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Pharmaceuticals suppress algal growth and microbial respiration and alter bacterial communities in stream biofilms

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Abstract. Pharmaceutical and personal care products are ubiquitous in surface waters but their effects on aquatic biofilms and associated ecosystem properties are not well understood. We measured in situ responses of stream biofilms to six common pharmaceutical compounds (caffeine, cimetidine, ciprofloxacin, diphenhydramine, metformin, ranitidine, and a mixture of each) by deploying pharmaceutical-diffusing substrates in streams in Indiana, Maryland, and New York. Results were consistent across seasons and geographic locations. On average, algal biomass was suppressed by 22%, 4%, 22%, and 18% relative to controls by caffeine, ciprofloxacin, diphenhydramine, and the mixed treatment, respectively. Biofilm respiration was significantly suppressed by caffeine (53%), cimetidine (51%), ciprofloxacin (91%), diphenhydramine (63%), and the mixed treatment (40%). In autumn in New York, photosynthesis was also significantly suppressed by diphenhydramine (99%) and the mixed treatment (88%). Pyrosequencing of 16S rRNA genes was used to examine the effects of caffeine and diphenhydramine on biofilm bacterial community composition at the three sites. Relative to the controls, diphenhydramine exposure significantly altered bacterial community composition and resulted in significant relative increases in *Pseudomonas* sp. and decreases in *Flavobacterium* sp. in all three streams. These ubiquitous pharmaceuticals, alone or in combination, influenced stream biofilms, which could have consequences for higher trophic levels and important ecosystem processes.

Key words: *caffeine; ciprofloxacin; diphenhydramine; ecosystem function.*

Introduction

As an ever greater number of pharmaceutical compounds are developed and distributed in greater quantities among a growing human population, these chemicals are increasingly entering natural ecosystems with largely unknown consequences. Synthetic compounds such as pharmaceutical and personal care products (e.g., fragrances, stimulants, analgesics, antibiotics, antihistamines, and hormones) are readily found in aquatic ecosystems (Kolpin et al. 2002, Kim et al. 2007, Focazio et al. 2008, Fick et al. 2009). Typically these compounds are found in very low concentrations (parts per trillion), but in some locations, such as the discharge waters of pharmaceutical production facilities, these compounds can be found at much higher concentrations (Larsson et al. 2007, Phillips et al. 2010). Most of these chemical substances are developed for human or veterinary uses and, as such, are designed to be pharmacologically active. Further, they are unique in their chemical composition and biological system target. Thus, pharmaceutical compounds found in aquatic environments may alter structural and functional relationships of aquatic ecosystems differently depending on the suite of compounds present (Monteiro and Boxall 2010). The occurrence of pharmaceuticals may act as a selective force acting on aquatic communities and may result in changes in species composition, which may in turn influence ecosystem function. For example, antibiotics may impact bacterial communities but have little direct effect on algae or fungi, although these groups could experience indirect effects that might decouple ecological interactions. The occurrence of pharmaceutical compounds may influence aquatic ecosystems in unexpected and non-additive ways. Pharmaceuticals frequently occur in locations with elevated nutrients (e.g., wastewater effluent, leaky infrastructure, or combined sewer overflows) and although increased nutrients may lead to increased rates of primary production or decomposition, the presence of pharmaceuticals that suppress these activities may influence the response of the system to elevated nutrients.

Although pharmaceuticals are ubiquitous in aquatic ecosystems, the influence of the wide variety of pharmaceuticals on stream biofilm structure and function is not currently known (Boxall et al. 2012, Rosi-Marshall and Royer 2012). Algae serve as a basal resource for higher

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TABLE 1. Name and description of pharmaceutical compounds added to diffusing substrates.

Compound	Chemical formula	Environmental concentration (μg/L)	CAS number	Medical use	Molar mass (g/mol)	log K_{ow}	pKa	log D (pH 5.5)
Caffeine	$C_8H_{10}N_4O_2$	0.0049–0.88†	58–08–2	stimulant	194.2	0.07	10.4	−0.63
Cimetidine	$C_{10}H_{16}N_6S$	0.074–0.58†	51 481–61–9	antihistamine (heartburn)	252.4	0.40	6.80	−1.28
Ciprofloxacin	$C_{17}H_{18}FN_3O_3$	ND–0.03†,‡	85 721–33–1	antibiotic	331.5	−1.08	6.09	−1.06
Diphenhydramine	$C_{17}H_{21}NO$	0.01–0.01§	58–73–1	antihistamine (allergens)	255.4	3.40	8.98	0.13
Metformin	$C_4H_{11}N_5$	0.11–0.15†	657–24–9	antidiabetic, cholesterol reducer	129.2	−3.39	12.4	−3.02
Ranitidine	$C_{13}H_{22}N_4O_3S$	0.0013–0.0385†	66 357–35–5	antihistamine (heartburn)	314.4	0.27	8.2	na

Note: K_{ow} (octanol water partitioning coefficient) is the ratio of the concentration of a compound dissolved in octanol relative to water. The acid dissociation constant, pKa, indicates the strength of an acid, and larger values indicate low dissociation in solution. D (pH 5.5) (distribution coefficient) is the ratio of all forms of the compound dissolved in both water and octanol at pH 5.5; this value indicates how compounds would be distributed in biological tissues (hydrophobic vs. hydrophilic). Key to abbreviations: ND, not detectable; na, not applicable.

† Found in Monteiro and Boxall (2010).

‡ A much higher concentration of up to 31 000 μg/L is reported in Larsson et al. (2007), as a result of pharmaceutical manufacturing facilities.

§ Found in Stackelberg et al. (2004).

trophic levels in many aquatic ecosystems (Minshall 1978), and heterotrophic microorganisms are responsible for decomposition of organic matter and influence numerous biogeochemical pathways. Primary production and respiration respond to various environmental, physical, and chemical conditions and can be valuable indicators of ecosystem-level responses to pharmaceuticals (Sabater et al. 2007). In addition, shifts in the composition of microbial communities may serve as an even more sensitive bioindicator of pharmaceutical effects and contribute to changes in functional processes. Studying the responses of ecosystems to pharmaceuticals that affect certain species in an aquatic ecosystem should enhance our understanding of the interactions among structure and function.

Exposure to certain pharmaceutical compounds can alter biofilms as indicated by various biomarkers of microbial activity (Aristilde et al. 2010, Bonnineau et al. 2010) and particular antibiotics can influence specific components of the microbial community (Cordova-Kreylos and Scow 2007). For example, triclosan, a commonly used antimicrobial compound, can alter the composition of both algae and bacteria in stream biofilms (Wilson et al. 2003, Lawrence et al. 2009, Ricart et al. 2010). In addition, tetracycline altered bacterial and algal assemblages and led to reduced biofilm growth rates (Quinlan et al. 2011). In contrast, cimetidine did not significantly affect primary production or respiration of stream biofilms (Hoppe et al. 2012). Measuring the effects of pharmaceuticals on aquatic ecosystems in situ is challenging because pharmaceuticals often co-occur with other pollutants such as nutrients from wastewater treatment plants.

We conducted a study to explore the effects of six common pharmaceuticals that are found in varying concentrations in the environment on stream biofilm structure and function (Table 1). Stream biofilms consist of algae, fungi, bacteria, and organic matter that develop on organic or mineral substrates. Because they occur at the interface of the physiochemical environment and the biological community, much of the biogeochemical activity in streams occurs in these biofilms (Wetzel 1993) and these biofilms support higher trophic levels in stream ecosystems (Allan and Castillo 2007). Here we present direct in situ measurements of the influence of these pharmaceuticals on community composition, algal growth, photosynthesis, and microbial respiration in stream biofilms. To measure the influence of these pharmaceuticals on biofilms, we adapted a method frequently used to study nutrient limitation to examine the effect of water-soluble pharmaceuticals on stream biofilms (Fairchild et al. 1985, Tank et al. 2006, Hoellein et al. 2010; see Fig. 1). We measured the effects of pharmaceutical compounds on biofilm chlorophyll *a* (chl *a*) content, rates of gross primary production (GPP) and respiration (R), and biofilm community composition in relation to an unamended reference condition. The diffusion rates out of the agar to which the biofilms are exposed will be dependent on compound properties, in-stream water velocity, and the development of biofilm communities. Therefore, we view this method as similar to assessing nutrient limitation and may be limited to identifying sensitive taxa and ecological processes, e.g., primary production or respiration. Other methods are more appropriate for establishing dose–response relationships, which we did not examine in this study.

We selected six pharmaceutical compounds that are commonly detected in surface waters across the United States (Kolpin et al. 2002). The suite of compounds consisted of four over-the-counter compounds, including two antihistamines used to treat heartburn (cimetidine, ranitidine), an antihistamine for allergens (diphenhydramine), and a stimulant (caffeine). We also chose two common prescription medications: an antibiotic (ciprofloxacin) and an antidiabetic (metformin). We selected these six compounds because they are all commonly detected in surface waters (Table 1), are

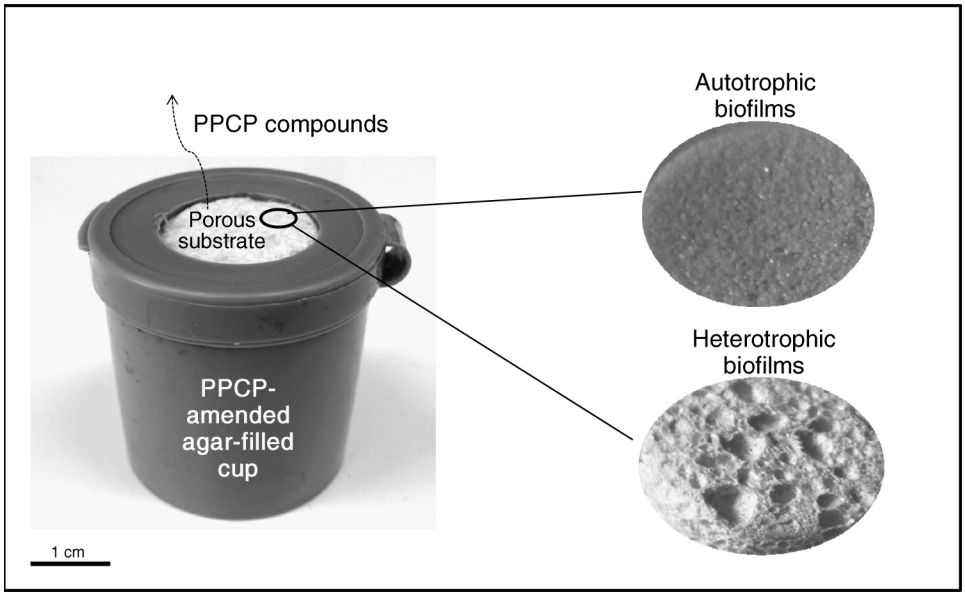

FIG. 1. Experimental design for measuring effects of water-soluble pharmaceutical compounds on stream biofilms. Inorganic substrates were used to focus on biofilm autotroph response, while organic substrates were used to better capture heterotroph response. Substrates were attached to vials filled with agar amended with water-soluble pharmaceuticals.

water soluble, and they represent a range of potential effects on aquatic biofilms. For example, we predicted that the antibiotic ciprofloxacin would inhibit bacterial activity in biofilms. Antihistamines are very commonly used and as such are commonly detected in surface waters; however, recent studies have shown no effect of antihistamines on algae (Brain et al. 2008, Gunnarsson et al. 2008), aquatic plants (Berninger et al. 2011) or primary production (Hoppe et al. 2012). Therefore, we predicted that the three antihistamines tested (diphenhydramine, cimetidine, and ranitidine) would not influence algal biomass or primary production but might more strongly affect heterotrophs. Our experimental design allowed rigorous testing of this prediction in situ, and should also provide a good assessment of the sensitivity (or lack thereof) of bacterial biofilms to these compounds. Metformin, an antidiabetic, has been detected in surface waters at concentrations of 130–1700 ng/L (Scheurer et al. 2009) and caffeine is commonly detected in surface waters sewage (e.g., Aufdenkampe et al. 2006). The potential effects of metformin and caffeine on aquatic biofilms are not known, so our study was designed to test the predictions that these compounds would alter the composition and function of stream biofilms.

Stream biofilms are complex communities that have interacting microbial populations, including both heterotrophs and autotrophs, and we hypothesized that the pharmaceuticals could influence the heterotrophic and autotrophic activity of biofilms. To test this hypothesis we measured the influence of the selected pharmaceuticals both individually and as a mixture of all compounds, on both autotrophic and heterotrophic components of biofilms in natural streams. We deployed pharmaceutical-diffusing substrates in a rural stream in New York and in suburban streams in Indiana and Maryland. These streams ranged in nutrient concentrations and degree of developed land in the basin. In addition, in temperate forested streams, the relative activity and composition of biofilms can vary throughout the year. For example, autotrophic activity can be high in the spring when the forest canopy is open while heterotrophic activity can be high in the autumn when terrestrial organic matter inputs are high. To encompass this potential variability, we conducted seasonal experiments in the rural stream in New York.

METHODS

We deployed pharmaceutical-diffusing substrata (PhaDS) in three similarly sized streams. East Branch Wappinger Creek (EBWC), in New York, USA, drains forest and agriculture and receives effluent from a small wastewater treatment plant that serves the village of Millbrook (~1300 people). Jack's Defeat Creek (JDC) in Indiana, USA, drains a low-intensity suburban area near Bloomington, Indiana and the site is below a wastewater treatment plant that serves approximately 4000 customers. Dead Run (DR), Maryland, USA, drains a suburban area, does not have a wastewater treatment plant in the drainage, and is a long-term site of the LTER Baltimore Ecosystem Study. In all of these streams, pharmaceutical compounds have been detected, but usually at low concentrations (<100 ng/L; E. J. Rosi-Marshall and T. V. Royer, *unpublished data*). During winter (December) 2009, spring (March) 2010,

and fall (November) 2010, we deployed PhaDS in the EBWC, in JDC, (spring only), and in DR (spring only).

We constructed PhaDS by adapting a method used to study nutrient limitation in streams via nutrient-diffusing substrata (Tank et al. 2006). We filled 30-mL polyethylene cups with a 2% (by mass) agar solution amended with six water-soluble pharmaceutical treatments: caffeine ($C_8H_{10}N_4O_2$, 0.015 mol/L), cimetidine ($C_{10}H_{16}N_6S$, 0.015 mol/L), ciprofloxacin ($C_{17}H_{18}FN_3O_3$, 0.013 mol/L), diphenhydramine ($C_{17}H_{21}NO$, 0.013 mol/L), metformin ($C_4H_{11}N_5$, 0.012 mol/L), ranitidine ($C_{13}H_{22}N_4O_3S$, 0.013 mol/L), and a combined treatment (additive mixture; hereafter Mix) of all of the compounds in the concentrations used above (Table 1). A control group (i.e., no pharmaceuticals added to agar) was used as well. Each cup was capped with either an inorganic substrate (fritted glass disc) to promote algal colonization or an organic substrate (cellulose sponge) to promote colonization of heterotrophic bacteria and fungi. Four replicates of each pharmaceutical and control treatment for both types of substrata were secured to the stream bottom on plastic L-bars (White PVC-1 Extruded Angle; Harvel Plastics, Lima, Ohio, USA) for 18 ± 1 days and replicates were dispersed randomly on the L-bars (Tank et al. 2006). Laboratory assays demonstrated that these compounds continue to diffuse out of the agar for 18 days and diffusion followed Fickian diffusion (L. E. Shaw and M. R. Grace, *unpublished data*). This method provides continued exposure to the compound, but not a concentration that can be controlled (Hadgraft 1979, Rugenski et al. 2008). For this reason, this method allows us to examine the influence of nominal, but not constant, concentrations on developing biofilms. After the incubation period, we transported the PhaDS from the stream to the laboratory and separated the substrata from the cups.

Functional responses of biofilms

We measured chl *a*, community respiration (*R*), and gross primary production (GPP; autumn 2010 only) on substrates. Each substrate was placed in a 50-mL centrifuge tube, filled with filtered stream water with known initial dissolved oxygen (DO) concentration, capped underwater to remove all air bubbles, and allowed to incubate in the light for 2–4 hours. We measured DO with an optical meter (ProODO meter; YSI, Yellow Springs, Ohio, USA). We included three to five "blank" tubes, which were filled with filtered stream water only, to correct for changes in background DO. All stream water used was filtered through GN-6 Metricel Membrane (Pall Corporation, Port Washington, New York, USA) filters with a pore size of 0.45 μm. After the incubation period, we measured the DO concentration in each of the tubes. We measured *R* on the same substrata by replenishing tubes with fresh water and incubating them in the dark for 2–4 hours. We calculated *R* as the change in DO per substratum area per time in the dark and calculated GPP as the change in DO per substratum area per time in the light plus the change in oxygen in the dark to account for respiration that occurred during the light treatment (Hill et al. 2002). We extracted chl *a* from inorganic substrata by freezing the substrata for at least 24 hours and immersing them in methanol in the dark for 24 hours. We measured chl *a* concentrations using a Turner Designs Model TD-700 fluorometer (Turner Designs, Sunnyvale, California, USA).

We measured response of biofilm metrics to the addition of pharmaceutical compounds (Mix, caffeine, cimetidine, ciprofloxacin, diphenhydramine, metformin, ranitidine) in autumn 2010 at the New York location, because other dates did not have all metrics or all compounds. We used a two-way analysis of variance (ANOVA) to test the effects of pharmaceuticals on each response metric (GPP, *R*, or chl *a*) and substrate type (inorganic, organic) for autumn 2010 from the New York location. If there was a significant interaction between compound and substrate, we then used a one-way ANOVA to analyze the effects of compounds on response metrics for each substrate separately. We examined the influence of pharmaceutical compounds on biofilm *R* on organic substrates and chl *a* or GPP on inorganic substrates. We used a two-way ANOVA to test response variables (*R* or chl *a*) to pharmaceutical compounds (caffeine, cimetidine, ciprofloxacin, diphenhydramine, metformin, ranitidine) with season or site (New York winter, spring, and autumn; Indiana spring; Maryland spring) and compared them to respective controls. We used inorganic substrates to analyze chl *a* responses and GPP and used organic substrates to analyze *R* responses.

Statistical analyses were performed using SYSTAT 12 (Systat Software, Richmond, California, USA) with a level of statistical significance set at $\alpha = 0.05$. We tested data using a Kolmogorov-Smirnov (Lilliefors) test for normality ($P > 0.05$). Non-normal data were transformed using natural logarithmic (chl *a*, *R*) or square root (GPP, *R*) transformations to meet assumptions of normality.

Heterotrophic bacterial community responses to caffeine and diphenhydramine

We imaged each organic substrate and calculated its surface area using ImageJ (Abramoff et al. 2004). DNA was isolated from each substrate using the PowerBiofilm DNA Isolation Kit (MoBio Laboratories, Carlsbad, California, USA). Successful DNA isolation was confirmed by agarose gel electrophoresis. The amount of DNA isolated from each sample was determined using the Quant-iT DNA Assay Kit (Invitrogen, Carlsbad, California, USA). DNA yields were normalized by the surface area of the substrate and DNA yields between samples were compared by two-way ANOVA using Systat version 13 (Systat Software, San Jose, California, USA). DNA from each substrate was sent to the Research and Testing Laboratory (Lubbock, Texas,

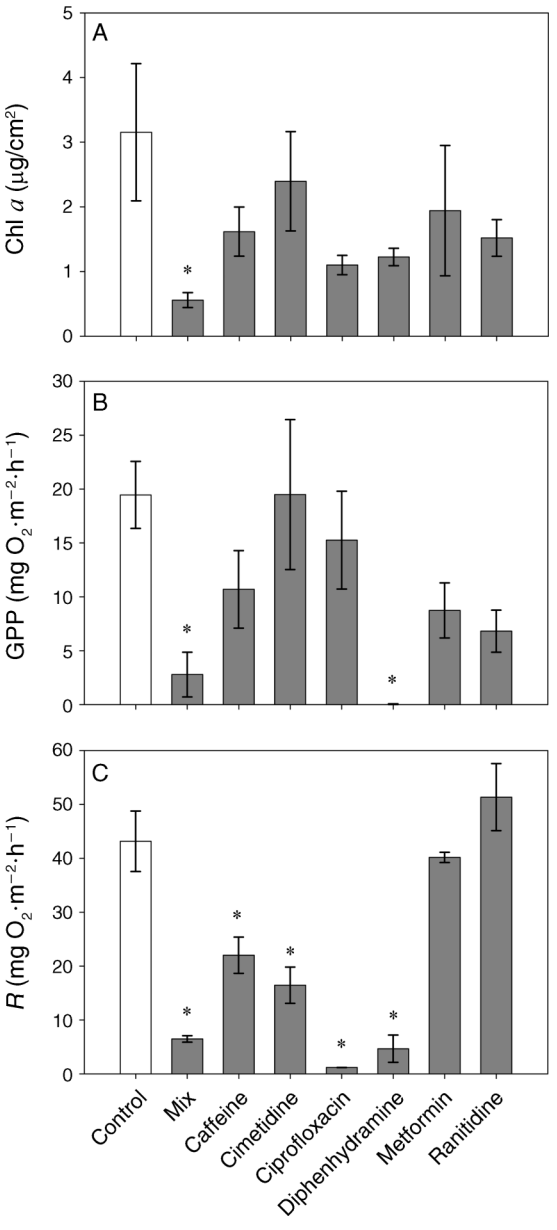

FIG. 2. Effect of pharmaceutical compounds on stream biofilms as indicated by (A) chlorophyll *a* (chl *a*), (B) gross primary production (GPP), and (C) respiration (*R*). Measurements of chl *a* and GPP are from inorganic substrata whereas *R* is from organic substrata. All data were collected in autumn 2010 in a stream in New York, USA (East Branch Wappinger Creek, EBWC). Mean and standard errors are illustrated, and asterisks indicate significant differences among the treatment and control ($P < 0.05$). We used four replicates per compound, per substrate and eight controls.

USA) for tag pyrosequencing of bacterial 16S rRNA genes. PCR amplification was performed using primers 530F and 1100R (Boon et al. 2002). The 530F primer was chosen in order to obtain sequences for the V4 hypervariable region, which has been shown to provide species richness estimates comparable to those obtained with the nearly full-length 16S rRNA gene (Youssef et al. 2009). Sequencing reactions utilized a Roche 454 FLX instrument (Roche, Indianapolis, Indiana, USA) with titanium reagents. Sequences were processed using MOTHUR v.1.22.2 (Schloss et al. 2009). Briefly, any sequences containing ambiguities or homopolymers longer than eight bases were removed. Remaining sequences were individually trimmed to retain only high quality sequence reads and sequences were aligned based on comparison to the SILVA-compatible bacterial alignment database available within MOTHUR. Aligned sequences were trimmed to a minimum length of 250 base pairs and chimeric sequences were removed using Uchime (Edgar et al. 2011) run within MOTHUR. Sequences were classified based on comparison to the RDP classifier data set (version 6) available through MOTHUR and chloroplast sequences were removed. After these pretreatment steps were completed the data set included a total of 213 851 high-quality sequences for an average of 5940 sequences per sample. Sequences were clustered into operational taxonomic units (OTUs) based on 97% sequence identity using the average neighbor algorithm. The community composition of the individual samples were compared by nonmetric multidimensional scaling (NMDS; Clarke and Warwick 2001) using the Primer V.5 software package (Primer-E, Plymouth, UK). Briefly, the relative abundance of each of the OTUs within each of the samples was imported into Primer and a similarity matrix was calculated using the Bray-Curtis coefficient (Bray and Curtis 1957). The NMDS procedure was then used to ordinate the similarity data following 100 random restarts. The analysis of similarity (ANOSIM) routine in Primer was used to determine if there were statistically significant differences between the experimental groups. The SIMPER routine in Primer was used to determine the relative contribution of each of the OTUs to differences between the experimental groups.

RESULTS

The concentration of chlorophyll *a* on inorganic substrates was significantly reduced by pharmaceuticals ($F_{7,27} = 3.12$, $P = 0.015$). In particular, the combined treatment had significantly lower amounts of chl *a* (Mix, $P = 0.006$, Fig. 2A) compared to the control. These findings translate into an 82% reduction in total algal biomass on the Mix treatment when compared to the control, indicating the potential to reduce both the functional role of algae and its availability as a resource. On inorganic substrates, GPP was significantly reduced in the presence one pharmaceutical compound ($F_{7,28} = 7.89$, $P < 0.001$). Specifically, GPP was 99.8% lower than the control in the presence of diphenhydramine and 85.7% lower on the Mix treatment ($P < 0.05$, Fig. 2B).

Respiration (*R*) decreased in the presence of pharmaceutical compounds on organic substrates ($F_{7,24} = 95.7$, $P < 0.001$, Fig. 2C) and was reduced by 49%, 62%, 97%, 89%, and 85% in the presence of caffeine, cimetidine,

TABLE 2. Two-way ANOVA results of response variables (respiration or chlorophyll *a*) to pharmaceutical compounds (treatment, Trt) compared to control by season or site (SS) and the change in response variables when exposed to compounds compared to controls.

	Chlorophyll *a*						Respiration					
	SS		Trt		SS × Trt	Change	SS		Trt		SS × Trt	Change
Compound	*P*	df	*P*	df	*P*	(%)	*P*	df	*P*	df	*P*	(%)
Caffeine	<0.001	4, 35	0.058	1, 35	0.98	−21.5	<0.001	3, 27	<0.001	1, 27	0.02	−52.7
Cimetidine	<0.001	4, 35	0.726	1, 35	0.78	ns	<0.001	3, 27	<0.001	1, 27	0.51	−51.2
Ciprofloxacin	<0.001	1, 17	0.025	1, 17	0.44	−4.3	0.003	1, 17	<0.001	1, 17	0	−91.4
Diphenhydramine	<0.001	4, 35	0.006	1, 35	0.69	−22.2	<0.001	3, 24	<0.001	1, 24	0.01	−62.6
Metformin	<0.001	4, 34	0.415	1, 34	0.81	ns	<0.001	3, 26	0.535	1, 26	0.01	ns
Mix	<0.001	2, 24	0.001	1, 24	0.01	−17.5	<0.001	2, 21	<0.001	1, 21	0	−39.5
Ranitidine	<0.001	4, 35	0.545	1, 35	0.6	ns	<0.001	3, 25	0.354	1, 25	0.29	ns

Notes: Season–site combinations are New York, winter 2009, spring 2010, autumn 2010; Indiana, spring 2010; Maryland, spring 2011. New York winter 2009 was removed from respiration analyses because of zero values. The abbreviation "ns" indicates no significant difference from the control.

ciprofloxacin, diphenhydramine, and the mixture of these compounds, respectively ($P < 0.05$ for each compound).

To explore whether the patterns we observed were similar across seasons and sites, we measured the responses of the biofilms across three seasons (spring, summer, autumn) in New York (EBWC) and compared responses in one season (spring) at two other stream sites in suburban areas in Indiana (JDC) and Maryland (DR). Chl *a* concentrations and respiration rates on control substrates were different among seasons and sites (Table 2) likely reflecting intersite and seasonal differences in environmental conditions. To account for this variability, we examined the response ratios (treatment : control) for all compounds across sites and seasons. However, relative to the controls the response to the compounds was similar across seasons and sites. Specifically, chl *a* concentrations were significantly lower on the diphenhydramine, ciprofloxacin, and Mix treatments compared to the control (Table 2). Biofilm *R* response ratio (treatment : control) was significantly less than 1, indicating suppression in response to caffeine, ciprofloxacin, cimetidine, and diphenhydramine across all seasons and sites (Fig. 3, Table 2). In contrast, the antihistamine ranitidine and the antidiabetic metformin did not significantly influence *R* at any time of the study.

To gain further insight into the effects of pharmaceuticals on bacteria within biofilms we used a molecular approach to examine the composition of bacterial communities on organic substrates from the control, caffeine, and diphenhydramine treatments from all three stream sites. There was only a small amount of substrate available for bacterial community analysis, and the physical integration of the bacterial communities into the organic substrate made obtaining counts of bacterial cells difficult, so DNA yield was used as a proxy for bacterial community size. There was no significant effect of pharmaceutical treatment ($P = 0.488$) on DNA yield from the substrates (Fig. 4). However, there was a significant effect of site on DNA yield ($P < 0.005$), with the substrates from JDC producing significantly higher DNA yields than DR and EBWC (Fig. 4). These data suggest that incubation of substrates in JDC resulted in significantly more bacterial colonization than incubation in DR or EBWC.

Analysis of the composition of the bacterial communities colonizing the substrates based on tag pyrosequencing of 16S rRNA genes revealed some significant differences. The most obvious difference in community composition was based on site, with the communities from DR being well separated on the NMDS ordination (Fig. 5) and significantly different in composition (Table 3) from the communities from EBWC and JDC. The communities from EBWC and JDC were also clustered separately from each other on the NMDS ordination

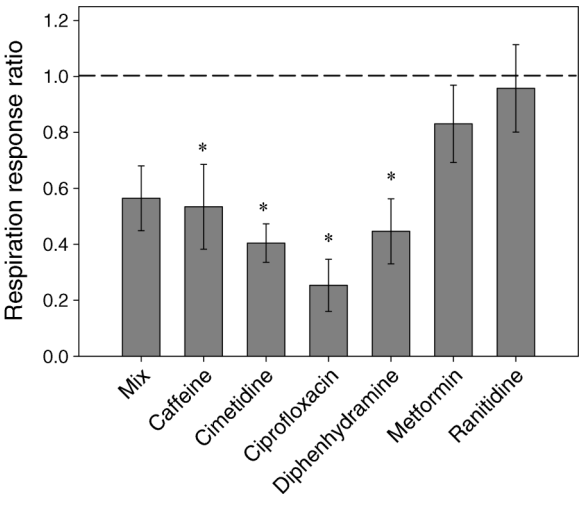

FIG. 3. Average effect of exposure to caffeine, cimetidine, ciprofloxacin, diphenhydramine, metformin, ranitidine, and a mixture of all of them (Mix) on respiration response ratios (treatment : control) of stream biofilms growing on organic substrata from all seasons and all site locations. Asterisks indicate significant differences ($P < 0.05$) among the treatments and the control. Error bars represent SE. We used the following number of replicates for each treatment: Mix $n = 11$, caffeine $n = 19$, cimetidine $n = 19$, ciprofloxacin $n = 8$, diphenhydramine $n = 19$, metformin $n = 18$, ranitidine $n = 15$, and control $n = 24$.

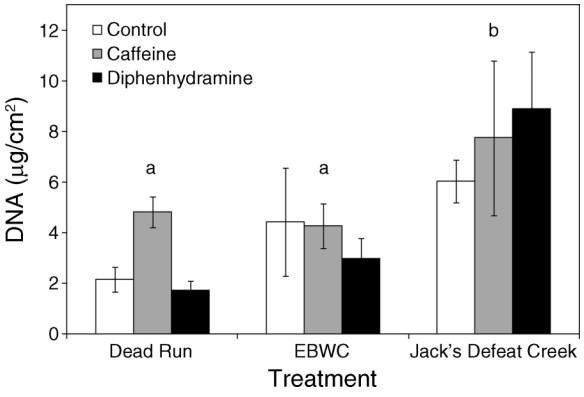

FIG. 4. DNA yield from substrates. Values are means and SE ($n = 4$ replicates except for Jack's Defeat Creek control and Dead Run control [$n = 5$]). ANOVA indicated a significant effect of site ($P < 0.005$) but no significant effect of pharmaceutical treatment ($P = 0.488$) and no significant interaction effect ($P = 0.533$). Different letters above bars indicate significant differences between sites based on Tukey's HSD test ($P < 0.05$).

(Fig. 5), and although the separation between communities from these two sites was not as large as the separation of communities from DR, there was a significant difference between the communities from EBWC and the communities from JDC (Table 3).

The NMDS did not reveal an overall separation of communities based on the pharmaceutical treatments (Fig. 5, Table 3). Interestingly, the comparison of all sites control vs. all sites diphenhydramine produced a significant P value (0.041), but its R statistic was very low (0.140). However, when each site was examined individually, there were significant differences between the communities from the diphenhydramine treatments and the communities from the control treatments at EBCW ($P < 0.05$), JDC ($P < 0.05$), and DR ($P < 0.10$; Fig. 5, Table 3). In contrast there were no significant differences between the communities from the caffeine treatments and the communities from the control treatments at any of the three sites (Fig. 5, Table 3).

Due to the differences observed between the control and diphenhydramine treatments, SIMPER analysis was used to compare the communities from all of the control and all of the diphenhydramine treatments. This analysis indicated that three OTUs accounted for 30% of the variation in the communities from these two treatments. Specifically, OTU 4 accounted for 14% of the variability, OTU 1 accounted for 12% of the variability, and OTU 25 accounted for 3% of the variability. Two-way ANOVA indicated a significant effect of diphenhydramine on OTU 1 ($P = 0.005$), with diphenhydramine treatment increasing the relative abundance of OTU 1 (Fig. 6A). There was no significant effect of site ($P = 0.191$) and no significant interaction ($P = 0.214$) for OTU 1. Comparison to the RDP classifier database identified OTU 1 as falling within the *Pseudomonas* genus. Two-way ANOVA indicated a significant effect of diphenhydramine on OTU 4 ($P < 0.001$), with diphenhydramine treatment decreasing the relative abundance of OTU 4 (Fig. 6B). There was also a significant effect of site on OTU 4 ($P < 0.001$) but no significant interaction ($P = 0.214$). Comparison to the RDP classifier database identified OTU 4 as falling within the *Flavobacterium* genus. Finally, two-way ANOVA indicated a significant effect of diphenhydramine on OTU 25 ($P = 0.001$), with diphenhydramine treatment decreasing the relative abundance of OTU 25 (Fig. 6C). There was also a significant effect of site on OTU 25 ($P < 0.05$) but no significant interaction ($P = 0.878$). Comparison to the RDP database identified OTU 25 as also falling within the *Flavobacterium* genus.

DISCUSSION

Our findings indicate that respiration, gross primary production, and composition of benthic microbial communities in streams are affected by some common pharmaceuticals, alone or in combination. The responses to pharmaceutical compounds were consistent across sites and seasons, suggesting that the responses may be common despite the fact that the composition of the bacterial communities colonizing the organic substrates varied among sites. Our data demonstrate that some processes, e.g., GPP, may be much more sensitive indicators than algal chl *a*.

The most striking result of our research was the strong indication of the effects of diphenhydramine on algal production, microbial respiration, and bacterial community composition. The pharmacokinetics of diphenhydramine in mammals and humans is well documented and it is known to bind to histamine (H1) receptors and alleviate an allergic reaction. In contrast, our under-

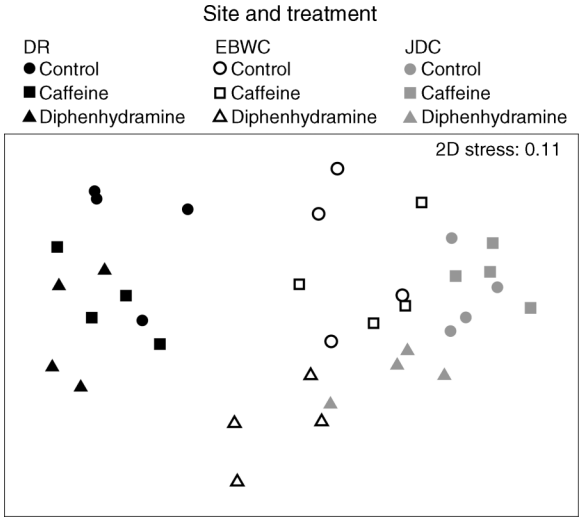

FIG. 5. Comparison of bacterial communities from substrates based on nonmetric multidimensional scaling (NMDS) ordination of 16S rRNA tag pyrosequencing data. Abbreviations are: JDC, Jack's Defeat Creek; DR, Dead Run; and EBWC, East Branch Wappinger Creek.

TABLE 3. Selected ANOSIM results for comparisons of bacterial community composition between groups based on 16S rRNA tag pyrosequencing data.

Comparison	Correlation coefficient	*P*
Dead Run vs. EBWC	0.892	0.001
Dead Run vs. Jack's Defeat Creek	0.994	0.001
EBWC vs. Jack's Defeat Creek	0.398	0.002
All sites control vs. all sites caffeine	0.020	0.504
All sites control vs. all sites diphenhydramine	0.140	0.041
All sites caffeine vs. all sites diphenhydramine	0.091	0.088
Dead Run control vs. Dead Run diphenhydramine	0.438	0.086
Dead Run control vs. Dead Run caffeine	0.281	0.143
Dead Run caffeine vs. Dead Run diphenhydramine	0.083	0.543
EBWC control vs. EBCW diphenhydramine	0.635	0.029
EBWC control vs. EBCW caffeine	0.083	0.657
EBWC caffeine vs. EBCW diphenhydramine	0.542	0.029
Jack's Defeat Creek control vs. Jack's Defeat Creek diphenhydramine	0.479	0.029
Jack's Defeat Creek control vs. Jack's Defeat Creek caffeine	0.198	0.143
Jack's Defeat Creek caffeine vs. Jack's Defeat Creek diphenhydramine	0.885	0.029

Note: EBWC stands for East Branch Wappinger Creek.

standing of the influence of diphenhydramine on nontarget organisms is less well understood, but diphenhydramine can have multiple effects on vertebrates (Berninger et al. 2011). Diphenhydramine had no observable effect on *Daphnia magna* (Crustacea: Cladocera) at a concentration of 0.12 μg/L, which is 40 times greater than the maximum concentrations measured in surface waters (Meinertz et al. 2010), suggesting little risk to zooplankton. In addition, although fathead minnows (*Pimephales promelas*) and *Daphnia* had both acute and chronic responses to diphenhydramine, the plant *Lemna gibba* was not sensitive to this compound. Previous research exploring likely modes of action suggests that plants and algae are not sensitive to antihistamines (Gunnarsson et al. 2008) and a mesocosm study of long-term exposure of the antihistamine cimetidine did not indicate significant effects on algae (Hoppe et al. 2012). However, diphenhydramine has been shown to be present and persistent in the environment; a study of degradation of diphenhydramine in experimental wetlands indicated no change in the concentration of diphenhydramine after 3 years (Walters et al. 2010). Our observations indicate there is a strong negative effect of diphenhydramine on both biomass and metabolism of freshwater algal and bacterial communities associated with stream biofilms, which could result in indirect effects on higher trophic levels as has been observed for other contaminants (Relyea 2009). Further research exploring the mechanisms behind our findings is warranted.

In addition to the algal response, diphenhydramine had a significant effect on bacterial communities colonizing the organic substrates. Diphenhydramine has antibacterial activity (Dastidar et al. 1976), and we found that diphenhydramine altered the species composition of the biofilm bacterial communities, specifically resulting in an increase in the relative abundance of *Pseudomonas* and a decrease in the relative abundance of *Flavobacterium*. *Pseudomonads* are Gram-negative bacteria that are common in a wide variety of habitats including freshwater ecosystems (Palleroni 2010). Bacteria from the genus *Pseudomonas* commonly produce biofilms (Heydorn et al. 2000) and several *Pseudomonas* species have high levels of antibiotic resistance due to the presence of multidrug efflux pumps (Poole et al. 1993). *Pseudomonads* are also well known for their ability to degrade a large variety of organic molecules, including many that are toxic to other microorganisms and to higher organisms (Palleroni 2010) such as benzene and toluene (Reardon et al. 2000), polycyclic aromatic hydrocarbons (Deziel et al. 1996), and polychlorinated biphenyls (Hickey and Focht 1990). The ability of *Pseudomonas* species to degrade toxic compounds can enable them to survive in contaminated environments (Palleroni 2010). Increased abundance of *Pseudomonas* species in biofilms on diphenhydramine substrates in this study indicates their ability to tolerate diphenhydramine.

Flavobacterium are Gram-negative bacteria that are widely distributed in nature, occurring mostly in aquatic ecosystems (Bernardet and Bowman 2006). Like *Pseudomonas*, bacteria from the genus *Flavobacterium* are commonly associated with biofilms (Basson et al. 2008). Within aquatic habitats the *Flavobacterium* are involved in the metabolism of various organic compounds, including carbohydrates and polysaccharides (Bernardet and Bowman 2006). Several *Flavobacterium* species have been shown to degrade cellulose derivatives (Bernardet and Bowman 2006) and recent evidence indicates that some *Flavobacteria* may be able to degrade cellulose compounds (Lednicka et al. 2000, Rodgers et al. 2003), which could explain their presence on our organic substrates. The suppression of *Flavobacteria*, algal biomass, GPP, and *R* indicates that that diphenhydramine poses a risk to stream biofilms.

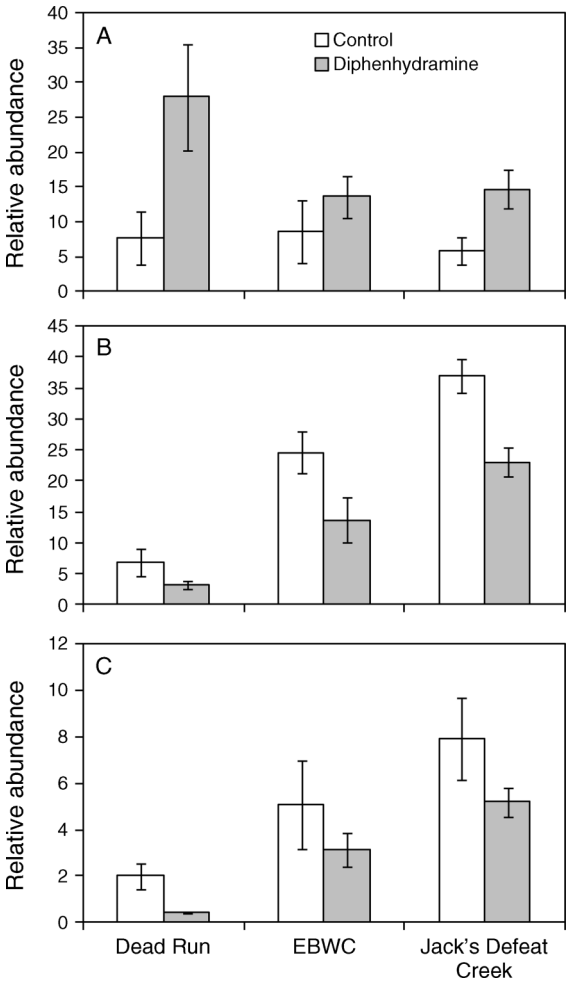

FIG. 6. Relative abundance of (A) operational taxonomic unit (OTU) 1, (B) OTU 4, and (C) OTU 25 within the 16S rRNA gene tag pyrosequencing data sets for the control and diphenhydramine treatments from each site. Values are mean ± SE ($n = 4$ replicates). OTU 4, OTU 1, and OTU 25 accounted for 14.33%, 12.25%, and 3.43% of the variation between the control and diphenhydramine treatments.

Caffeine, ciprofloxacin, and cimetidine also had significant effects on respiration of heterotrophic microbial biofilms. These compounds are commonly found in surface waters and the consequences of reduced microbial respiration on ecosystem-level processes have not been previously explored. It is not surprising that ciprofloxacin, a powerful antibiotic, significantly reduced microbial respiration. It remains to be seen whether a reduction in whole-system respiration can be attributed to this compound in nature, though it has been detected in many areas and below manufacturing facilities it reaches concentrations in the mg/L range (Larsson et al. 2007). We explored the potential influence of caffeine on respiration and bacterial community structure and, although caffeine consistently suppressed respiration, unlike diphenhydramine, it did not act as a selective agent for the bacterial communities. Our results thus indicate that different pharmaceuticals can alter bacterial function in similar ways while having distinct effects on bacterial community structure. The contrast in findings for diphenhydramine and caffeine indicate interesting relationships among structure and function of stream biofilms. In the case of caffeine, the reduced respiration was apparently not tied to changes in the composition of the community, but changed function. In contrast, the bacterial community composition change in response to diphenhydramine indicates that some pharmaceuticals can alter function through changes in composition. These findings indicate that exploring the influence of the compounds on aquatic biofilms should provide insight into the relationships among structure and function in aquatic biofilms.

Stream biofilms are the base of the food web for streams, and negative impacts on these microbial communities by exposure to pharmaceuticals may affect both nutrient cycling in an ecosystem and higher trophic levels that depend on biofilms. Although pharmaceuticals likely co-occur with other stressors, we were able to detect, in situ, the effects of exposure to compounds that have potential consequences for ecosystem function and higher trophic levels. Similar to other stressors that disrupt ecological interactions (e.g., nutrients, land use change) the occurrence of pharmaceutical compounds may influence aquatic ecosystems in interesting and non-additive ways and result in novel community structure, altered function and important higher trophic level responses (Davis et al. 2010). In addition, pharmaceuticals likely interact with other stressors, such as nutrients (Fulton et al. 2009, 2010), and research that explores the interactions among nutrients and pharmaceuticals is warranted. The use of this method in a wider variety of habitats with other pharmaceuticals has the potential to expand our understanding of the effects of these ubiquitous compounds on stream ecosystem structure and function. The method we present here could be readily applied to a number of sites and to explore potentially sensitive processes and taxa and could provide the foundation for generating hypotheses about the ecosystem-scale consequences of pharmaceuticals present in aquatic ecosystems.

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