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The effects of low-level pharmaceuticals on stream biofilm structure and function across a land-use gradient in streams of the Huron River watershed

Elizabeth Mae Stover

The Effects of Low-Level Pharmaceuticals on Stream Biofilm Structure and Function Across a Land-Use Gradient in Streams of the Huron River Watershed

by

Elizabeth Mae Stover

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Thesis Committee:
Kristin Judd, Ph.D., Chair
Steven Francoeur, Ph.D.
Daniel Clemans, Ph.D.

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Abstract

Pharmaceuticals and personal care products (PPCPs) are a contaminant class of worldwide concern. Their environmental omnipresence indicates they may be a potential source of global change, and ecosystem-scale impacts at non-lethal levels have not been fully explored. We used stream biofilms to assess ecosystem responses to PPCPs. Biofilms were cultivated in streams draining areas of different land use and then exposed to triclosan, diphenhydramine, and sulfamethoxazole-trimethoprim treatments. We found evidence that low levels of these PPCPs affected some, but not all, aspects of biofilm processes and bacterial community composition. Bacterial carbon uptake was reduced ($p = 0.06$) and we found shifts in biofilm community composition following treatments. However, maximum photosynthetic efficiency, decomposition, and microbial physiological profiles showed no significant effect of PPCPs. Still, changes in bacterial activity and composition suggest that PPCPs may act as ecological disruptors at low levels, and further research is needed to assess ecosystem-scale effects.

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Introduction

Freshwater ecosystems impact environmental, economic, and human health and provide important ecosystem services (Millennium Ecosystem Assessment 2005). With increasing urbanization, such systems are often contaminated with pollutants, the long-term ecological impacts of which are largely unknown (Malmqvist and Rundle 2002). Understanding how pollutants impact ecosystem processes is critical to conserving freshwater resources. Further, as the human population increases globally and landscapes shift to urban and agricultural uses, synthetic compound production and release into the environment has increased and has even outpaced known drivers of global change such as rising atmospheric CO_2 and nutrient pollution (Bernhardt et al. 2017). As these synthetic compounds become more prevalent in waterways, it is important to understand their ecosystem-scale impacts.

Pharmaceuticals and personal care products (PPCPs) are a class of pollutants of emerging concern (e.g., Daughton and Ternes 1999; Kolpin et al. 2002; Bernot et al. 2016; Bernhardt et al. 2017). As these compounds intended for medicinal, industrial and personal use become more widespread, their presence in waterways becomes more prevalent. PPCPs enter freshwater systems through a variety of pathways, such as agricultural and livestock runoff, leaking sewer infrastructure in urban landscapes, and industrial and domestic waste (Ebele et al. 2017). Most wastewater treatment facilities are ill-equipped to remove these diverse organic compounds (Joss et al. 2006). As a result, a multitude of compounds have been detected in stream water across the United States and globally (Kolpin et al. 2002; Bernot et al. 2016; Bernhardt et al. 2017). The omnipresence of pharmaceutical contaminants in waterways at low, non-lethal concentrations demonstrates their potential to impact ecosystems through chronic exposure and bioaccumulation (Kolpin et al. 2002; Bernot et al. 2016).

The bioactive nature and chronic presence of PPCPs makes them potential ecological disruptors with the capacity to alter ecological processes (Daughton and Ternes 1999; Richmond et al. 2017). While the ecotoxicology of many of these compounds is understood, few studies have explored their ecological disrupting effects at environmentally relevant, non-lethal levels, and those that have largely focused on effects on animal behavior (Richmond et al. 2017). The impacts of PPCPs at non-lethal concentrations on ecosystem processes, such as photosynthesis and decomposition, is not well understood.

In freshwater systems, particularly low-order streams, biofilms play an integral role in ecosystem processes (Figure 1). Composed mainly of algae, bacteria, and fungi, biofilms comprise a large source of primary production in stream ecosystems and form the foundation of the stream food web, providing food for stream macroinvertebrates and protozoa. These microorganism collectives are responsible for facilitating nutrient cycling, such as decomposition and nitrogen fixation. Biofilms likewise play an important role in dissolved organic carbon (DOC) dynamics and other biogeochemical processes in streams (e.g., Battin et al. 2003; Romani et al. 2004).

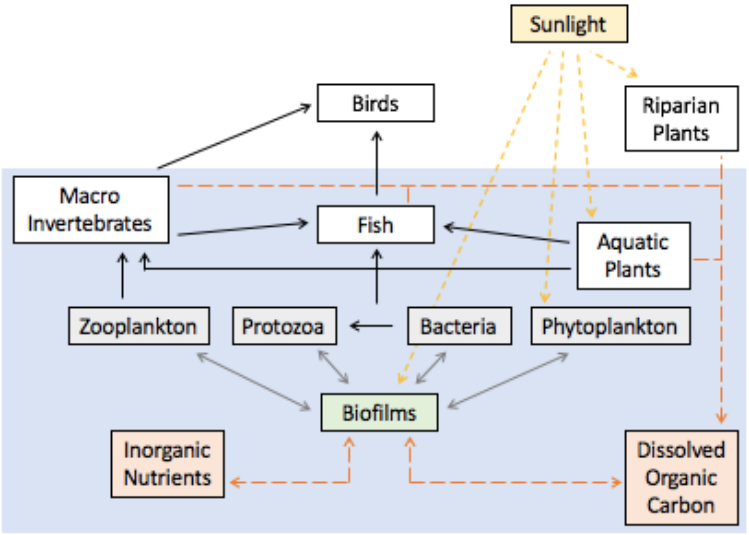


Figure 1. The diverse roles of biofilms in stream ecosystems, adapted from "World's water streams affected by pharmaceutical pollution" (Puiu 2013).

Recent studies focusing on biofilm response to pharmaceuticals have found measurable impacts, such as reduced microbial respiration and altered bacterial and algal communities (Wilson et al. 2003; Rosi-Marshall et al. 2013). For example, the antibiotics sulfamethoxazole-trimethoprim and triclosan suppress algal growth (Teixeira and Granek 2017, Xin et al. 2017), and diphenhydramine, an antihistamine, suppresses algal growth and microbial respiration in biofilms (Rosi-Marshall et al. 2013). Bacterial richness and diversity are lower in rivers where pharmaceuticals are present, and triclosan reduces bacterial diversity, suggesting a shift in bacterial community composition (Drury et al. 2013; Drury et al. 2013). There is also evidence for the selection of antimicrobial-resistant microbes in the environment where pharmaceuticals are present (Magalhaes et al. 2016; Rosi et al. 2018). Previous research has documented the effects of PPCPs on aquatic systems, but few have investigated the effects at very low, environmentally relevant levels.

Impacts of PPCPs on ecological processes may be influenced by interactions with other environmental stressors, such as nutrient and contaminant input as a result of different surrounding land uses. Land development can cause hydrologic alterations, nutrient enrichment and contaminant pollution, creating a number of stressors (Allan 2004). Agricultural land use may introduce excess nitrogen and phosphorus to streams through fertilizers, and streams with urban land use are subject to increased salinity and flashiness due to road salts and high amounts of impervious surfaces. Riparian clearing in land development can increase stream temperatures, and sedimentation can decrease the suitability of substrate for biofilm communities (Allan 2004). Further, previous research indicates that streams in less urban areas are more susceptible to pharmaceutical contaminants, possibly due to resistance from continuous exposure to contaminants in urban waterways (Rosi et al. 2018). The variable stressors of different land uses

shape microbial communities and therefore may influence their response to pharmaceutical exposure.

The purpose of this study was to investigate the ecological effects of low doses of three common pharmaceuticals, triclosan, diphenhydramine, and sulfamethoxazole-trimethoprim, on stream biofilm structure and function in streams across a land use gradient. We hypothesized that due to the bioactive nature of pharmaceutical pollutants, biofilms exposed to such pollutants in low, environmentally relevant concentrations would show reduced bacterial activity, decomposition, photosynthetic potential, and reduced bacterial community diversity compared to controls. Additionally, because biofilm communities in agricultural and urban streams are more likely to be impacted from chronic exposure to other environmental stressors, we hypothesized that these communities would exhibit less of a response than communities from unimpacted, or natural, streams. To test these hypotheses, we collected biofilms on tiles in streams of variable land use and examined the bacterial community structure and function of biofilms following exposure to very low levels of pharmaceuticals in laboratory microcosm experiments.

Methods

Experimental Set-Up

To test the effects of PPCPs on ecological processes, we cultivated biofilms from streams of different land use on tiles and cotton strips to conduct manipulative experiments. We used microcosm experiments to assess the effects of three common pharmaceuticals on the ecological functioning of stream biofilms across varying land uses. Dissolved organic carbon (DOC) uptake, Pulse-Amplitude-Modulated (PAM) chlorophyll fluorescence, cotton strip decomposition experiments, and community-level physiological profiles were conducted to assess functional responses of biofilms to treatments. DNA analysis of the bacterial community was used to compare bacterial community composition of biofilms from different streams and after treatment with PPCPs.

Study Sites

Biofilms were cultivated in six streams of the Huron River watershed that differed in land use. The streams were categorized as natural, agricultural, or urban based on the dominant land use. Intact natural area and impervious surface data made available in creekshed reports by the Huron River Watershed Council (HRWC) were used to categorize streams (Figure 2, Table 1). Millers Creek and Malletts Creek were classified as urban streams due to a high percentage of impervious surfaces (30% and 40%) and urban land use (80% and 85%). The streams categorized as agricultural and natural each had relatively high intact natural areas (30-42%) with low impervious surfaces (4-7%) and low urbanization (12-35%). The agricultural streams, Portage Creek and Mill Creek, had a higher percent of agricultural land use (33% and 47%). Hay Creek and Honey Creek were classified as natural streams with lower areas of agricultural use

(8% and 22%). Millers Creek and Malletts Creek have the smallest creekshed size (6.2 km^2 and 28.5 km^2) and Portage Creek and Mill Creek have the largest creekshed size (204.6 km^2 and 370.4 km^2).

Twice during the summer of 2018, at each site, stream discharge, temperature, conductivity, and oxygen measurements were made, and water samples were collected for nutrient analysis in order to characterize the sites (Table 2). Millers Creek and Malletts Creek had the lowest stream discharge and notably higher conductivity (3.488 ms/cm and 2.487 ms/cm) than other sites.

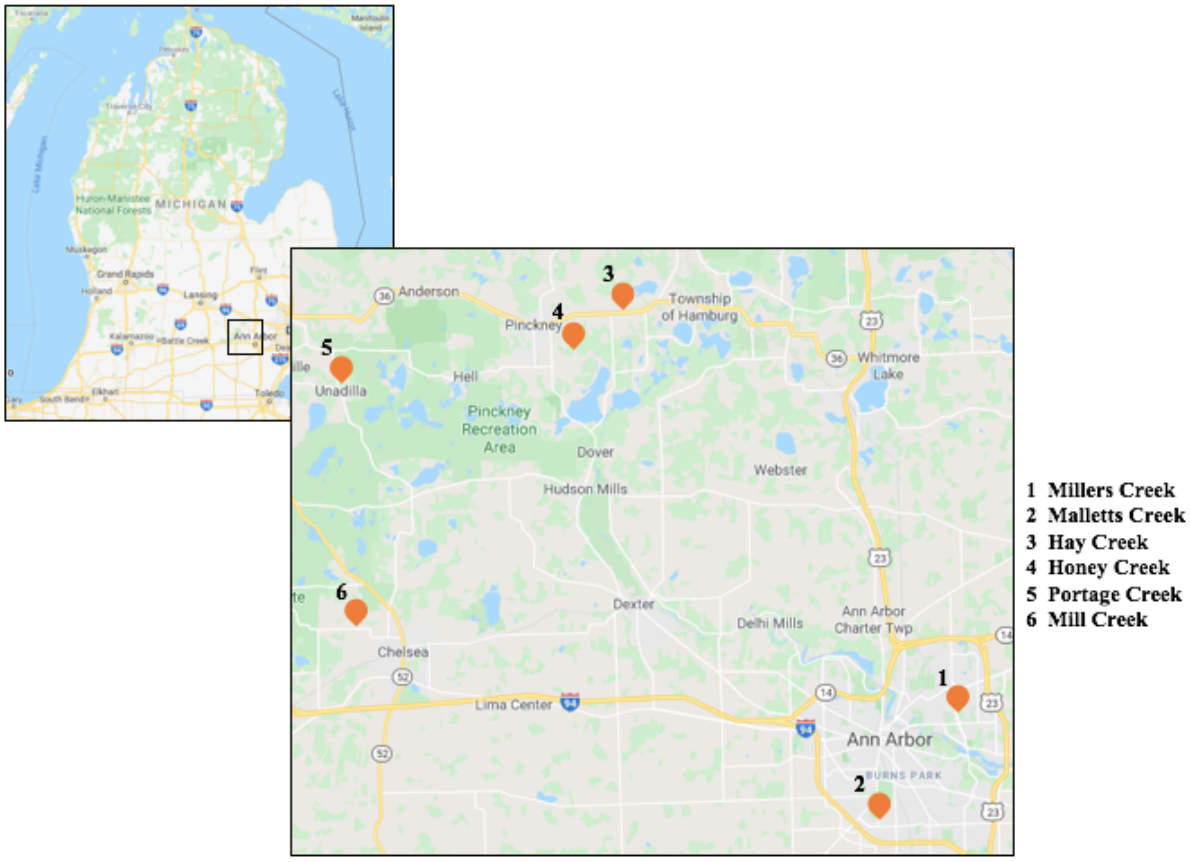


Figure 2. Map of study sites in the Huron River Watershed.

Table 1. Land use data for streams available through the Huron River Watershed Council and land use classification.

	Creekshed Size	Intact Natural	Agriculture	Urban	Impervious Surfaces	Land Use Classification
Hay Creek	34.7 km^2	30%	8%	35%	7%	Natural
Honey Creek (Livingston)	69.9 km^2	40%	22%	19%	4%	Natural
Portage Creek	204.6 km^2	42%	33%	12%	5%	Agricultural
Mill Creek	370.4 km^2	31%	47%	18%	4%	Agricultural
Millers Creek	6.2 km^2	10%	-	80%	30%	Urban
Malletts Creek	28.5 km^2	8%	2%	85%	40%	Urban

Table 2. Stream characterization measurements during brick-tile placements and retrievals.*

	Date	Hay Creek	Honey Creek	Portage Creek	Mill Creek	Millers Creek	Malletts Creek
Stream Discharge (m^3/s)	*26-Jun*	0.0909	0.7812	0.6800	0.1082	0.0039	0.0079
	17-Jul	0.0244	0.3582	0.3563	0.0522	-	-
Temperature (°C)	*26-Jun*	17.45	19.24	22.07	21.15	14.58	19.58
	17-Jul	20.17	22.34	21.67	18.68	16.86	21.1
pH	*26-Jun*	8.04	8.01	8.21	8.10	7.58	7.94
	17-Jul	8.16	7.97	8.06	8.14	7.68	7.95
Conductivity (ms/cm)	*26-Jun*	1.30	1.48	1.62	1.39	3.48	2.48
	17-Jul	1.80	2.38	3.23	2.45	3.54	4.75
Oxygen (mg/L)	*26-Jun*	8.84	9.39	8.27	8.70	7.69	8.51
	17-Jul	8.82	8.36	7.26	9.37	7.47	8.20
Nitrogen (NO_3) (µg/L)	*26-Jun*	254.89	236.88	420.78	66.40	450.87	230.99
	17-Jul	308.38	277.30	186.84	127.39	256.85	190.69
Phosphorus (PO_4) (µg/L)	*26-Jun*	3.65	3.40	3.67	3.60	4.01	6.67
	17-Jul	4.06	3.91	5.29	4.13	3.63	7.05

*Dashes (-) indicate missing data.

Biofilm Cultivation

Bricks were affixed with eighteen 2 cm x 2 cm unglazed ceramic tiles using inert rubber cement (VersaChem Mega Black O.E.M. High-Temp Silicone Gasket Maker). The brick-tile fixtures were autoclaved to ensure sterility and transported to streams inside autoclaved lidded plastic bins. The brick-tile fixtures were submerged at each stream site (four per site) and carefully pinned into place using landscape staples. Four bricks were placed in each of the six streams. Brick-tile fixtures remained in streams for 3 weeks to allow adequate biofilm growth. Following this period, they were retrieved and tiles were removed from bricks using sterilized pliers to prevent biofilm contamination between sites.

Cotton strips prepared using the method described by Tiegs et al. (2013) were also submerged at each site, secured using zip ties and polypropylene twine, and held in place with steel rebar. Cotton strips remained in streams for a period of 1 week in order to establish adequate biofilm inoculation and to prevent too much decomposition of the cotton strips before experimental laboratory use. The strips were retrieved and transported in separate ziplock bags to prevent cross contamination.

Pharmaceutical Treatments

The pharmaceutical treatments for this experiment included 5 µg/L triclosan (an antibacterial agent), 5 µg/L diphenhydramine (an antihistamine), and 2.5:2.5 µg/L sulfamethoxazole-trimethoprim (antibiotics frequently prescribed together) (Table 3). All pharmaceuticals were chosen based on their common presence in streams and their concentrations to reflect commonly observed levels, as few studies have explored the ecological disrupting effects at environmentally relevant, nonlethal concentrations (Kolpin et al. 2002;

Larsson et al. 2007; Bernot et al. 2016; Bradley et al. 2017; Richmond et al. 2017). Triclosan is a broad-spectrum antibacterial and antifungal that inhibits the growth of bacteria by blocking lipid synthesis (McMurry et al. 1998). Diphenhydramine is a common antihistamine that blocks the H1 histamine receptor and may inhibit some bacteria and alter bacterial communities (Wolfson et al. 2018). Sulfamethoxazole is a sulfonamide and trimethoprim inhibits folic acid synthesis. Together with trimethoprim, this combination inhibits two steps in the enzymatic pathways required for DNA synthesis in bacteria (Tenover 2006).

Pharmaceuticals were prepared as 10 mg/L solutions, then diluted to µg/L doses (Table 3). Diphenhydramine, sulfamethoxazole and trimethoprim were dissolved in water, and we used 5 mL of ethanol per 1 L stock solution to facilitate dissolving triclosan into solution to a final negligible ethanol experimental concentration of 0.00025%.

Table 3. Characteristics of pharmaceutical treatments.

Name	Chemical Formula	CAS	Use	Experimental Concentration
Triclosan	$C_{12}H_7Cl_3O_2$	3380-34-5	Antimicrobial	5µg/L
Diphenhydramine	$C_{17}H_{21}NO$	58-73-1	Antihistamine	5µg/L
Sulfamethoxazole-trimethoprim	$C_{10}H_{11}N_3O_3S$, $C_{14}H_{18}N_4O_3$	723-46-6 738-70-5	Antibiotic combination	2.5:2.5µg/L

Effects of Pharmaceuticals on DOC Uptake

DOC uptake assays were conducted by measuring the difference in DOC uptake between treatments and controls as a proxy for bacterial activity. Inoculated tiles were placed in individual conical tubes; dosed with triclosan, diphenhydramine, or sulfamethoxazole-trimethoprim treatment; and compared to controls. By measuring the change in DOC

concentration over the three-week incubation period, we compared differences in biofilm DOC uptake among controls (no pharmaceuticals added) and pharmaceutical treatments.

Individual 50 mL sterile plastic conical tubes filled with 40 mL of sterile filtered stream water (Grade GF/F glass microfiber filter, followed by 0.22 μm membrane filtered to remove bacteria) and a tile containing cultivated biofilm were spiked with DOC (n = 5; Table 4). This DOC spike established a starting concentration for the biofilm community to consume. To prepare the DOC spike, 10 g of black maple leaves were added to 1 L megapure water and kept on a shaker table at 90 rpm for 24 hours. The leachate was filtered with a Grade GF/F glass microfiber filter, followed with a 0.22 μm filter to sterilize. Four milliliters of filtered leachate was added to tubes of 40 mL stream water for a ratio of 1:10.

Table 4. Stream DOC concentrations for each site and experimental concentrations with DOC leachate spike.

	26-Jun Conc. (ppm)	17-Jul Conc. (ppm)	Experimental DOC Concentration with leachate (ppm)
Hay Creek (natural)	9.28	4.24	83.69
Honey Creek (natural)	7.33	4.01	90.59
Portage Creek (agricultural)	15.05	12.60	82.29
Mill Creek (agricultural)	10.57	6.01	84.05
Millers Creek (urban)	4.46	2.84	91.92
Malletts Creek (urban)	7.51	11.90	87.88

Triclosan (5 µg/L), diphenhydramine (5 µg/L), and sulfamethoxazole-trimethoprim (2.5:2.5 µg/L) treatments were added and tubes were loosely capped to prevent evaporation and debris from contaminating tubes. Tubes were kept continuously on a shaker plate in the dark to ensure solutions were properly mixed, to allow sufficient airflow, and to prevent carbon production of photosynthetic microbes. Following a three-week incubation period, water samples were taken from each tube, filtered (Grade GF/F glass microfiber filter) and acidified with 20 mL trace metal grade hydrochloric acid. DOC concentrations were measured via wet combustion as non-purgeable organic carbon on a Shimadzu TOC-5000 model total organic carbon analyzer, taking measurements in triplicates. DOC concentrations were determined by comparing values to known potassium hydrogen phthalate standards. DOC uptake was calculated as

$$DOC_{uptake} = DOC_{initial} - DOC_{final}$$

where tubes containing 0.22 µm filtered stream water and filtered leachate established the starting DOC concentration. The control for this experiment included biofilms absent of treatment to determine DOC uptake under normal conditions. We also included controls of stream water absent of biofilm or treatment, and DI water absent of biofilm or treatment to determine if some compound present in the tile or stream water was responsible for changes in DOC. Experimental biofilm tiles from Portage Creek and Malletts Creek were saved for DNA analysis.

Effects of Pharmaceuticals on Maximum Photosynthetic Efficiency

To assess changes in algal community physiology in response to low levels of pharmaceuticals, we measured the photosynthetic potential of biofilms in treatments compared to controls. We determined photosynthetic potential using chlorophyll fluorescence, which uses

light re-emitted by chlorophyll molecules to measure the maximum efficiency of PSII photochemistry (Consalvey et al. 2005). Individual tiles were placed in petri dishes and 15 mL Grade GF/F glass microfiber filtered stream water was added until the surface of the tile was submerged (n = 5). Triclosan (5 µg/L), diphenhydramine (5 µg/L), and sulfamethoxazole-trimethoprim (2.5:2.5 µg/L) treatments were added with 5 replicates. The petri dishes were incubated at 25 °C under 11.25 µmol/s·m^2 controlled light conditions. Chlorophyll fluorescence measurements were taken 2 days and 6 days following treatment (or control). None of the tiles showed any change in photosynthetic potential on Day 2, so we gave them an additional designated pharmaceutical dose of increasing strength (5, 10, 20, 40 and 80 µg/L) (Table 5). We then took measurements 6 days after the initial dose (4 days after additional dose).

Table 5. Additional dose of designated pharmaceutical treatments for chlorophyll fluorescence.*

Replicate	Initial Dose (µg/L)	Final Dose (µg/L)
1	5	5
2	5	10
3	5	20
4	5	40
5	5	80

*Sulfamethoxazole-trimethoprim doses were 2.5:2.5 µg/L per 5 µg/L dose.

Effects of Pharmaceuticals on Decomposition

To measure the effects of pharmaceutical exposure on decomposition rates, we used the cotton strip assay (Tiegs et al. 2013). We focused on a single pharmaceutical, triclosan, due to its antimicrobial properties, common occurrence in waterways, and presence in a large number of

common household products, such as antimicrobial hand soaps, cleaning products, and a wide variety of other consumer products (Bedoux et al. 2011). Cotton strips prepared using the method described by Tiegs et al. (2013) were submerged at each site, secured using zip ties and polypropylene twine, and held in place with steel rebar. After 1 week, cotton strips were collected from the field and placed in 50 mL conical tubes with 0.22 µm filtered stream water and 5 µg/L triclosan treatment (or control without triclosan; n = 5). Cotton strips were suspended in solution and capped loosely. Tubes were incubated at in the dark at 25 °C for 3 weeks, then removed, cleaned with an ethanol wash, and dried thoroughly at 40 ºC. Tensile strength was tested using a tension meter (Chatillon LTCM-5 motorized test stand and Chatillon DFI-100 digital force gauge) in order to determine the impact of pharmaceutical treatments on decomposition rates. Percent tensile strength loss per day for each stream was then calculated as

$$\%\ Strength\ loss\ per\ day = \left[\left(1 - \frac{Strength\ treatment/control\ strips}{Strength\ reference\ strips}\right) \times 100\right] \div Time$$

By assessing the rate of tensile strength loss, we were able to determine the decomposition rate of the cotton strip.

Effects of Pharmaceuticals on Community-Level Physiological Profiles

We used BIOLOG Microbial Community Analysis EcoPlates (Catalog No. 1506; https://www.biolog.com/products-portfolio-overview/microbial-community-analysis-with-ecoplates/) to determine changes in biofilm community functioning following exposure to pharmaceuticals. EcoPlates contain 31 different common carbon sources and the pattern of use of these substrates can provide a physiological profile of the community via color development of the wells for each substrate.

Biofilm slurries were incubated with pharmaceutical treatments and added to EcoPlates. Biofilm slurries were made by carefully scraping five tiles from each site using sterile technique into a combined slurry and thoroughly mixing using a vortex to ensure identical starting bacterial communities. Three milliliters biofilm slurry and 10 mL filtered stream water were added to 15 mL conical tubes. Triclosan (5 µg/L), diphenhydramine (5 µg/L), and sulfamethoxazole-trimethoprim (2.5:2.5 µg/L) treatments were added to the slurry and incubated in the dark at 20 °C for 1 week. After 1 week, biofilm slurries were aliquoted into the EcoPlates (n = 1). Control groups without pharmaceuticals added had two replicates and pharmaceutical treatments groups had no replication. Plates were incubated at 20 °C and measurements taken at 5 days. Average Metabolic Response (AMR) was determined by calculating the mean value for plate color development and Shannon-Wiener diversity (H') was calculated using

$$H' = -\Sigma p_i(lnp_i)$$

to quantify biofilm response, where p_i is the proportion of total well development for each substrate. A multivariate principal components analysis (PCA) on the covariance matrix was used to determine patterns in substrate usage across treatments and land-use variables.

Effects of Pharmaceuticals on Bacterial Community Composition

To determine the effects of land use and pharmaceutical exposure on bacterial community composition, 16S rRNA genes were sequenced using a general bacterial primer (515F forward and 806R reverse), which targets the V4 region of the 16S small subunit rRNA. We analyzed biofilms samples from all six streams to assess patterns related to land use and samples from pharmaceutical treatments and controls of Portage Creek and Malletts Creek in the DOC uptake assay to assess patterns related to treatments. Tiles were carefully scraped with a

razor blade into a slurry using sterile technique. The slurry for each stream and treatment (or control) contained the pooled biofilms of five tiles (n = 1). DNA was isolated using QIAGEN PowerSoil DNA Isolation Kit, and samples were sent to MR DNA sequencing services for analysis (http://www.mrdnalab.com, Shallowater, TX, USA). The samples were amplified using single-step 30 cycle PCR with QIAGEN HotStarTaq Plus Master Mix Kit, sequenced using the Ion S5 XL torrent platform and data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA) and QIIME analysis, providing bacterial identification counts and relative abundance data. We assessed the overall bacterial community by calculating richness (S) as the total number of genera (not species) present and Shannon-Wiener diversity (H') as

$$H' = -\Sigma p_i(lnp_i)$$

where p_i is the proportion of total counts for each genus to quantify biofilm response. We looked for trends in the bacterial community composition between treatments and controls as well as across land use, focusing on notable community composition changes in class and genus.

Statistical Analyses

We used a mixed-model analysis of variance (ANOVA) to test the effects of pharmaceuticals and land use on responding variables (DOC uptake, chlorophyll fluorescence, and decomposition responses) (Myers and Well 2002). If there were no significant interactions between pharmaceutical and land-use, we performed post-hoc tests to determine the effects. In particular, a Bonferroni-based post-hoc t-test was used to determine the effects of land use on DOC uptake. Statistical analyses were performed using Systat 10.2 Software with a statistical significance level of $\alpha = 0.05$ and a Bonferroni-based t-test significance level of $\alpha = 0.017$. Data that did not meet normality or equality of variance assumptions were transformed using

exponential transformations (x^4 for photosynthetic potential and x^3 for decomposition) in order to meet assumptions.

Results

Effects of Pharmaceuticals on DOC Uptake

Control biofilms consumed DOC by an average of 65.45 ppm, or 2.88 mg (0.14 mg/day) for a 2 cm x 2 cm area. Our results showed that land use had a significant effect on DOC uptake ($p = 0.02$), with biofilms from agricultural streams consuming the most DOC and biofilms from natural streams consuming the least. Pharmaceutical treatments reduced DOC uptake ($p = 0.06$). In all streams except Mill Creek, DOC uptake was reduced by triclosan treatments by as much as 6.9% (Millers Creek) and diphenhydramine treatments by as much as 7.7% (Honey Creek) compared to controls. In all sites except Mill Creek and Millers Creek, DOC uptake was reduced in sulfamethoxazole-trimethoprim treatments by as much as 3.5% (Portage Creek) compared to controls (Figure 3). There was no interaction between land use and pharmaceutical treatment ($p = 0.32$), indicating that land use did not influence biofilm response to pharmaceutical treatments. Data for additional controls is available in Appendix A.

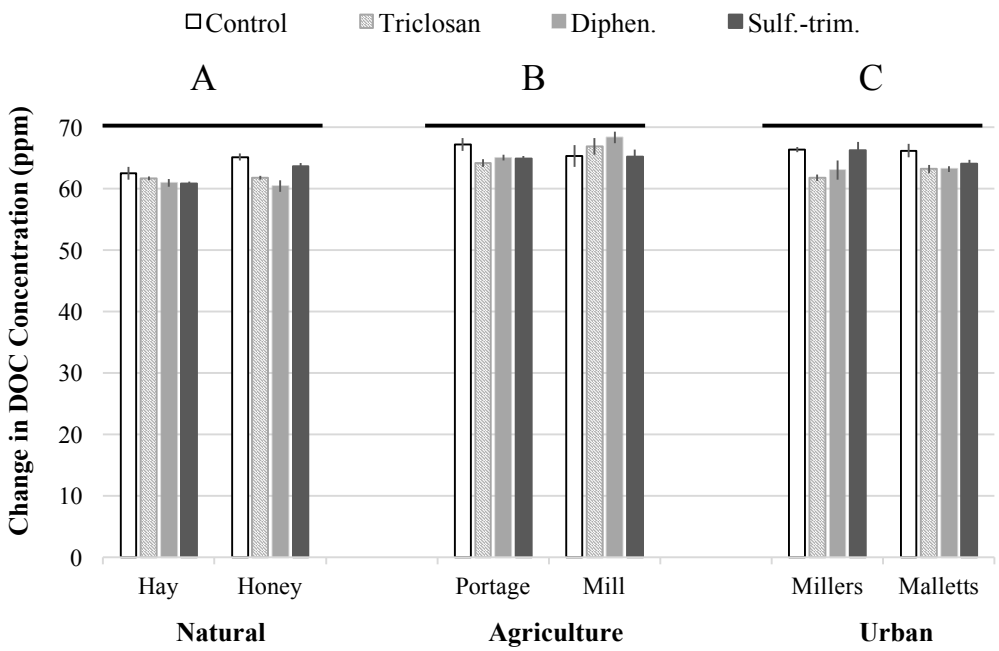


Figure 3. DOC uptake in biofilms exposed to triclosan, diphenhydramine, sulfamethoxazole-trimethoprim, and control conditions across natural, agricultural, and urban land uses. Error bars indicate standard error. Letters indicate significant differences. n = 5, except for triclosan at Honey Creek (n = 4).

Effects of Pharmaceuticals on Maximum Photosynthetic Efficiency

We found no significant effect of pharmaceuticals or land use on maximum photosynthetic efficiency after two days of exposure ($p = 0.28$ and $p = 0.68$, respectively). Other than a notable low fluorescence in the Hay Creek control group, no streams showed a difference between treatments and controls (Figure 4). Following the extra pharmaceutical dose to observe the effect of different concentrations, no clear pattern in fluorescence with pharmaceutical concentration emerged across pharmaceutical or land use (Figure 5; for example, $R^2 = 0.00061$, $R^2 = 0.16339$, and $R^2 = 0.0269$ for triclosan, diphenhydramine and sulfamethoxazole-trimethoprim treatments respectively in Honey Creek). The data for each site and treatment is available in Appendix A.

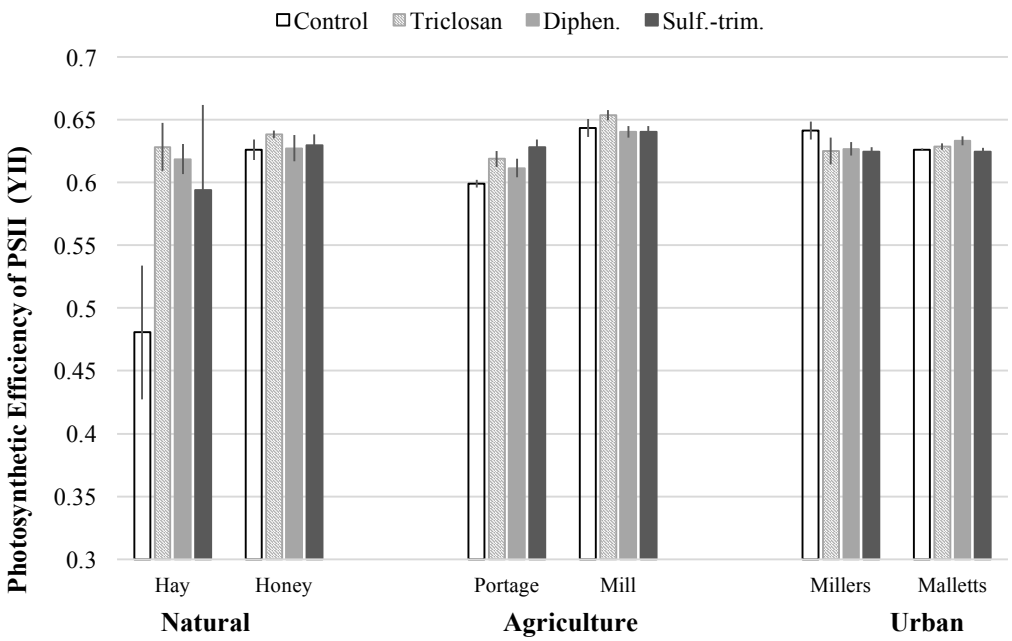
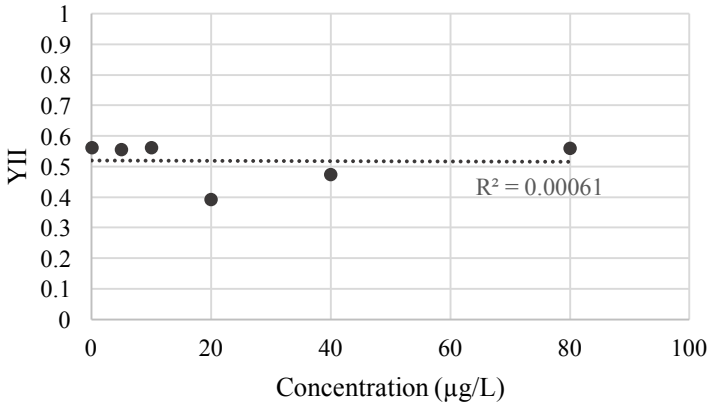
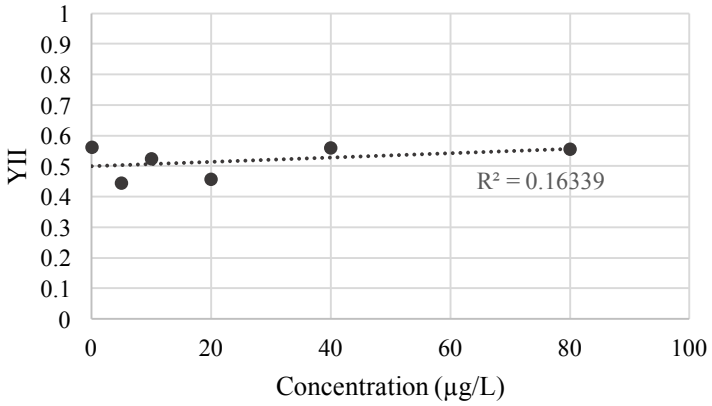


Figure 4. Maximum photosynthetic efficiency of photosystem II (YII) in biofilms 2 days following exposure to triclosan, diphenhydramine, sulfamethoxazole-trimethoprim, and control conditions across natural, agriculture, and urban land uses. Error bars indicate standard error. n = 5.

(a) *Triclosan*



(b) *Diphenhydramine*



(c) *Sulfamethoxazole-trimethoprim*

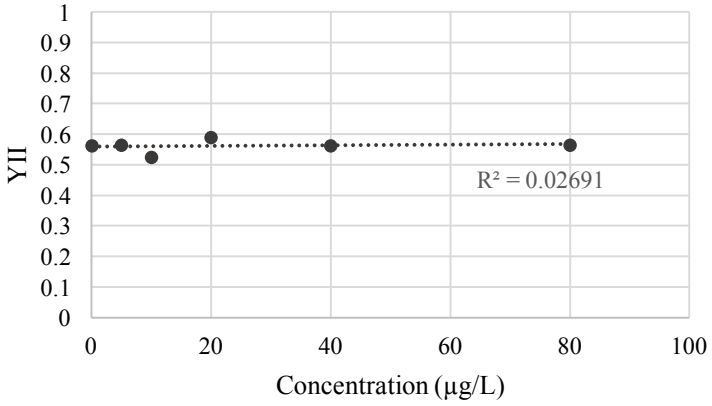


Figure 5. Regression of maximum photosynthetic efficiency of photosystem II (YII) of **(a)** triclosan, **(b)** diphenhydramine, and **(c)** sulfamethoxazole-trimethoprim treatments in Honey Creek biofilms 4 days post-treatment spike (6 days post initial treatment).

Effects of Pharmaceuticals on Decomposition

Exposure to pharmaceuticals had no significant effect on decomposition of cotton strips ($p = 0.34$). While triclosan exposure decreased decomposition activity by 59% in Mill Creek (agricultural) and 19% in Millers Creek (urban), there was no difference in loss of tensile strength (decomposition) between controls and triclosan treatments incubated in the other streams (Figure 6). Further, no clear pattern emerged among land use ($p = 0.33$).

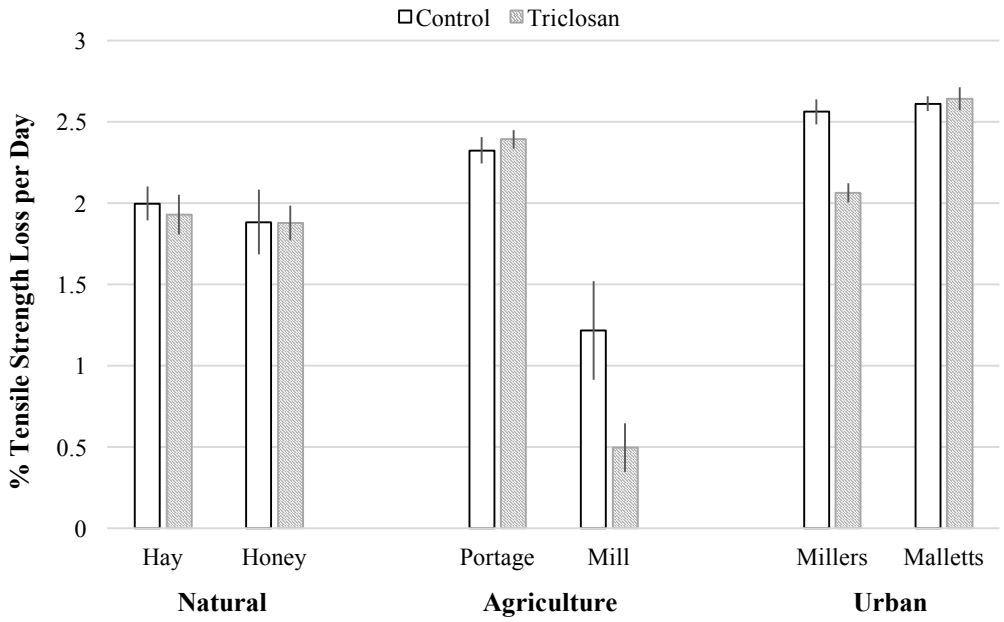


Figure 6. Percent loss of tensile strength per day in controls and triclosan treatments across natural, agricultural, and urban land uses. Lines represent standard error. n = 5.

Effects of Pharmaceuticals on Community-Level Physiological Profiles

A principal components analysis of substrate utilization showed no clear groupings across pharmaceutical treatment or land use (Figure 7). At Day 5 measurements, the percent variance explained by the first two axes was 43%. Average metabolic response (AMR) and Shannon-Wiener diversity (H') also showed no clear pattern across pharmaceutical or land use (Table 6). AMR in Honey Creek biofilms was 17% and 18% lower in triclosan and

diphenhydramine treatments compared to controls (Table 6). In contrast, the sulfamethoxazole-trimethoprim treatment was 47% higher than the control, but there was little difference in AMR at other sites.

Table 6. Day 5 average metabolic response (AMR) and Shannon-Wiener diversity (H') in biofilms across pharmaceutical treatments and land use.

	Natural		**Agricultural**		**Urban**	
AMR	**Hay**	**Honey**	**Portage**	**Mill**	**Millers**	**Malletts**
Control	1.04	1.79	1.07	1.22	1.59	1.25
	1.11	1.70	1.30	1.54	1.56	1.19
Triclosan	1.24	1.44	1.18	1.33	1.62	1.25
Diphen.	1.05	1.43	1.07	1.21	1.69	1.32
Sulf.-trim.	1.30	1.45	1.23	1.31	1.55	1.49
	Natural		**Agricultural**		**Urban**	
H'	**Hay**	**Honey**	**Portage**	**Mill**	**Millers**	**Malletts**
Control	3.00	3.36	3.12	3.08	3.32	3.13
	3.08	3.35	3.22	3.22	3.27	3.13
Triclosan	3.13	3.23	3.28	3.07	3.28	3.15
Diphen.	2.95	3.26	3.14	3.17	3.28	3.17
Sulf.-trim.	3.08	3.18	3.24	3.05	3.29	3.18

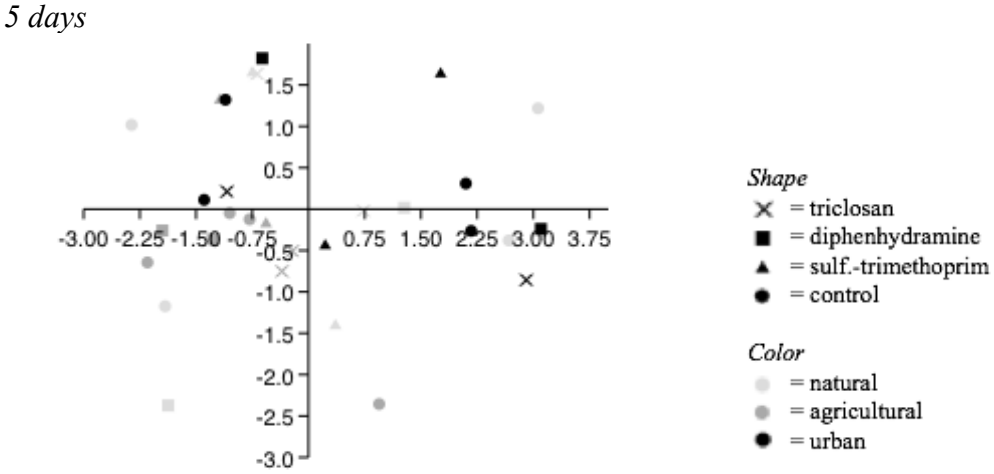


Figure 7. Multivariate principal components analysis (PCA) on the covariance matrix of EcoPlate substrate utilization of biofilm slurry exposed to triclosan, diphenhydramine, sulfamethoxazole-trimethoprim, and controls after 5 days.

Effects of Pharmaceuticals on Bacterial Community Composition

Across the six streams, there was no consistent pattern in community composition with land use. Genera in Hay Creek (natural) and Malletts Creek (urban) had the lowest value of richness (S), and Portage Creek (agricultural) had the highest (Table 7). While Malletts Creek had the lowest value, Shannon-Wiener diversity (H') in genera did not differ substantially across land use (Table 7).

Pharmaceutical treatments showed little overall change in S compared to controls, with the exception of a much lower S in the triclosan treatment of Portage Creek (Table 8). Surprisingly, we found a slightly lower H' in control biofilms than in pharmaceutical-treated biofilms in both Portage Creek and Malletts Creek.

Table 7. Richness (S) and Shannon-Wiener diversity (H') in bacterial genera across land use collected from each site.

Natural	**S**	**H'**
Hay Creek	742	5.28
Honey Creek	786	5.27
Agricultural		
Portage Creek	822	5.27
Mill Creek	774	5.32
Urban		
Millers Creek	779	5.36
Malletts Creek	723	4.89

Table 8. Richness (S) and Shannon-Wiener diversity (H') in bacterial genera across pharmaceutical treatments from the DOC uptake assay.

Portage Creek (agricultural)	**S**	**H'**
Control	648	4.53
Triclosan	602	4.64
Diphenhydramine	680	4.81
Sulf.-trimethoprim	675	4.88
Malletts Creek (urban)		
Control	671	4.57
Triclosan	673	4.96
Diphenhydramine	664	5.02
Sulf.-trimethoprim	644	5.02

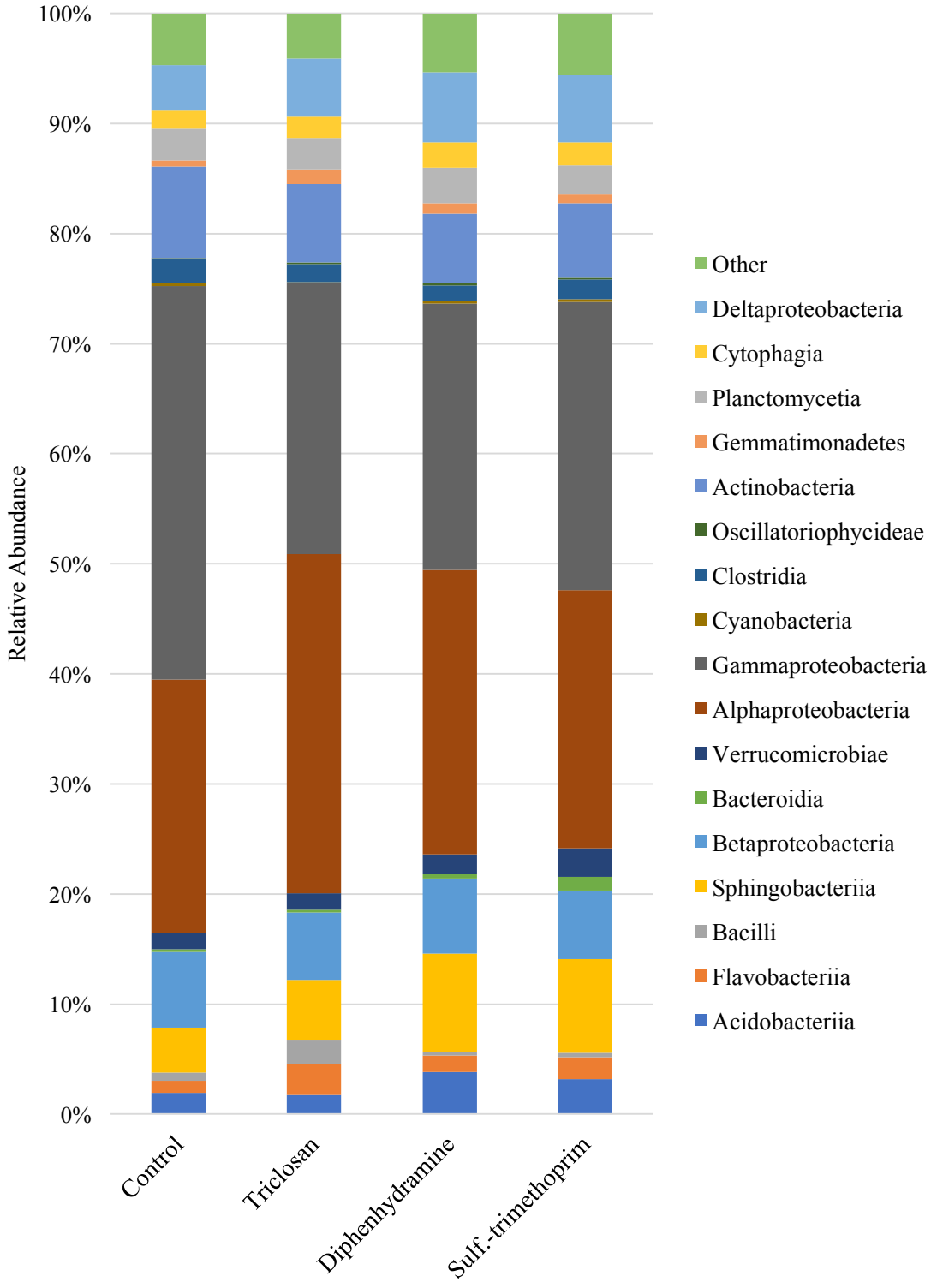
There were some differences in community composition at the taxonomic resolution of class across sites, but differences in bacterial abundance were site-specific rather than consistent

with land use categories. Malletts Creek (urban) had over 1,000% more Oscillatoriophycideae relative to other sites, 72-81% less Actinobacteria, and 60-80% less Deltaproteobacteria. Millers Creek (urban) had 40-49% less Alphaproteobacteria.

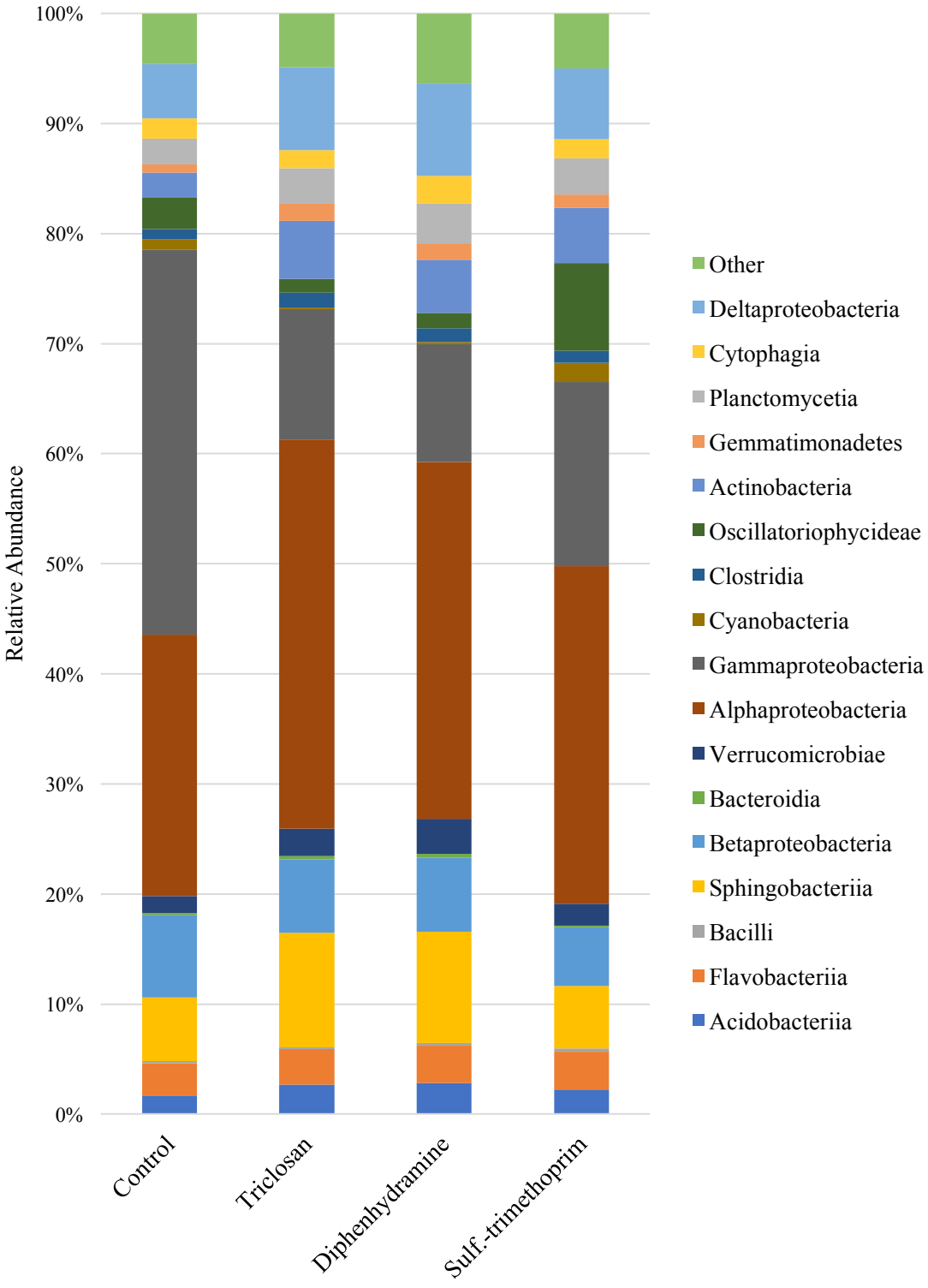
Notable changes in bacterial community composition were observed between control and pharmaceutical treatments, most notably in triclosan and diphenhydramine treatments. The class Gammaproteobacteria was 31%, 32%, and 27% lower in Portage Creek biofilms and 66%, 69%, and 52% lower in Malletts Creek biofilms after exposure to triclosan, diphenhydramine and sulfamethoxazole-trimethoprim treatments, respectively (Figures 8a and 8b). Several genera within this class were consistently lower in treatment groups compared to controls, while other genera were higher with pharmaceutical exposure (Figures 8c and 8d). In particular, *Alcanivorax* was 95%, 85%, and 67% lower in Portage Creek biofilms and > 99%, > 99%, and 74% lower in Malletts Creek biofilms after exposure to triclosan, diphenhydramine and sulfamethoxazole-trimethoprim treatments, respectively (Table 9). Similarly, *Halioglobus* was 95%, 71%, and 37% lower in Portage Creek biofilms and > 99%, > 99%, and 74% lower in Malletts Creek biofilms after exposure to triclosan, diphenhydramine and sulfamethoxazole-trimethoprim treatments, respectively.

The genus *Pseudomonas* was notably higher in treatments than controls, 169%, 146%, and 92% higher in triclosan, diphenhydramine and sulfamethoxazole-trimethoprim treatments in Portage Creek biofilms (Table 9). Malletts Creek also had a higher percentage of *Pseudomonas* in triclosan and diphenhydramine-treated biofilms (70% and 21%), but *Pseudomonas* was 39% lower than controls in the sulfamethoxazole-trimethoprim treatment. The full list of class and genus data are available in Appendix B.

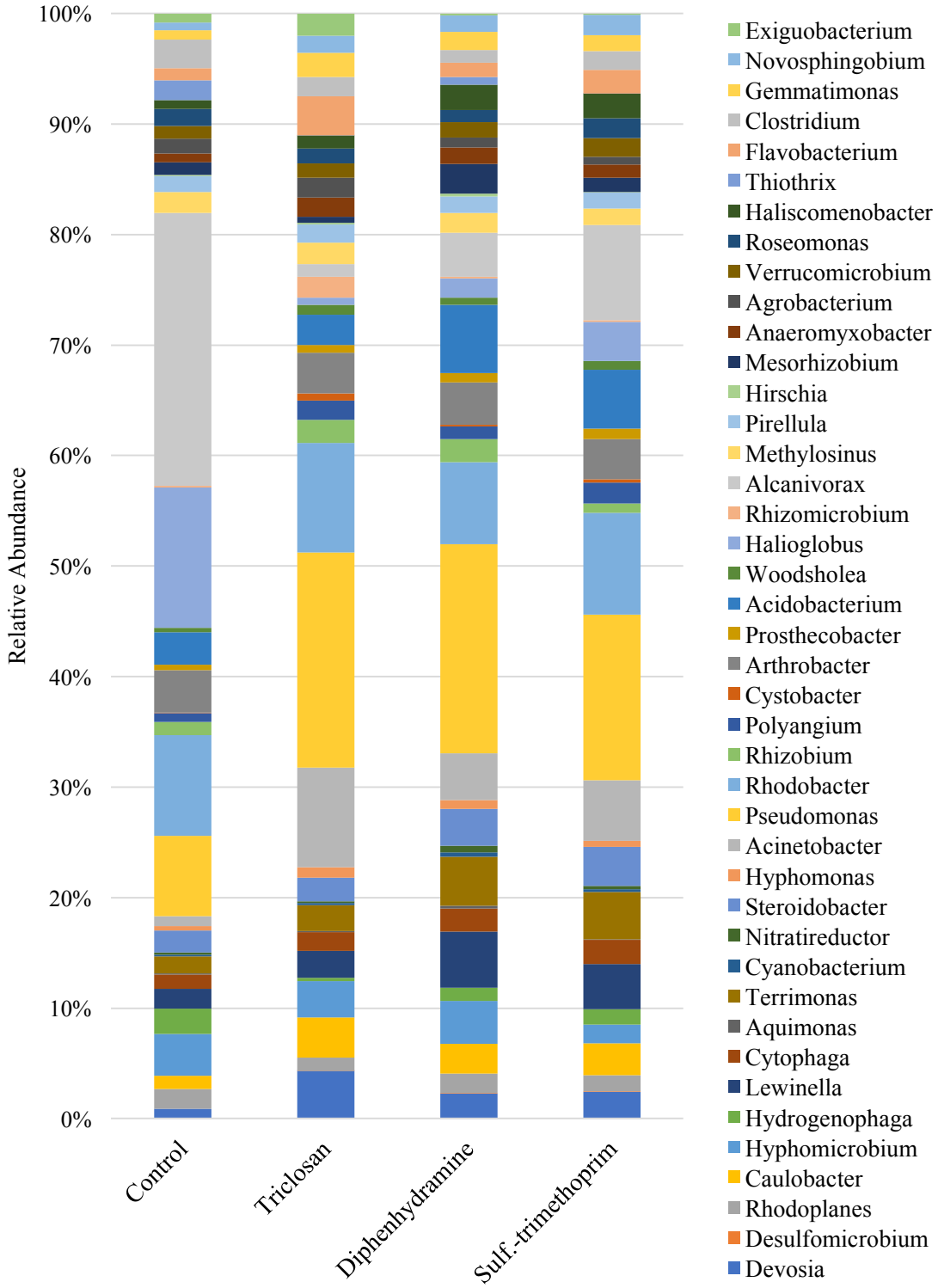
(a) ***Portage Creek, class***



(b) *Malletts Creek, class*



(c) ***Portage Creek, genus (excluding genera < 1% for all groups))***



(d) ***Malletts Creek, genus (excluding genera < 1% for all groups)***

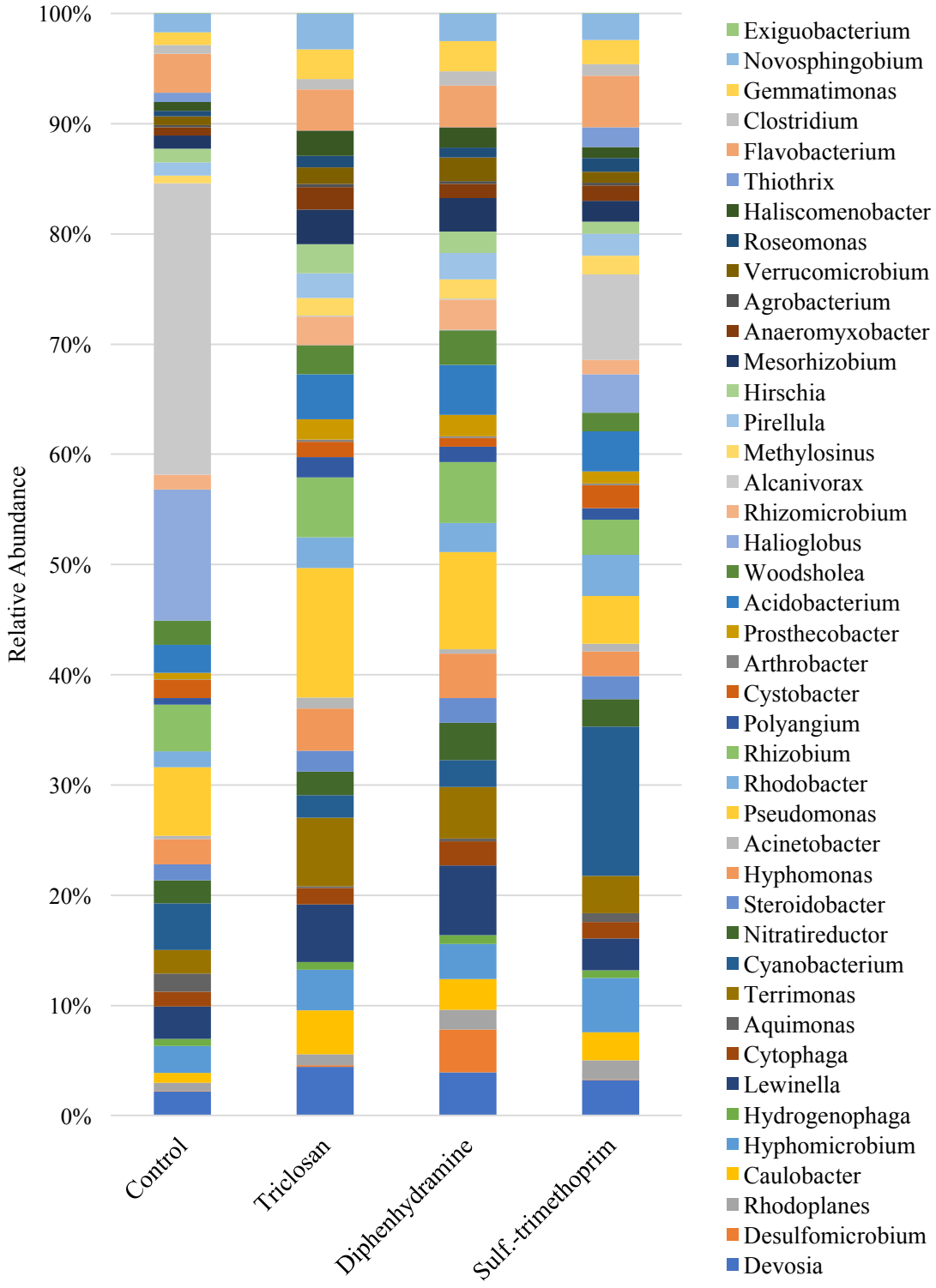


Figure 8. Bacterial community composition in **(a)** Portage Creek (class), **(b)** Malletts Creek (class), **(c)** Portage Creek (genus), and **(d)** Malletts Creek (genus) biofilms following exposure to pharmaceutical treatments.

Table 9. Total genus percentages (%) for *Alcanivorax*, *Halioglobus*, and *Pseudomonas* following exposure to pharmaceutical treatments.

	Control	Triclosan	Diphenhydramine	Sulf.-trimethoprim
Portage Creek (agricultural)				
Alcanivorax	14.9	0.7	2.3	4.9
Halioglobus	7.7	0.4	1.0	2.0
Pseudomonas	4.4	11.8	10.8	8.4
Malletts Creek (urban)				
Alcanivorax	16.5	0.1	0.1	4.2
Halioglobas	7.4	0.0	0.0	1.9
Pseudomonas	3.9	6.6	4.7	2.4

Discussion

We assessed the effects of pharmaceutical compounds on biofilm communities across a land use gradient using five different response variables and found some evidence of reduced function and change in bacterial community composition in biofilms exposed to pharmaceuticals, although we found no evidence to suggest that land use influences the response. Decomposition, photosynthetic potential, and community-level physiological profiles were not affected by pharmaceutical treatment or land use, but we found a significant response in DOC uptake to land use ($p = 0.02$) and a decrease in DOC uptake with pharmaceutical treatment that was nearly significant ($p = 0.06$). In addition, metagenomic sequencing results showed several distinct shifts in bacterial community composition in response to low doses (5 μg/L) of pharmaceuticals. These results suggest that PPCPs may impact some, but not all, aspects of stream biofilm structure and function.

Effects of Pharmaceutical Treatments on Biofilm Functioning

Our findings suggest that the bacterial activity of stream biofilms has the potential to be affected by PPCP exposure. The effect of pharmaceuticals on bacterial activity via DOC uptake was nearly significant ($p = 0.06$), indicating that biofilms may be sensitive to these compounds. These results suggest that many streams may be negatively affected by low-level pharmaceutical exposure. The effect size, while small (a maximum effect size of 7.25%), extrapolated over larger areas could impact stream carbon budgets. Our control biofilms on 2 cm x 2 cm tiles consumed an average of 0.137 mg carbon (C) per day. Extrapolated to a 1 km reach of a 10 m wide stream, a 5% reduction in DOC consumption would decrease DOC uptake by 5.14 kg C

over a 30-day period, or by 30.8 kg C for a 6-month growing season, increasing organic carbon export downstream.

Our results are consistent with other findings that biofilm microbial respiration is suppressed in response to pharmaceutical exposure, both alone or in combination with other pharmaceuticals, although other studies have found much larger responses. For example, the antidepressants fluoxetine and citalopram reduced biofilm respiration by more than 43%, and caffeine, diphenhydramine, the antacid cimetidine, and the antibiotic ciprofloxacin suppressed respiration by 49-97% (Rosi-Marshall et al. 2013; Richmond et al. 2016). Our results from 5 µg/L concentrations suggest that bacterial activity may be impacted at even very low concentrations, although the higher concentrations used in other studies (≥ 40µg/L) elicited a stronger effect.

We found no effect of pharmaceuticals on photosynthetic potential, as measured by the maximum photosynthetic efficiency of PSII, even at higher pharmaceutical concentrations (80 µg/L). Previous research indicates that some, but not all, pharmaceutical compounds suppress the processes of algal assemblages. While low amounts of triclosan do not appear to impact final algal biomass, diphenhydramine reduces gross primary production and sulfamethoxazole-trimethoprim reduces algal growth (Wilson et al. 2003; Rosi-Marshall et al. 2013; Teixeira and Granek 2017). We did not see similar effects in photosynthetic potential, and one possibility for our results may be the mechanism by which the pharmaceuticals in question affect photosynthetic microorganisms. Our method assessed photosynthetic potential using PAM chlorophyll fluorescence by using light re-emitted by chlorophyll molecules to measure PSII photochemistry efficiency (Consalvey et al. 2005). This suggests that the impacts pharmaceutical contaminants have on photosynthetic microorganisms do not target specific photosynthetic

machinery. Rather than impacting the mechanisms of photosynthesis directly, pharmaceuticals may indirectly affect photosynthesis by suppressing algal growth. This toxicity would nonetheless disrupt the flow of energy, potentially altering food web dynamics. Other investigators have reported mixed results of the effects of PPCPs on algae, with only certain pharmaceuticals or mixtures of pharmaceuticals impacting algal processes (Wilson et al. 2003; Rosi-Marshall et al. 2013), or only some parameters of algal processes being affected and not others (Richmond et al. 2016). This suggests that algae may only be susceptible to certain pharmaceuticals or that some parameters may be more sensitive indicators of algal functioning than others.

While there was no significant effect of triclosan or land use on decomposition, one urban (Millers Creek) and one agricultural (Mill Creek) stream showed a strong negative response to triclosan. Decomposition in triclosan treatments was reduced by 19% in Millers Creek and 59% in Mill Creek. Triclosan is a broad-spectrum antimicrobial which affects both bacteria and fungi, so these results suggest that some factor specific to those streams may be affecting how biofilms respond to triclosan. While we did not assess fungal community composition, a difference in fungi decomposers in these sites may account for these differences, particularly if fungi are more sensitive to triclosan than bacteria.

Community-level physiological profiles showed little effect of pharmaceutical treatments and land use. AMR and Shannon-Wiener diversity (H') did not differ between treatments and controls. However, DNA results showed noticeable shifts in treatments compared to control groups, such as *Alcanivorax* and *Halioglobus* in the class Gammaproteobacteria lower in pharmaceutical treatments, and *Pseudomonas* (also of the class Gammaproteobacteria) higher in treatments. This shift in community composition without a similar shift in physiological profiles

indicates that while the bacterial community may be altered, similarly-functioning groups may compensate for the loss of any single group. However, another possibility for the lack of response in microbial function may be the short duration of pharmaceutical exposure. The community-level physiological profile experiment consisted of a short-term exposure of 7 days, while we found greater changes in the long-term bacterial activity experiment, which ran for 21 days. Similarly, previous research has found that longer-term (21+ days) exposures to stressors impact biofilm communities more than short-term exposures (Teixeira and Granek 2017; Romero et al. 2019). This may be explained by the extracellular polymeric substance (EPS), which provides structural support and a protective barrier for biofilm communities. The polysaccharides and proteins that make up this barrier confers tolerance against antimicrobial agents (Flemming and Wingender 2010). Therefore, the protective elements in EPS could prevent contaminants at low concentrations from penetrating the community within for a short period.

Effects of Pharmaceuticals on Bacterial Community Composition

The lack of a consistent pattern between genera richness (S) and Shannon-Wiener diversity (H') in pharmaceutical treatments indicates that while overall diversity of bacterial genera may not be impacted, the relative abundances of distinct groups may be influenced by pharmaceuticals. We found little difference in S between pharmaceutical treatments and controls in Malletts Creek biofilms and lower S in triclosan-treated Portage Creek biofilms. H' was lower in control and sulfamethoxazole-trimethoprim-treated biofilms than in triclosan and diphenhydramine-treated biofilms in Malletts Creek, and was only slightly lower in control and

triclosan-treated biofilms in Portage Creek. The discrepancy between S and H' results suggests that relative abundance is being impacted.

Several bacterial groups showed profound differences in abundance between controls and treatments. The class Gammaproteobacteria was lower by 27% to 32% in Portage Creek and 52% to 69% in Malletts Creek. Within the Gammaproteobacteria class, we found that the notable shifts in the abundance of distinct genera. *Alcanivorax* and *Halioglobus* showed notably lower abundance in treatments compared to controls. *Alcanivorax* was lower by 67% to 95% in Portage Creek and 74% to > 99% in Malletts Creek biofilms in response to pharmaceutical treatments, and *Halioglobus* was lower by 37% to 95% in Portage Creek and 74% to > 99% in Malletts Creek biofilms. The genus *Pseudomonas*, also within the Gammaproteobacteria class, was more abundant in treatments, with the most substantial changes by an increase of 169% and 146% in response to triclosan and diphenhydramine treatments in Portage Creek biofilms. Our results are consistent with previous findings, showing that *Pseudomonas* and its parent class, Gammaproteobacteria, were abundant following pharmaceutical treatments (Collado et al. 2013; Rosi-Marshall et al. 2013). The *Pseudomonas* genus is a common environmental bacteria frequently associated with biofilms (Cole 1982). This genus is known for its metabolic diversity and ability to persist in environmental conditions that other bacteria cannot tolerate, and antibiotic resistance has been observed in the pathogenic *Pseudomonas aeruginosa* in streams receiving wastewater effluent (Magalhaes et al. 2016). Our results are consistent with the idea that *Pseudomonas* thrives in an environment other bacterial genera may not be able to tolerate, and that pharmaceutical contaminants act as a pressure for the selection of antimicrobial-resistant bacteria. Pharmaceutical contaminants, even in very low concentrations, can therefore effectively alter bacterial community composition.

Effects of Land Use

DOC uptake differed across land use ($p = 0.02$) but did not predict biofilm response to pharmaceuticals. The DOC uptake assay was conducted under controlled laboratory conditions during incubation, so this result is not site-specific but may be attributed to differences in the biofilms across land use. We did not measure biomass, but a greater biomass on tiles from agricultural streams may account for their higher rates of DOC consumption. Another possibility may be differences in bacterial community composition across different land uses. While we found no direct interactive effects of land use on bacterial activity, previous research indicates that microbial communities in urban waterways show pharmaceutical resistance due to continuous contaminant exposure (Rosi et al. 2018). However, the complex effects of pharmaceuticals and land use may influence bacterial activity in ways difficult to predict. Aquatic systems are often subject to multiple pollutants, particularly in urban watersheds, and the effects of multiple stressors are complex and difficult to anticipate (Romero et al. 2019). Furthermore, while individual PPCP contaminants are typically found in low concentrations, they are often detected in large combinations with other PPCPs (Kolpin et al. 2003; Bartelt-Hunt et al. 2009; Bernot et al. 2016). Each contaminant has unique chemical properties which influence its ease of removal, persistence in the environment, biological pathways it impacts, and interactive effects in combination with other contaminants. Combinations of individual compounds to form "chemical cocktails" may have unexpected interactive effects not anticipated in analysis of their individual effects (Kaushal et al. 2018). Our knowledge is limited on the ways in which multiple stressors interact with synthetic compounds to impact ecological processes, and future research should focus on how combinations of such stressors interact with PPCPs to provide a more comprehensive approach to studying impacts on ecological processes.

Conclusions

We found that low levels of PPCPs have the potential to impact DOC uptake and bacterial community composition, although we did not detect effects on maximum photosynthetic efficiency, decomposition or physiological profiles. One important ecosystem function, DOC consumption, decreased (nearly significant; $p = 0.06$) following exposure to trace amounts of single pharmaceutical contaminants and significantly differed across land-use types. Responses at extremely low treatment levels suggests that these compounds have the capacity to impact ecosystem processes, particularly as PPCPs are often detected in combinations in the environment. However, further research is required to determine the specific mechanisms by which they impact biofilms and the extent to which these processes may be affected. The three tested pharmaceuticals did not impact PSII in photosynthesis, but they may impact photosynthetic microorganisms through some other mechanism, such as affecting algal biomass or chlorophyll *a*. Bacterial community composition may be influenced by the presence of pharmaceuticals, as we observed decreases in *Alcanivorax* and *Halioglobus* genera and an increase in the *Pseudomonas* genera following treatments indicate a shift to more drug-tolerant groups. Community composition changes in response to PPCPs may impact bacterial function, but additional information is required to understand whether a change in diversity would impact ecological processes. The long-term impacts of PPCPs on biofilm communities may be stronger than short-term effects, and the complex relationships of multiple stressors combined with PPCPs on stream ecosystems is not well understood and should be further explored.

PPCPs and their metabolites pose a risk for aquatic ecosystems in a multitude of ways, from aquatic organisms to energy and nutrient transformation. Few studies have explored the ecological disrupting effects of these contaminants at environmentally relevant, non-lethal levels.

Other variables such as time scale and interactions with other environmental stressors should be investigated. Exploring the ecological costs of pharmaceutical contaminants in aquatic ecosystems will better inform regulations and remediations for the health of aquatic systems.

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APPENDICES

Appendix A: Biofilm Functioning Experimental Data

Table 10. DOC uptake assay data of DOC consumption concentrations (ppm).*

	Natural		Agricultural		Urban	
	Hay	**Honey**	**Portage**	**Mill**	**Millers**	**Malletts**
Control	59.38	64.30	64.16	66.54	64.83	68.60
	63.05	65.82	68.78	59.63	66.96	66.48
	64.47	66.56	68.72	64.93	66.31	63.76
	64.50	65.54	69.03	64.95	66.64	68.23
	60.79	63.27	65.10	70.68	66.99	63.71
Triclosan	62.26	61.51	64.41	66.23	61.98	62.05
	60.86	61.94	66.07	67.52	60.53	64.99
	60.93	61.01	64.25	66.50	60.76	64.12
	61.70	62.38	62.95	70.43	62.41	61.33
	62.23	-	62.92	62.08	63.03	63.35
Diphenhydramine	59.30	60.88	63.53	67.63	62.74	61.77
	59.60	62.58	66.21	69.64	60.55	63.61
	61.74	60.81	65.36	66.92	60.60	64.01
	61.99	56.70	65.38	66.20	62.09	62.49
	61.84	59.58	64.35	71.09	69.04	63.46
Sulfamethoxazole-trimethoprim	61.47	62.86	64.77	66.97	68.27	62.48
	61.41	64.55	65.05	65.69	67.52	65.31
	60.79	63.70	64.44	63.11	61.71	65.52
	60.35	62.08	66.43	62.16	68.87	63.87
	59.89	64.80	63.52	67.95	64.92	62.84

*A dash (-) indicates missing data.

Table 11. Stream DOC concentrations with DOC spike, additional controls.

Filtered stream water + DOC spike (ppm)	**Control - Filtered stream water, no biofilm, no treatment (ppm)**	**Control - DI water, no biofilm, no treatment (ppm)**
(Hay) 82.29	(Hay) 74.97	1.61
(Honey) 84.05	(Honey) 75.30	1.44
(Portage) 91.92	(Portage) 85.85	1.18
(Mill) 87.88	(Mill) 78.9051	1.26
(Millers) 83.69	(Millers) 70.95	1.76
(Malletts) 90.59	(Malletts) 84.16	-

Table 12. PAM chlorophyll fluorescence data (YII).

	Natural		Agricultural		Urban	
	Hay	**Honey**	**Portage**	**Mill**	**Millers**	**Malletts**
Control	0.64	0.62	0.61	0.64	0.62	0.63
	0.37	0.65	0.60	0.66	0.64	0.62
	0.46	0.65	0.60	0.63	0.64	0.63
	0.45	0.62	0.59	0.63	0.67	0.63
	0.68	0.61	0.59	0.66	0.64	0.63
Triclosan	0.57	0.63	0.61	0.67	0.62	0.64
	0.61	0.64	0.64	0.65	0.61	0.63
	0.67	0.64	0.63	0.64	0.63	0.63
	0.65	0.65	0.61	0.65	0.67	0.63
	0.66	0.64	0.61	0.66	0.60	0.62
Diphenhydramine	0.61	0.60	0.62	0.65	0.64	0.63
	0.65	0.61	0.63	0.64	0.62	0.63
	0.62	0.63	0.61	0.65	0.62	0.65
	0.58	0.65	0.59	0.63	0.62	0.63
	0.63	0.64	0.61	0.63	0.64	0.64
Sulfamethoxazole-trimethoprim	0.66	0.63	0.62	0.64	0.62	0.63
	0.71	0.60	0.64	0.64	0.64	0.62
	0.33	0.62	0.62	0.63	0.63	0.62
	0.64	0.65	0.65	0.65	0.62	0.63
	0.63	0.64	0.62	0.64	0.62	0.63

Table 13. Cotton strip assay data (percent loss tensile strength per day).

	Natural		Agricultural		Urban	
	Hay	**Honey**	**Portage**	**Mill**	**Millers**	**Malletts**
Control	2.31	2.17	2.32	1.68	2.33	2.56
	1.81	2.44	2.20	0.79	2.52	2.72
	1.72	1.92	2.18	1.04	2.51	2.47
	2.13	1.41	2.29	0.45	2.67	2.70
	2.02	1.47	2.63	2.13	2.78	2.62
Triclosan	1.96	2.13	2.21	0.22	2.02	2.85
	2.14	1.61	2.56	0.87	2.15	2.61
	1.46	2.09	2.42	0.21	2.01	2.44
	2.11	1.68	2.39	0.86	1.89	2.73
	1.97	1.88	2.37	0.32	2.23	2.56

Table 14. Community-level physiological profile BIOLOG plate Shannon-Wiener diversity (H') data.

		Natural		Agricultural		Urban	
		Hay	Honey	Portage	Mill	Millers	Malletts
Day 0	Control	2.13	1.68	0.25	2.63	0.00	2.68
		0.49	2.53	1.89	0.00	2.70	2.93
	triclosan	3.05	2.71	0.82	2.79	0.00	2.07
	Diphen.	0.00	2.29	2.60	0.60	2.73	2.62
	Sulf.-trim.	1.55	1.68	2.85	2.78	2.68	0.00
Day 3	Control	2.89	3.25	3.02	2.99	3.22	2.99
		2.91	3.26	3.07	3.09	3.18	2.96
	Triclosan	0.00	3.14	3.13	2.99	3.26	3.04
	Diphen.	2.87	3.09	3.03	3.06	3.22	3.05
	Sulf.-trim.	2.98	3.02	3.02	2.99	3.17	3.06
Day 5	Control	3.00	3.36	3.12	3.08	3.32	3.13
		3.08	3.35	3.22	3.22	3.27	3.13
	Triclosan	3.13	3.23	3.28	3.07	3.28	3.15
	Diphen.	2.95	3.26	3.14	3.17	3.28	3.17
	Sulf.-trim.	3.08	3.18	3.24	3.05	3.29	3.18

Table 15. Community-level physiological profile BIOLOG plate average metabolic response (AMR) data.

		Natural		Agricultural		Urban	
		Hay	Honey	Portage	Mill	Millers	Malletts
Day 0	Control	0.01	-0.01	-0.01	0.02	-0.06	0.04
		-0.06	0.03	0.01	-0.04	0.02	0.04
	Triclosan	0.03	0.03	-0.06	0.03	-0.08	0.02
	Diphen.	-0.04	0.03	0.03	-0.04	0.01	0.03
	Sulf.-trim.	0.00	0.01	0.04	0.02	0.04	-0.06
Day 3	Control	0.83	1.35	0.91	1.01	1.25	0.87
		0.68	1.30	0.92	1.17	1.24	0.92
	Triclosan	0.96	1.10	1.03	1.17	1.31	0.96
	Diphen.	0.75	1.14	0.91	0.95	1.32	0.96
	Sulf.-trim.	1.00	1.10	0.89	0.98	1.12	1.06
Day 5	Control	1.04	1.79	1.07	1.22	1.59	1.25
		1.11	1.70	1.30	1.54	1.56	1.19
	Triclosan	1.24	1.44	1.18	1.33	1.62	1.25
	Diphen.	1.05	1.43	1.07	1.21	1.69	1.32
	Sulf.-trim.	1.30	1.45	1.23	1.31	1.55	1.49

Appendix B: Bacterial Community Experimental Data

Table 16. Relative abundance of bacterial classes (%) from tiles at the end of the DOC uptake assays in controls and pharmaceutical treatments in Portage Creek. Samples were pooled from 5 tiles (n = 1).

Class	Control	Triclosan	Diphenhydramine	Sulf.-trimethoprim
Acidobacteriia	1.93	1.76	3.83	3.19
Flavobacteriia	1.08	2.81	1.47	1.98
Bacilli	0.76	2.18	0.35	0.39
Sphingobacteriia	4.08	5.45	8.92	8.52
Betaproteobacteria	6.86	6.10	6.82	6.21
Bacteroidia	0.26	0.27	0.41	1.26
Verrucomicrobiae	1.44	1.48	1.80	2.61
Alphaproteobacteria	23.06	30.85	25.85	23.43
Gammaproteobacteria	35.77	24.62	24.17	26.19
Cyanobacteria	0.28	0.08	0.23	0.27
Clostridia	2.14	1.64	1.42	1.79
Oscillatoriophycideae	0.11	0.13	0.24	0.15
Actinobacteria	8.34	7.12	6.29	6.76
Gemmatimonadetes	0.52	1.32	0.94	0.79
Planctomycetia	2.90	2.83	3.21	2.62
Cytophagia	1.63	1.96	2.32	2.13
Deltaproteobacteria	4.16	5.29	6.37	6.13
Other (<1% for all groups)	4.68	4.08	5.33	5.58

Table 17. Relative abundance of bacterial classes (%) from tiles at the end of the DOC uptake assays in controls and pharmaceutical treatments in Malletts Creek. Samples were pooled from 5 tiles (n = 1).

Class	Control	Triclosan	Diphenhydramine	Sulf.-trimethoprim
Acidobacteriia	1.71	2.68	2.82	2.24
Flavobacteriia	2.93	3.21	3.40	3.44
Bacilli	0.19	0.19	0.23	0.27
Sphingobacteriia	5.79	10.39	10.10	5.70
Betaproteobacteria	7.44	6.67	6.76	5.32
Bacteroidia	0.20	0.29	0.32	0.16
Verrucomicrobiae	1.54	2.53	3.20	2.00
Alphaproteobacteria	23.77	35.34	32.44	30.66
Gammaproteobacteria	34.97	11.85	10.69	16.73
Cyanobacteria	0.93	0.15	0.20	1.73
Clostridia	0.93	1.35	1.27	1.11
Oscillatoriophycideae	2.85	1.24	1.37	7.99
Actinobacteria	2.30	5.28	4.84	5.00
Gemmatimonadetes	0.73	1.51	1.47	1.20
Planctomycetia	2.32	3.28	3.66	3.29
Cytophagia	1.88	1.62	2.48	1.75
Deltaproteobacteria	4.90	7.53	8.35	6.39
Other (<1% for all groups)	4.61	4.89	6.40	5.02

Table 18. Relative abundance of bacterial classes (%) from tiles from each stream. Samples were pooled from 5 tiles (n = 1).

Class	Natural		Agricultural		Urban	
	Hay	Honey	Portage	Mill	Millers	Malletts
Nitrospira	1.25	1.71	0.89	1.01	0.51	0.20
Acidobacteriia	3.95	3.59	3.02	3.51	1.75	1.39
Flavobacteriia	3.88	5.44	3.07	2.84	4.42	4.24
Bacilli	1.03	1.45	0.62	0.86	0.58	0.23
Sphingobacteriia	7.19	5.11	8.06	9.20	7.57	13.71
Betaproteobacteria	9.64	7.64	10.36	9.96	10.31	7.52
Bacteroidia	1.42	1.13	1.54	2.55	3.08	0.41
Verrucomicrobiae	4.00	2.63	2.97	3.16	2.31	3.28
Alphaproteobacteria	18.33	19.00	19.67	18.31	10.96	21.70
Gammaproteobacteria	8.07	8.75	10.55	7.72	13.16	8.93
Cyanobacteria	2.15	5.21	5.34	0.48	0.35	5.93
Clostridia	1.89	2.11	2.57	2.93	2.73	2.23
Oscillatoriophycideae	0.88	0.98	0.59	0.45	0.29	11.30
Actinobacteria	11.14	9.42	7.60	10.34	10.09	2.14
Gemmatimonadetes	0.99	0.84	0.68	0.62	0.78	0.97
Planctomycetia	3.11	3.51	4.57	4.21	2.50	3.50
Anaerolineae	3.01	2.29	2.18	2.55	1.96	0.27
Cytophagia	3.98	3.91	3.88	5.37	6.52	6.06
Deltaproteobacteria	8.53	8.63	6.86	7.68	13.84	2.73
Other (<1% for all groups)	5.55	6.63	4.96	6.27	6.31	3.25

Table 19. Relative abundance of bacterial genera (%) from tiles at the end of the DOC uptake assays in controls and pharmaceutical treatments in Portage Creek. Samples were pooled from 5 tiles (n = 1).

Genus	Control	Triclosan	Diphenhydramine	Sulf.-trimethoprim
Devosia	0.53	2.62	1.31	1.37
Desulfomicrobium	0.00	0.00	0.03	0.04
Rhodoplanes	1.10	0.74	0.99	0.81
Caulobacter	0.72	2.21	1.53	1.62
Hyphomicrobium	2.29	2.00	2.24	0.95
Hydrogenophaga	1.36	0.19	0.67	0.78
Lewinella	1.08	1.47	2.92	2.31
Cytophaga	0.79	1.02	1.20	1.23
Aquimonas	0.07	0.07	0.15	0.04
Terrimonas	0.91	1.44	2.53	2.39
Cyanobacterium	0.11	0.12	0.23	0.16
Nitratireductor	0.10	0.07	0.32	0.15
Steroidobacter	1.23	1.31	1.93	1.99
Hyphomonas	0.22	0.58	0.45	0.31
Acinetobacter	0.55	5.48	2.42	3.09
Pseudomonas	4.39	11.83	10.82	8.44
Rhodobacter	5.52	6.02	4.23	5.17
Rhizobium	0.72	1.30	1.21	0.49
Polyangium	0.48	1.05	0.64	1.05
Cystobacter	0.02	0.39	0.10	0.19
Arthrobacter	2.32	2.26	2.20	2.05
Prosthecobacter	0.29	0.41	0.49	0.54
Acidobacterium	1.77	1.68	3.53	2.99
Woodsholea	0.25	0.54	0.35	0.45
Halioglobus	7.69	0.40	1.00	1.98
Rhizomicrobium	0.08	1.16	0.10	0.07
Alcanivorax	14.92	0.69	2.25	4.86
Methylosinus	1.14	1.18	1.03	0.84
Pirellula	0.90	0.99	0.88	0.80
Hirschia	0.04	0.10	0.13	0.03
Mesorhizobium	0.69	0.34	1.55	0.73
Anaeromyxobacter	0.49	1.07	0.85	0.69
Agrobacterium	0.81	1.08	0.52	0.39
Verrucomicrobium	0.71	0.79	0.80	0.94
Roseomonas	0.92	0.82	0.63	1.01
Haliscomenobacter	0.49	0.72	1.31	1.26
Thiothrix	1.08	0.01	0.40	0.01

Flavobacterium	0.66	2.14	0.74	1.21
Clostridium	1.57	1.07	0.64	0.95
Gemmatimonas	0.52	1.32	0.94	0.79
Novosphingobium	0.41	0.96	0.87	1.04
Exiguobacterium	0.50	1.21	0.09	0.07
Other (<1% for all groups)	39.56	39.17	42.78	43.73

Table 20. Relative abundance of bacterial genera (%) from tiles at the end of the DOC uptake assays in controls and pharmaceutical treatments in Malletts Creek. Samples were pooled from 5 tiles (n = 1).

Genus	Control	Triclosan	Diphenhydramine	Sulf.-trimethoprim
Devosia	1.36	2.49	2.09	1.77
Desulfomicrobium	0.04	0.10	2.07	0.02
Rhodoplanes	0.46	0.54	0.94	0.96
Caulobacter	0.54	2.26	1.49	1.39
Hyphomicrobium	1.53	2.08	1.70	2.69
Hydrogenophaga	0.41	0.38	0.42	0.40
Lewinella	1.82	2.95	3.36	1.56
Cytophaga	0.83	0.84	1.17	0.83
Aquimonas	1.04	0.10	0.12	0.43
Terrimonas	1.33	3.51	2.50	1.86
Cyanobacterium	2.62	1.15	1.28	7.42
Nitratireductor	1.31	1.20	1.82	1.35
Steroidobacter	0.89	1.07	1.19	1.15
Hyphomonas	1.44	2.18	2.12	1.23
Acinetobacter	0.17	0.56	0.22	0.38
Pseudomonas	3.88	6.61	4.68	2.38
Rhodobacter	0.88	1.57	1.40	2.03
Rhizobium	2.66	3.08	2.94	1.76
Polyangium	0.34	1.04	0.75	0.58
Cystobacter	1.03	0.78	0.42	1.15
Arthrobacter	0.05	0.11	0.08	0.09
Prosthecobacter	0.37	1.04	1.03	0.58
Acidobacterium	1.57	2.33	2.42	2.00
Woodsholea	1.36	1.47	1.65	0.94
Halioglobus	7.40	0.02	0.03	1.91
Rhizomicrobium	0.85	1.45	1.46	0.70
Alcanivorax	16.45	0.07	0.06	4.25
Methylosinus	0.43	0.90	0.93	0.93
Pirellula	0.73	1.26	1.27	1.10
Hirschia	0.78	1.49	1.04	0.61
Mesorhizobium	0.74	1.76	1.61	1.03
Anaeromyxobacter	0.49	1.14	0.70	0.76
Agrobacterium	0.10	0.17	0.11	0.13
Verrucomicrobium	0.49	0.83	1.16	0.56
Roseomonas	0.33	0.59	0.48	0.68
Haliscomenobacter	0.50	1.29	0.97	0.53
Thiothrix	0.52	0.01	0.01	0.97

Flavobacterium	2.20	2.10	1.99	2.57
Clostridium	0.49	0.53	0.69	0.57
Gemmatimonas	0.73	1.51	1.47	1.20
Novosphingobium	1.04	1.83	1.31	1.31
Exiguobacterium	0.02	0.01	0.02	0.01
Other (<1% for all groups)	37.79	43.56	46.83	45.20

Table 21. Relative abundance of bacterial genera (%) from tiles from each stream. Samples were pooled from 5 tiles (n = 1).

Genus	Natural		Agricultural		Urban	
	Hay	Honey	Portage	Mill	Millers	Malletts
Syntrophus	0.95	1.12	0.62	0.56	0.31	0.07
Rhodoplanes	1.41	1.18	0.99	1.71	0.63	0.17
Desulfobacterium	0.12	0.13	0.15	0.06	1.56	0.04
Dechloromonas	0.03	0.09	0.22	0.06	1.68	0.24
Luteolibacter	1.50	0.45	0.53	0.65	0.26	1.61
Mariniflexile	0.00	1.16	0.00	0.00	0.09	0.05
Myxosarcina	0.00	0.48	1.37	0.00	0.00	0.16
Burkholderia	1.02	1.24	1.73	1.50	0.64	0.53
Sphingobacterium	0.51	0.57	1.01	0.58	0.64	0.56
Hyphomicrobium	1.87	2.98	2.70	1.85	0.83	1.69
Arenimonas	0.27	0.17	0.32	0.51	0.44	1.52
Lewinella	1.70	1.13	2.44	2.64	1.40	2.80
Cytophaga	1.48	1.87	2.07	1.88	3.98	2.57
Haliea	0.36	0.70	1.87	0.72	2.15	0.87
Chamaesiphon	1.46	1.53	2.27	0.24	0.00	1.15
Terrimonas	0.79	0.44	1.21	1.65	0.98	2.85
Cyanobacterium	0.82	0.92	0.59	0.42	0.26	10.39
Chroococcidiopsis	0.00	0.01	0.04	0.00	0.06	1.32
Steroidobacter	1.09	1.36	1.91	1.65	1.22	0.75
Pseudomonas	0.32	0.42	0.42	0.34	1.65	0.31
Rhodobacter	2.24	2.55	2.99	2.28	0.93	3.65
Lacibacter	0.14	0.09	0.09	0.31	0.11	1.75
Rhizobium	0.84	0.79	0.60	0.62	0.63	1.06
Leadbetterella	1.17	0.50	0.40	1.86	0.19	1.00
Bacteroides	0.65	0.47	0.70	1.00	0.94	0.21
Acidobacterium	3.62	3.45	2.88	3.17	1.69	1.24
Pelobacter	1.77	1.81	0.92	1.28	1.33	0.35
Chelatococcus	1.03	1.50	0.29	0.70	0.02	0.48
Nitrospira	1.13	1.57	0.83	0.94	0.47	0.19
Methylobacter	0.62	0.48	1.05	0.44	0.19	0.09
Pseudanabaena	0.34	1.59	0.73	0.03	0.00	0.31
Pleurocapsa	0.09	1.24	0.26	0.00	0.00	0.16
Methylosinus	0.51	0.65	1.23	0.63	0.16	0.48
Pirellula	0.98	1.22	1.58	1.49	1.08	1.41
Geobacter	1.75	1.52	1.50	1.42	4.12	0.33
Anaeromyxobacter	1.02	0.96	0.63	1.14	0.92	0.13
Pedosphaera	0.58	0.54	0.51	0.51	1.06	0.37

Solitalea	0.18	0.20	0.23	0.15	1.33	0.27
Tetrasphaera	0.13	0.11	0.04	0.20	1.05	0.14
Verrucomicrobium	1.43	1.17	1.15	1.32	0.63	0.70
Gaiella	1.27	0.85	0.72	1.39	0.27	0.09
Haliscomenobacter	1.61	1.46	1.12	1.91	1.09	2.76
Levilinea	1.39	0.78	0.77	1.19	0.77	0.08
Flavobacterium	2.92	3.38	1.94	2.02	2.91	3.21
Clostridium	0.75	0.81	1.55	1.40	1.55	1.52
Conexibacter	1.71	1.44	0.79	1.52	1.11	0.32
Novosphingobium	1.11	0.73	0.54	0.60	0.29	1.54
Sediminibacterium	0.92	0.24	0.53	0.30	0.15	1.55
Other (<1% for all groups)	52.38	49.95	50.99	53.15	56.22	44.98