogens in biofilter effluents was 0.022% and the
282 average number of potential pathogen species was approximately six which was
283 comparable to stormwater influent (Table S2). The fecal markers HF183 and Entero1A
284 in biofilter effluents were either not detectable or at similar levels as the stormwater
285 influent (Table S3).

286 Sewage-enriched bacteria and specific genera were abundant in the effluents of
287 biofilters C3 and C4 when receiving mixed influent (Fig. 1 and 2), and the most
288 abundant genera in the effluents were also abundant in sewage (Fig. S4). As such, the
289 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 Entero1A 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 Entero1A 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).

SourceTracker and FEAST analyses

The contributions of stormwater, raw sewage, and biofilter soil to bacterial communities in biofilter effluents were simulated by SourceTracker and FEAST (Fig. S6). The results of these two software approaches were well correlated (Spearman test, all $\rho>0.890$, all $p<0.0001$). Stormwater bacterial communities dominated the effluents of all 4 biofilters when stormwater was the influent, with the average percentage being 40.0% and 38.7% by SourceTracker and FEAST, respectively. In contrast, the average proportion of the effluent bacterial community sourced from biofilter soil was 11.8% and 17.4% in 4 biofilters by SourceTracker and FEAST, respectively. Such contributions of stormwater or soil to effluents simulated by SourceTracker and FEAST correlated well with the total relative abundances of bacterial genera sourced from stormwater or soil in the effluents of 4 biofilters (Fig. S5) (Spearman test, all $\rho>0.839$, all $p<0.0001$). When using mixed influent, sewage became the predominant source of effluent bacterial populations in biofilters C3 and C4, with the average proportion up to 58.6% and 41.8% by SourceTracker and FEAST, respectively. In contrast, contributions of stormwater and soil to effluents of biofilters C3 and C4 were comparably trivial, with the average percentages of 4.89% and 4.70% for stormwater, and 2.79% and 4.06% for soil by SourceTracker and FEAST, respectively. The predicted percentages of sewage in effluents of biofilters C3 and C4 when using mixed influent correlated well with the total relative abundance of sewage-sourced bacterial genera in effluents (Fig. S5) (Spearman test, both $\rho>0.930$, both $p<0.0001$ for 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, ρ=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 Entero1A 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

m^3, or about 1.6 pore volumes), may effectively dilute to extinction any soil bacterial populations released during infiltration events with influent bacterial populations. Based on total biomass (Table S3) and 16S rRNA gene qPCR results (data not shown), microbial biomasses in the effluents were quantitatively similar to those in the influents of either stormwater or sewage-mixed stormwater. The proportions of potential human pathogens in effluents were also in the same range as the influent, indicating that the removal efficiency of human pathogens in biofilters was limited, at least based on molecular methods used here. This is consistent with the human fecal marker HF183 and *Enterococci* marker Entero1A qPCR results, which indicate that log10 reduction values achieved during filtration in biofilters were generally less than 0.5 (Table S3), and thus lower than previous reports.[15] It should be noted that potential human pathogens identified in this study were based on sequence similarities, and thus we cannot infer the viability and pathogenicity of the identified species. While the removal capacity of different stormwater treatment systems including biofilters is variable, previously studied systems achieved 0.5 to 1 log10 reduction for FIB and bacterial pathogens, such as *Campylobacter* spp. (0.78-0.90 log10 reduction) and *Clostridium perfringens* (3.20 log10 reduction).[15] The performance of stormwater treatment systems is likely site specific, owing to variations in influential factors including physiochemical characteristics, the selection of plants, incorporation of submerged zones, amendments to the medium, and operations under wet or dry conditions.[20,38] Also, our experiments were conducted at the field scale under realistic (transient) flow conditions[25] 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

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

433 Bacterial community analysis data were complementary and confirmatory of qPCR
434 results, suggesting the value of both approaches to understanding biofilter pathogen
435 removal. High throughput sequencing-based bacterial community characterization has
436 been applied to distinguish environmental sources of microbial inputs, such as drinking
437 water, river water, stormwater runoff, groundwater, and sediments.[43-46] SourceTracker
438 has allowed for discerning source contributions to bacterial communities in ecological
439 patches with high accuracy, sensitivity, and specificity.[23,47] The more recently-
440 established FEAST software estimates proportions of source contributions using an
441 expectation-maximization algorithm with much higher computational efficiency.[24] In
442 this study, the fates of overall bacterial communities during infiltration were similarly
443 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 Entero1A 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 Entero1A, 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

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

476 In summary, four full-scale biofilters conveying the flow of several realistic storms,
477 both with and without sewage contamination, appeared to be mostly pass-through
478 systems for bacterial communities including potential pathogens based on qPCR and
479 sequencing methods. This determination was made by holistic quantitative examination
480 of microbial communities entering, exiting, and persisting in the biofilters. Because a
481 subset of the entering sewage-associated microbial contaminants were potential
482 pathogen taxa, the dynamics of these biofilters with regards to actual bacterial filtration
483 might not bode well for biofilters achieving stated goals of pathogen removal from
484 stormwater. More research, such as using viability PCR with propidium monoazide
485 (PMA) to allow preferential detection of membrane intact bacteria, is needed.[49] The
486 removal that did occur was limited to a narrow surface soil lens, consistent with the
487 predictions of clean bed filtration theory[17] 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 Entero1A.

Acknowledgements

Funding was provided by the University of California Office of the President, Multicampus Research Programs and Initiatives, Grant ID MRP-17-455083. We thank the Orange County Public Works, California and Proposition 84 funding for use of biofilters, logistical support, soil coring, and use of the onsite laboratory. We thank the Orange County Sanitation District, CA for providing raw sewage. We also thank the

510 Southern California Coastal Water Research Project and Brandon Steets from
511 Geosyntec for feedback on the study design and results. Assistance in filtering samples
512 onsite was provided by Timnit Kefela, Yizhen Shao, Jian Peng, Anda Zhang, Douglas
513 Gramajo and Sumant Avasarala. Jesse Kurylo, Rich Ambrose, and Lisa Levin
514 contributed expert opinions during the planning phase of the study design and study
515 implementation.

References

1. Barbosa, A. E.; Fernandes, J. N.; David, L. M. Key issues for sustainable urban stormwater management. *Water Res*. **2012**, 46, 6787-6798.

2. Moore, T. L.; Rodak, C. M.; Vogel, J. R. Urban stormwater characterization, control, and treatment. *Water Environ Res*. **2017**, 89, 1876-1927.

3. Moore, T. L.; Rodak, C. M.; Ahmed, F.; Vogel, J. R. Urban stormwater characterization, control and treatment. *Water Environ Res.* **2018**, 90, 1821-1871.

4. Luthy, R. G.; Sharvelle, S.; Dillon, P. Urban stormwater to enhance water supply. *Environ Sci Technol*. **2019**, 53, 5534-5542.

5. Grant, S. B.; Saphores, J. D.; Feldman, D. L., Hamilton, A. J.; Fletcher, T. D.; Cook, P. L.; Stewardson, M.; Sanders, B. F.; Levin, L. A.; Ambrose, R. F.; Deletic, A.; Brown, R.; Jiang, S. C.; Rosso, D.; Cooper, W. J.; Marusic, I. Taking the 'waste' out of 'wastewater' for human water security and ecosystem sustainability. *Science* **2012**, 337, 681-686.

6. Roy, A. H.; Wenger, S. J.; Fletcher, T. D.; Walsh, C. J.; Ladson, A. R.; Shuster, W. D.; Thurston, H. W.; Brown, R. R. Impediments and solutions to sustainable, watershed-scale urban stormwater management: lessons from Australia and the United States. *Environ Manage*. **2008**, 42, 344-359.

7. Grant, S. B.; Fletcher, T. D.; Feldman, D.; Saphores, J. D.; Cook, P. L.; Stewardson, M.; Low, K.; Burry, K.; Hamilton, A. J. Adapting urban water systems to a changing climate: Lessons from the Millennium Drought in Southeast Australia. *Environ. Sci. Technol*. **2013**, 47, 10727-10734.

538 8. Rippy, M. A. Meeting the criteria: linking biofilter design to fecal indicator bacteria
539 removal. Wiley Interdiscip. Rev. Water **2015**, 2, 577-592.
540 9. Bratieres, K.; Fletcher, T. D.; Deletic, A.; Zinger, Y. Nutrient and sediment removal
541 by stormwater biofilters: A large-scale design optimisation study. *Water Res*. **2008**,
542 42, 3930–3940.
543 10. Endreny, T.; Burke, D. J.; Burchhardt, K. M.; Fabian, M. W.; Kretzer, A. M.
544 Bioretention column study of bacteria community response to salt-enriched
545 artificial stormwater. *J Environ Qual.* **2012**, 41, 1951-1959.
546 11. Hatt, B. E.; Fletcher, T. D.; Deletic, A. Hydraulic and pollutant removal
547 performance of fine media stormwater filtration systems. *Environ. Sci. Technol.*
548 **2008**, 42, 2535–2541.
549 12. Grebel, J. E.; Mohanty, S. K.; Torkelson, A. A.; Boehm, A. B.; Higgins, C. P.;
550 Maxwell, R. M.; Nelson, K. L.; Sedlak, D. L. Engineered infiltration systems for
551 urban stormwater reclamation. *Environ. Eng. Sci.* **2013**, 30, 437−454.
552 13. Henderson, C.; Greenway, M.; Phillips, I. Removal of dissolved nitrogen,
553 phosphorus and carbon from stormwater by biofiltration mesocosms. *Water Sci*
554 *Technol.* **2007**, 55, 183-191.
555 14. Ulrich, B. A.; Vignola, M.; Edgehouse, K.; Werner, D.; Higgins, C. P. Organic
556 carbon amendments for enhanced biological attenuation of trace organic
557 contaminants in biochar-amended stormwater biofilters. *Environ Sci Technol.* **2017**,
558 51, 9184-9193.

559 15. Ahmed, W.; Hamilton, K.; Toze, S.; Cook, S.; Page, D. A review on microbial
560 contaminants in stormwater runoff and outfalls: Potential health risks and
561 mitigation strategies. *Sci Total Environ.* **2019**, 692, 1304-1321.
562 16. Chandrasena, G. I.; Deletic, A.; McCarthy, D. T. Survival of *Escherichia coli* in
563 stormwater biofilters. *Environ Sci Pollut Res Int.* **2014**, 21, 5391-5401.
564 17. Parker, E. A; Rippy, M. A.; Mehring, A. S.; Winfrey, B. K.; Ambrose, R. F.; Levin,
565 L. A.; Grant, S. B. Predictive power of clean bed filtration theory for fecal indicator
566 bacteria removal in stormwater biofilters. *Environ. Sci. Technol.* **2017**, 51, 5703-
567 5712.
568 18. Huang, X.; Rippy, M. A.; Mehring, A. S.; Winfrey, B. K.; Jiang, S. C.; Grant, S. B.
569 Shifts in dissolved organic matter and microbial community composition are
570 associated with enhanced removal of fecal pollutants in urban stormwater wetlands.
571 *Water Res.* **2018**, 137, 310-323.
572 19. Afrooz, N.; Pitol, A.; Kitt, D.; Boehm, A. Role of microbial cell properties on
573 bacterial pathogen and coliphage removal in biochar-modified biofilters. *Environ*
574 *Sci Water Res Technol.* **2018**, 4, 2160–2169.
575 20. Chandrasena, G. I.; Deletic, A.; McCarthy, D. T. Biofiltration for stormwater
576 harvesting: comparison of *Campylobacter* spp. and *Escherichia coli* removal under
577 normal and challenging operational conditions. *J. Hydrol.* **2016**, 537, 248–259.
578 21. Li, D.; Van De Werfhorst, L. C.; Dunne, T.; Devarajan, N.; Ayala, T. G.; Holden,
579 P. A. Surf zone microbiological water quality following emergency beach

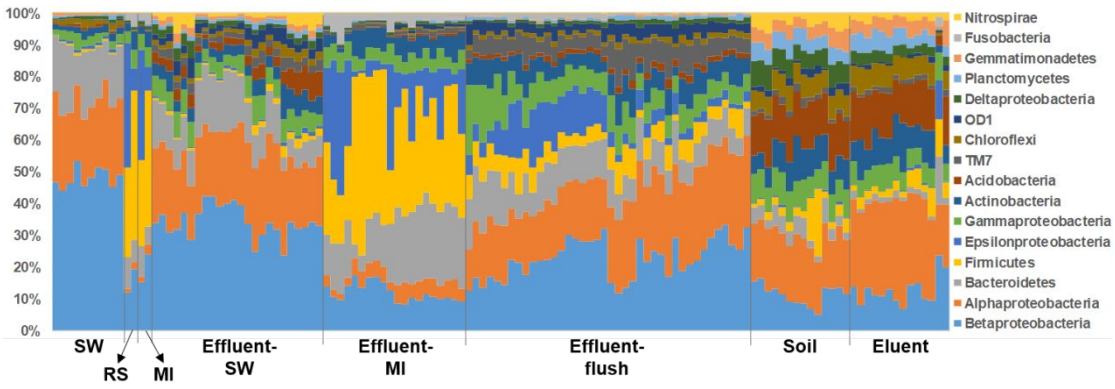
580 nourishment using sediments from a catastrophic debris flow. *Water Res.* **2020**,
581 176, 115733.
582 22. Segata, N.; Izard, J.; Waldron, L.; Gevers, D.; Miropolsky, L.; Garrett, W. S.;
583 Huttenhower, C. Metagenomic biomarker discovery and explanation. *Genome Biol*
584 **2011**, 12, R60.
585 23. Knights, D.; Kuczynski, J.; Charlson, E. S.; Zaneveld, J.; Mozer, M. C.; Collman,
586 R. G.; Bushman, F. D.; Knight, R.; Kelley, S. T. Bayesian community-wide
587 culture-independent microbial source tracking. *Nat. Methods* **2011**, 8, 761−U107.
588 24. Shenhav, L.; Thompson, M.; Joseph, T. A., Briscoe, L.; Furman, O.; Bogumil, D.;
589 Mizrahi, I.; Pe'er, I.; Halperin, E. FEAST: fast expectation-maximization for
590 microbial source tracking. *Nat. Methods* **2019**, 16, 627-632.
591 25. Parker, E. A.; Grant, S. B.; Cao, Y.; Rippy, M. A.; McGuire, K. J.; Holden, P. A.;
592 Feraud, M.; Avasarala, S.; Liu, H.; Hung, W. C.; Rugh, M.; Jay, J.; Peng, J.; Shao,
593 S.; Li, D. Predicting solute transport through green stormwater infrastructure with
594 unsteady transit time distribution theory. *Water Resour. Res.* **2021**
595 https://doi.org/10.1029/2020WR028579.
596 26. Boehm, A. B.; Griffith, J.; McGee, C.; Edge, T. A.; Solo-Gabriele, H. M.; Whitman,
597 R.; Cao, Y.; Getrich, M.; Jay, J. A.; Ferguson, D.; Goodwin, K. D.; Lee, C. M.;
598 Madison, M.; Weisberg, S. B. Faecal indicator bacteria enumeration in beach sand:
599 a comparison study of extraction methods in medium to coarse sands. *J Appl*
600 *Microbiol.* **2009**, 107, 1740-1750.

601 27. Haugland, R. A.; Siefring, S.; Lavender, J.; Varma, M. Influences of sample
602 interference and interference controls on quantification of enterococci fecal
603 indicator bacteria in surface water samples by the qPCR method. *Water Res.* **2012**,
604 46, 5989-6001.
605 28. Green, H. C.; Haugland, R. A.; Varma, M.; Millen, H. T.; Borchardt, M. A.; Field,
606 K. G.; Walters, W. A.; Knight, R.; Sivaganesan, M.; Kelty, C. A.; Shanks, O. C.
607 Improved HF183 quantitative real-time PCR assay for characterization of human
608 fecal pollution in ambient surface water samples. *Appl Environ Microb* **2014**, 80,
609 3086-3094.
610 29. Caporaso, J. G.; Kuczynski, J.; Stombaugh, J.; Bittinger, K.; Bushman, F. D.;
611 Costello, E. K.; Fierer, N.; Pena, A. G.; Goodrich, J. K.; Gordon, J. I.; Huttley, G.
612 A.; Kelley, S. T.; Knights, D.; Koenig, J. E.; Ley, R. E.; Lozupone, C. A.;
613 McDonald, D.; Muegge, B. D.; Pirrung, M.; Reeder, J.; Sevinsky, J. R.; Turnbaugh,
614 P. J.; Walters, W. A.; Widmann, J.; Yatsunenko, T.; Zaneveld, J.; Knight, R.
615 QIIME allows analysis of high-throughput community sequencing data. *Nat*
616 *Methods* **2010**, 7, 335-336.
617 30. Miao, J. J.; Han, N.; Qiang, Y. J.; Zhang, T. T.; Li, X. W.; Zhang, W. 16SPIP: a
618 comprehensive analysis pipeline for rapid pathogen detection in clinical samples
619 based on 16S metagenomic sequencing. *BMC Bioinformatics* **2017**, 18, 568.
620 31. Clarke, K. R.; Gorley, R. N. PRIMER v6: User Manual/Tutorial, PRIMER-E,
621 Plymouth. **2006**.

622 32. Babicki, S.; Arndt, D.; Marcu, A.; Liang, Y.; Grant, J. R.; Maciejewski, A.; Wishart,
623 D. S. Heatmapper: web-enabled heat mapping for all. *Nucleic Acids Res.* **2016**, 44,
624 W147-W153.
625 33. Liu, Z. P.; Wang, B. J.; Liu, Y. H.; Liu, S. J. *Novosphingobium taihuense* sp. nov.,
626 a novel aromatic-compound-degrading bacterium isolated from Taihu Lake, China.
627 *Int J Syst Evol Microbiol.* **2005**, 55, 1229-1232.
628 34. Eberspächer, J. Phenylobacterium. Bergey's Manual of Systematics of Archaea and
629 Bacteria. John Wiley & Sons, Ltd: **2015**, 1–12.
630 35. Kristensen, J. M.; Nierychlo, M.; Albertsen, M.; Nielsen, P. H. Bacteria from the
631 genus *Arcobacter* are abundant in effluent from wastewater treatment plants. *Appl*
632 *Environ Microbiol.* **2020**, 86, e03044-19.
633 36. Shanks, O. C.; Newton, R. J.; Kelty, C. A.; Huse, S. M.; Sogin, M. L.; McLellan,
634 S. L. Comparison of the microbial community structures of untreated wastewaters
635 from different geographic locales. *Appl Environ Microbiol.* **2013**, 79, 2906-2913.
636 37. Vanderlinde, E. M.; Harrison, J. J.; Muszyński, A.; Carlson, R. W.; Turner, R. J.;
637 Yost, C. K. Identification of a novel ABC transporter required for desiccation
638 tolerance, and biofilm formation in *Rhizobium leguminosarum* bv. viciae 3841.
639 *FEMS Microbiol Ecol.* **2010**, 71, 327-340.
640 38. Barrett, M. E.; Limouzin, M.; Lawler, D. F. Effects of media and plant selection on
641 biofiltration performance. *J. Environ. Eng.-ASCE* **2013**, 139, 462-470.
642 39. Mukerji, K. G.; Manoharachary, C.; Singh, J. Microbial Activity in the Rhizosphere.
643 Berlin Springer. **2006**.

644 40. Burtchett, J. M.; Mallin, M. A.; Cahoon, L. B. Micro-zooplankton grazing as a
645 means of fecal bacteria removal in stormwater BMPs. *Water Sci Technol.* **2017**,
646 75, 2702-2715.
647 41. Zhang, L.; Seagren, E. A.; Davis, A. P.; Karns, J. S. The capture and destruction of
648 *Escherichia coli* from simulated urban runoff using conventional bioretention
649 media and iron oxide-coated sand. *Water Environ Res* **2010**, 82, 701–714.
650 42. Brussaard, L. Soil fauna, guilds, functional groups and ecosystem processes. *Appl*
651 *Soil Ecol.* **1989**, 9, 123–135.
652 43. Duvert, C.; Priadi, C. R.; Rose, A. M.; Abdillah, A.; Marthanty, D. R.; Gibb, K. S.;
653 Kaestli, M. Sources and drivers of contamination along an urban tropical river
654 (Ciliwung, Indonesia): Insights from microbial DNA, isotopes and water chemistry.
655 *Sci Total Environ.* **2019**, 682, 382-393.
656 44. Liu, G.; Zhang, Y.; van der Mark, E.; Magic-Knezev, A.; Pinto, A.; van den Bogert,
657 B.; Liu, W.; van der Meer, W.; Medema, G. Assessing the origin of bacteria in tap
658 water and distribution system in an unchlorinated drinking water system by
659 SourceTracker using microbial community fingerprints. *Water Res.* **2018**, 138, 86-
660 96.
661 45. Wyckoff, K. N.; Chen, S.; Steinman, A. J.; He, Q. Impact of roadway stormwater
662 runoff on microbial contamination in the receiving stream. *J Environ Qual.* **2017**,
663 46, 1065-1071.
664 46. Zhang, W.; Gu, J.; Li, Y.; Lin, L.; Wang, P.; Wang, C.; Qian, B.; Wang, H.; Niu,
665 L.; Wang, L.; Zhang, H.; Gao, Y.; Zhu, M.; Fang, S. New insights into sediment

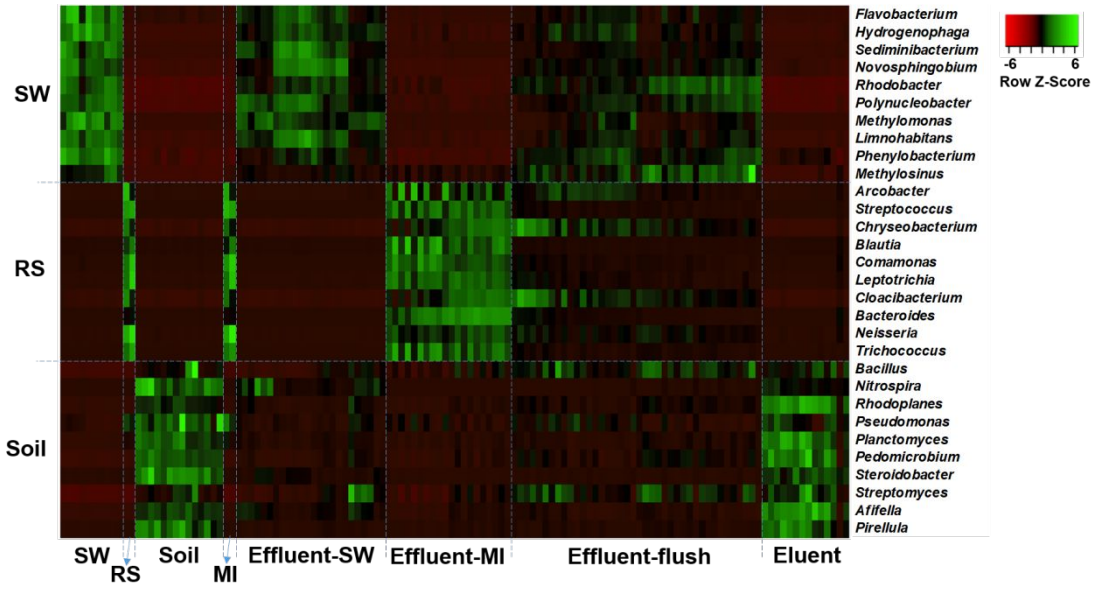
666 transport in interconnected river-lake systems through tracing microorganisms.
667 *Environ Sci Technol.* **2019**, 53, 4099-4108.
668 47. Staley, C.; Kaiser, T.; Lobos, A.; Ahmed, W.; Harwood, V. J.; Brown, C. M.;
669 Sadowsky, M. J. Application of SourceTracker for accurate identification of fecal
670 pollution in recreational freshwater: a double-blinded study. *Environ Sci Technol.*
671 **2018**, 52, 4207-4217.
672 48. Klappenbach, J. A.; Saxman, P. R.; Cole, J. R.; Schmidt, T. M. rrndb: the Ribosomal
673 RNA Operon Copy Number Database. *Nucleic Acids Res.* **2001**, 29, 181-184.
674 49. Seidel, L.; Strathmann, M.; Nocker, A. The feasibility of improved live-dead
675 distinction in qPCR-based microbial source tracking. *J Microbiol Methods.* **2017**,
676 140, 23-31.
677 50. Water Research Foundation. International stormwater BMP database. **2020**,
678 http://bmpdatabase.org/.
679



680

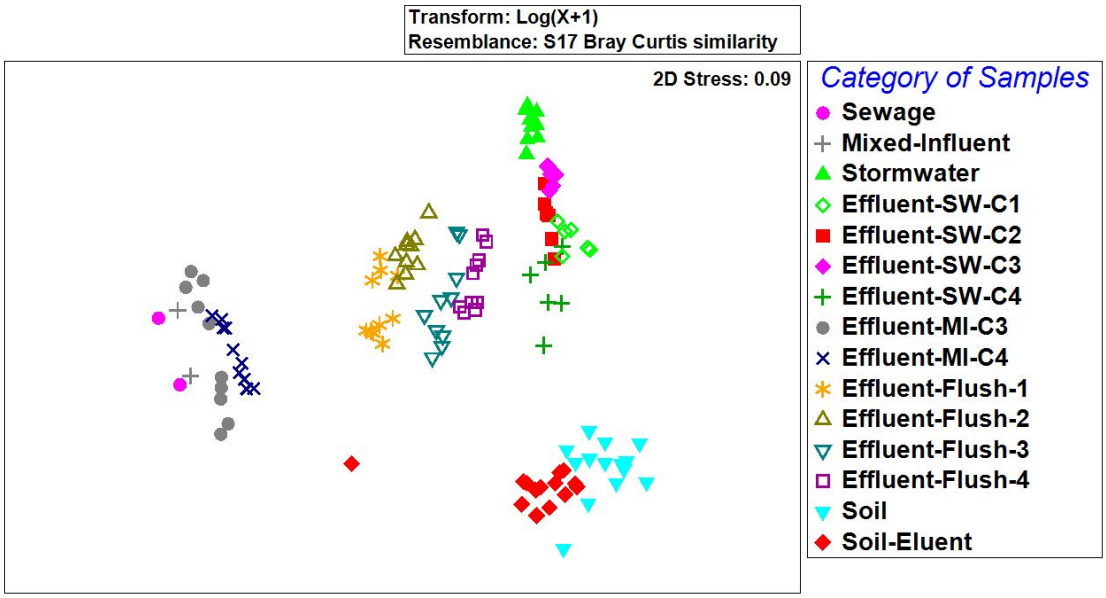
681 Fig. 1 Relative abundances of major bacterial phyla and super classes in all stormwater
682 (SW), raw sewage (RS), mixed influent (MI), effluents associated with stormwater
683 influent in biofilters C1, C3, C2, and C4 (Effluent-SW), effluents resulting from mixed
684 influent in biofilters C3 and C4 (Effluent-MI), effluents during 4 rounds of stormwater
685 flushing in biofilter C4 (Effluent-flush), soil from biofilters C1, C3, C2, and C4 (Soil),
686 and soil eluent from biofilters C1, C3, C2, and C4 (Eluent). The results of stormwater,
687 sewage, mixed influent, and effluents from each biofilter across all challenge
688 experiments are in the order, from left to right, of sampling time. The soil and soil eluent
689 results are presented (left to right) in the order of depth from shallow (0-10 cm) to deep
690 (20-30 cm or 50-60 cm) zone across the biofilters. For simplicity, the several simulated
691 storm events and coring events are not marked in the figure.

692



693

694 Fig. 2 Heat map of relative abundances of bacterial genera individually significantly
695 associated with stormwater (SW), raw sewage (RS), and soil in all samples of this study.
696 For each category of samples, the top 10 bacterial genera with the highest average
697 relative abundance were selected and are shown in order from top to bottom. In total,
698 30 bacterial genera are shown. From left to right: stormwater (SW), raw sewage (RS),
699 soil of biofilters C1, C3, C2, and C4, mixed influent (MI), effluents associated with
700 stormwater influent in biofilters C1, C3, C2, and C4 (Effluent-SW), effluents resulting
701 from mixed influent to biofilters C3 and C4 (Effluent-MI), effluents during 4 cycles of
702 stormwater flushing in biofilter C4 (Effluent-flush), and soil eluent from biofilters C1,
703 C3, C2, and C4. Results for stormwater, sewage, mixed influent, and effluents from
704 each biofilter are presented in order (left to right) of sampling time. The soil and soil
705 eluent results are presented in order of depth from shallow (0-10 cm) to deep (20-30 cm
706 or 50-60 cm) zone across the biofilters.
707



708

709 Fig. 3 Non-metric multidimensional scaling (NMDS) analysis of bacterial community

710 composition in all samples of this study including raw sewage, mixed influent,

711 stormwater, effluents associated with stormwater influent from biofilters C1-C4

712 (Effluent-SW-C1 through -C4), effluents with mixed influent from biofilters C3 and C4

713 (Effluent-MI-C3 and C4), effluents during 4 cycles of stormwater flushing in biofilter

714 C4 (Effluent-Flush-1 through -4), soil, and soil eluent (ANOSIM test global R = 0.866,

715 $p = 0.001$).

716

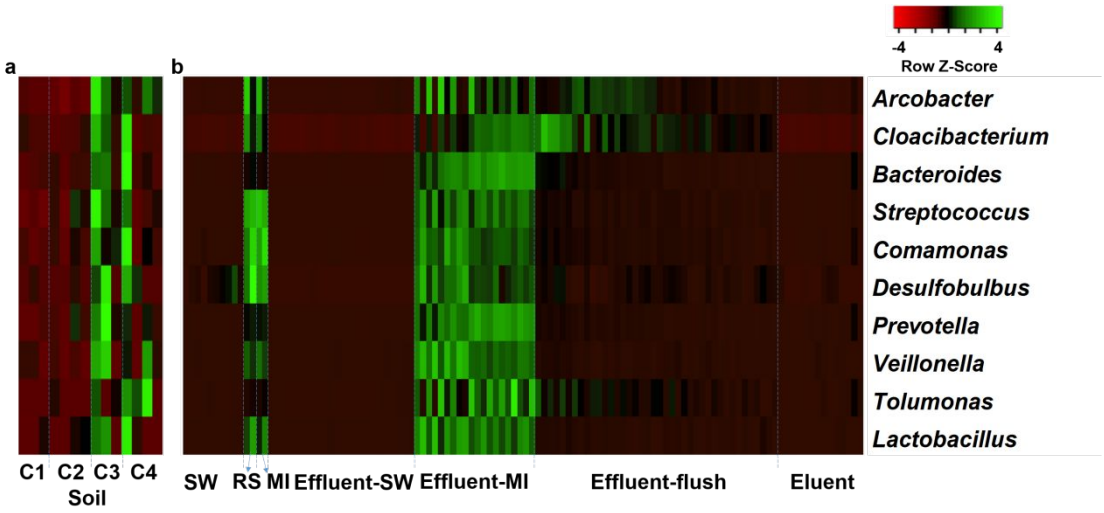


Fig. 4 Heat map of relative abundances of bacterial genera specifically associated with soils of biofilters C3 and C4 receiving mixed influent (MI), compared to biofilters C1 and C2 receiving only stormwater (SW). The top 10 bacterial genera with the highest average relative abundance in soil samples 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.