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Effects of Pharmaceutical Contamination on Sedment Microbial Communities in Streams

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EFFECTS OF PHARMACEUTICAL CONTAMINATION ON SEDIMENT MICROBIAL COMMUNITIES IN STREAMS

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ABSTRACT

The objective of this study was to examine the interaction of pharmaceuticals and personal care products (PPCP) and microbial communities in freshwater ecosystems. This study included two components. The first component consisted of an artificial stream study in which sediment was exposed to environmentally relevant concentrations of three different pharmaceuticals (diphenhydramine, ciprofloxacin, and fluoxetine) individually and as a mixture. Pharmaceutical exposure had no effect on denitrification rates or respiration rates in the sediments, and sediment microbial community composition showed only slight differences between streams dosed with pharmaceuticals and control streams. The second component consisted of a field study in which 5 field sites on 3 different streams were sampled. One stream received no wastewater treatment plant (WWTP) effluent. The two other streams received direct inputs of WWTP effluent, and two sites on each of these streams, one upstream of the effluent point and one downstream, were sampled in order to determine if the effluent was influencing the streams communities. Based on the physical characteristics, nitrate, SRP, and temperature, the WWTP effluent affected the stream with the highest effluent input. Microbial communities from both streams demonstrated compositional differences when comparing downstream to upstream sites. One of the microbes that contributed to the composition change is a group that can degrade complex aromatic compounds. Future work will include analysis of antibiotic

resistance genes present within the microbial communities and quantification of specific PPCPs in these streams.

CHAPTER 1

BACKGROUND ON PHARMACEUTICALS PERSONAL CARE PRODUCTS AND FRESHWATER ECOSYSTEM STUDIES

Introduction

Pharmaceuticals and personal care products (PPCPs), which include stimulants, analgesics, antibiotics, antiseptics, disinfectants, antihistamines, and other medications, are biologically active compounds that have been found to be prevalent in aquatic ecosystems throughout the world (Monteiro & Boxall, 2010). With the increasing human population, the amounts of PPCPs being produced and used around the world are expected to increase (Beek et al., 2016). PPCPs can enter aquatic habitats through point sources including leaky sewer systems, sewer overflow, and wastewater treatment plant effluent (Rosi-Marshall & Royer, 2012). Urban streams are especially prone to PPCP contamination because their watersheds include high density human populations and extensive sewer infrastructure. Urban streams often serve as critical sources of drinking water, food, and recreation for urban communities, so protecting these resources from degradation is important. The United States Geological Survey (USGS) conducted a study in 1999 in order to quantify the presence of specific PPCPs in surface waters in the United States. The USGS found that organic contaminants, including many PPCPs, were found in 80% of the streams studied in 30 states, with most streams containing multiple contaminants (Kolpin et al., 2002).Domestic wastewater is a potential route by which PPCPs can

enter the environment. Domestic wastewater is water that has become contaminated by human use, including restroom usage, washing, bathing, food preparation and laundry. Domestic wastewater should not be directly released into the environment in high volumes due to its high concentration of nutrients, which can harm the natural environment, and due to the possible presence of pathogens, which pose a risk to public health. In the United States domestic wastewater is therefore generally treated before it is released into aquatic ecosystems. Wastewater treatment plants (WWTPs) are the most common method to treat domestic wastewater, serving over 75% of the United States population (EPA, 2004). WWTPs are not designed to remove PPCPs and the incidental removal rate differs drastically based on the specific PPCP (Aga, 2007). Due to the fact that PPCPs are only removed incidentally, WWTPs can be point sources of PPCPs to the environment.

Many PPCPs found in the environment are unregulated or considered low risk (Richmond et al., 2017). These compounds are seen as safe as they are tested on model organisms at much higher concentrations than found in the stream environments. However this methodology does not take into account non-model organisms and non-lethal effects of the PPCPs (Richmond et al., 2017). Studies that examine the non-lethal effects of PPCPs are necessary to understand the implications throughout the food web.

Previous research has suggested that PPCPs can affect microbial communities in streams, for example by suppressing key ecosystem functions, including primary production and respiration (Bunch & Bernot, 2011; Richmond et al., 2016; Rosi-Marshall & Royer, 2012). For example, contamination of marine sediment with the antibiotic ciprofloxacin decreased microbial CO_2 production (Näslund, Hedman, and Agestrand 2008). Microbial communities are key

components of stream ecosystems because of the roles they play in nutrient cycling and as food for higher trophic levels. Therefore, it is imperative to study how PPCPs affect microbial communities in stream ecosystems, especially in urban streams.

The effects of PPCPs on stream microbial communities are largely unknown, but recent studies have reported that PPCPs can have effects on microbes that are drastically different than in humans (Rosi-Marshall et al., 2013). When designing a study to examine the effects of PPCPs on microbial communities it is not always feasible to control all variables such as weather, temperature, river flow, and rainfall. Without control of these variables, effects from independent variables (i.e. PPCP dosage) may not have as pronounced as an effect. Therefore researchers have developed methods to control as many variables as possible, including the use of artificial stream mesocosms. These systems are usually colonized with material from a natural stream and have a continuous flow of water which is recirculated constantly. This allows researchers to control PPCP levels in ways that are not as feasible in natural streams.

An alternative approach to artificial mesocosms is conducting a field study by sampling existing streams in nature. These studies can take a spatial or temporal focus. Spatial studies compare different sites or different streams to each other. For example, sampling upstream of a wastewater treatment plant effluent point, and sampling downstream and comparing the communities to see if there is a difference. A temporal study looks into patterns over a span of time. For example, comparing community composition in spring to summer at the same site to see patterns. Field studies can be harder to influence as opposed to artificial mesocosms due to the increased number of variables.

In order to understand if the addition of PPCPs to freshwater ecosystems is having an impact the effects of these compounds on the ecosystem must be measured. A common method is to measure gross primary production (GPP) of benthic microbial communities. GPP is a measure of the total amount of carbon fixed by a community through photosynthesis and other autotrophic processes, which is a useful indicator of the biological productivity of an ecosystem. Other functions that are commonly measured include respiration rates and denitrification rates, which represent the two dominant heterotrophic processes and are thus useful indicators of the overall heterotrophic activity within a microbial community.

Analysis of the taxonomic composition of microbial communities is another useful indicator of possible PPCP effects. Previously, researchers would have had to culture bacteria to see which microbes were in a specific environment. With the adoption of the 16s rRNA ribosomal subunit as a taxonomic classifier it is possible to study more microbes in a shorter amount of time (Hugenholtz & Pace, 1996). 16s amplicon studies can determine taxonomic information; however, they are unable to determine functional capacity of the microbes observed. A second approach is to sequence all of the DNA obtained when performing DNA extraction, which has been named ‘shotgun sequencing’ for its varying length of DNA fragments resembling a shotgun blast. The resulting reads can then be used to answer two main questions: 1) what taxa are there? 2) what genes are there? The first question, who are there?, is similar to the question asked in 16s amplicon studies, however instead of one reference gene, 16s, shotgun metagenomic studies can leverage a larger number of genes for identification. The larger number of genes can provide a better classification as opposed to amplicon comparisons. Before finding functional information about microbes, especially in soil environmental samples, assembly of

reads is important. Assembly takes short reads usually 100-250bp and constructs contiguous sequences (contigs) which can sometimes be assembled into nearly full genomes. To determine what the microbes are doing, there are two approaches: 1) gene prediction and 2) functional annotation. Gene prediction uses biological rules to find potential genes in contigs. These potential genes are then annotated or given information from a database based on homology. Through this method it is possible to determine novel genes.

This thesis is composed of two chapters. The first chapter is based on an artificial stream study where the streams were dosed with three different pharmaceuticals. To observe changes in the microbes, denitrification and respiration were measured at two points during the experiment. At the end of the experiment, the sediment was collected and frozen. DNA from the sediment was extracted and the microbial communities were profiled to determine if dosing the artificial streams shifted the communities. The second chapter describes a field study in the Chicago metro area aiming to determine if wastewater treatment plant effluent shifted microbial communities from upstream of the effluent to downstream. This was accomplished by collecting sediment and water samples. DNA was extracted from the sediment samples, and profiled using 16s amplicon sequencing, and constructing shotgun metagenomic libraries. Concentrations of PPCPs were measured from the water samples to determine if concentration increased downstream and compare to marker genes in the shotgun metagenomic sequences.

CHAPTER 2

EFFECTS OF PHARMACEUTICALS ON SEDIMENT MICROBIAL COMMUNITY COMPOSITION AND FUNCTION

Introduction

Pharmaceuticals and personal care products (PPCPs) are biologically active compounds that have become ubiquitous in aquatic ecosystems throughout the world (Monteiro & Boxall, 2010). With the ongoing increases in urbanization and human population, the amount of PPCPs in the environment is expected to increase (Beek et al., 2016). Normal use of PPCPs results in their entry into domestic wastewater, and PPCPs can be released to the environment through point sources including leaky sewer systems, combined sewer overflows, and wastewater treatment plant (WWTP) effluent (Rosi-Marshall & Royer, 2012). For example, the antibiotic ciprofloxacin, the antihistamine diphenhydramine, and the antidepressant fluoxetine have all been detected in surface waters in North America (Bartelt-Hunt et al., 2009; López-Serna et al., 2010; Metcalfe et al., 2010), with wastewater a likely route of entry for these compounds. In a review of 41 WWTPs in North America, 68% had ciprofloxacin concentrations in their effluent that exceeded the 100 ng/L predicted no effect concentration (Kelly & Brooks, 2018). Ciprofloxacin has some sensitivity to photodegradation, however it also has a tendency to absorb to suspended particles, which would suggest the potential for ciprofloxacin to accumulate in stream sediments (Cardoza et al., 2005) Antihistamines, such as diphenhydramine, have been

found to not degrade well in WWTPs (Kosonen & Kronberg, 2009), leading to their release in treated wastewater. Fluoxetine has been detected in surface waters from the ng/L to ug/L levels (Weinberger & Klaper, 2014), and a previous study reported that fluoxetine will move from being dissolved in water to sediment and will persist in the sediment (Kwon & Armbrust, 2006).

The concentrations of pharmaceuticals in WWTP effluent and stream ecosystems are generally below levels that would be lethal to microbes and other organisms, so regulatory agencies consider them to be low risk. However, previous research has demonstrated that PPCPs have the capability to affect stream microorganisms, for example suppressing key ecosystem functions such as primary production and respiration (Bunch & Bernot, 2011; Richmond et al., 2016, 2017). These types of sub-lethal effects of PPCPs have not been extensively studied, but they could have significant implications for ecosystem function (Richmond et al., 2017).

The aims of this study were to 1) measure functional responses (denitrification and respiration rates) of sediment microbial communities to pharmaceutical exposure, and to 2) evaluate the composition and diversity of the sediment microbial communities to determine if there is an impact of pharmaceutical exposure on bacterial community composition. To achieve these aims we worked with collaborators to conduct a 20-day artificial stream study where streams were dosed with environmentally relevant concentrations of ciprofloxacin, fluoxetine, and diphenhydramine. Sediment packs colonized by native stream microbial communities were incubated in these streams and we measured denitrification and respiration rates and analyzed the taxonomic composition of the microbial communities within these sediments to assess pharmaceutical impacts.

Methods

Experimental Design

Our collaborators conducted an artificial stream study in June and July 2017 consisting of 20 artificial streams within a greenhouse at the Cary Institute of Ecosystem Studies in Millbrook, New York. Each artificial stream was filled with 60 L of groundwater collected on site which was recirculated within the streams at a speed of 0.41 m s^{-1} using paddlewheels powered by Dayton DC gear motors and speed controllers (Dayton Electric Manufacturing Company, Niles, Illinois). Multiple substrate types were included in the artificial streams, but the focus of this chapter is on the sediment packs. Sediment packs were constructed by mixing washed silica sand with 1.5% weight/weight organic matter (finely-ground, dried *Acer rubrum* (Red Maple), leaves). Each pack consisted of 50 g of this sand and organic matter mixture packed into nylon mesh and shaped into a sphere with a 55 mm diameter. Sediment packs were tethered to stakes placed in a local stream, Wappinger Creek, and incubated for 5 days to allow microbial colonization. The microbially colonized sediment packs were then removed from the creek and placed in the artificial streams. Each stream had 8 sediment packs, with 4 placed in full sunlight conditions and 4 placed in PVC pipes to allow exposure to water but not to sunlight. The artificial streams were covered with 25-mm mesh netting to keep insects out.

The 20 artificial streams were separated into 5 treatments with 4 replicates of each treatment. The treatments were: control (no pharmaceuticals added), ciprofloxacin (140 ng/L), diphenhydramine (300 ng/L), fluoxetine (20 ng/L) and a mixture of all three pharmaceuticals at the concentrations listed above. The concentration of pharmaceuticals used in the study was based on global median concentrations (Bartelt-Hunt et al., 2009; López-Serna et al., 2010;

Metcalfe et al., 2010; Roberts et al., 2016; Watkinson et al., 2009). The streams were dosed on day 0 and then every other day for the rest of the 20 days. Ammonium and phosphate were also added to keep the concentrations at levels similar to Wappinger Creek, with NH_4^+ at 40 ug/L and PO_4^{3-} at 2.5 ug/L.

Denitrification and respiration rate measurements

Denitrification and respiration rates were measured for all sediment packs from all streams on days 4 and 16. On day 4, all sediment packs were placed in sealed glass jars filled with oxygenated water with no air bubbles and the jars were placed in a water bath at 25°C and kept in the dark until sampling. The water in the jars was then measured for levels of dissolved dinitrogen (N_2), dioxygen (O_2), and argon with a membrane inlet mass spectrometer (MIMS) at 0, 2, 4, and 6 hours. Sediment packs were then returned to their artificial streams until day 16 when they were measured again. A standard of oxygenated distilled water kept at 25°C was measured after every 4 samples as a control. MIMS readings were corrected for instrumental drift and pressure in the lab at time of sampling (Reisinger et al., 2016). Linear regression was used to calculate denitrification rates (based on N_2 production) and respiration rates (based on O_2 consumption).

DNA Extraction and Sequencing

After the measurement of denitrification and respiration rates on day 16, sediment packs were homogenized, a 0.5 ml subsample of each pack was transferred to a 2 ml microcentrifuge tube, and the tubes were stored at -80°C. The tubes were then shipped on dry ice to the Kelly lab at Loyola University Chicago where they were stored at -80°C prior to microbial community analysis. DNA was extracted from sediment samples using the Qiagen DNeasy Power Soil

Extraction kit (Qiagen, Inc., Hilden, Germany). Kits without samples were run as contamination controls. Successful extraction was confirmed with gel electrophoresis, and extracted DNA was quantified with Nanodrop (Thermo Fisher, Rockland DE). Polymerase Chain Reaction (PCR) was performed on each sample using 515F and 806R primers targeting the V4 hypervariable region of the 16S ribosomal RNA gene (Caporaso et al., 2012). Successful amplification was confirmed with gel electrophoresis. No bands were observed for kit controls, confirming the kits were not a source of contamination. Amplicons were sequenced in a 2 x 150 paired-end format with the MiSeq platform (illumina®, San Diego, California; Caporaso et al., 2012) by the DNA Services Facility, University of Illinois at Chicago. All sequence data analyzed in this paper can be downloaded from the National Center for Biotechnology Information Sequence Read Archive with accession number (PRJNA666340).

Analysis of Amplicon Sequence Data

Amplicon sequences were processed with mothur V.1.42.2 (Schloss et al., 2009) following the MiSeq Standard Operating Procedure (Kozich et al., 2013). Briefly, paired reads were assembled and demultiplexed, and any sequences with ambiguities or homopolymers >8 bases were removed from the data set. Sequences were aligned with the SILVA-compatible alignment database available within mothur. Chimeric sequences were identified with UCHIME (Edgar et al., 2011) and removed from the data set. Sequences were classified with the mothur-formatted version of the RDP training set (v.9) and any unknown (i.e., not identified as bacterial), chloroplast, mitochondrial, archaeal, and eukaryotic sequences were removed. Sequences were then clustered into operational taxonomic units (OTUs) based on 97% sequence identity. We randomly subsampled the entire dataset to 15,923 sequences per sample to avoid

biases associated with uneven numbers of sequences across samples. Bacterial communities were compared by calculating dissimilarities for each pair of samples based on theta index (Yue and Clayton 2005) in mothur and visualizing the resulting dissimilarity matrix using principal coordinates analysis (PCOA) run in R (v.3.6.1). Statistical significance of differences in communities between sampling sites based on the theta index was assessed by analysis of molecular variance (AMOVA), a nonparametric analog of traditional analysis of variance (Excoffier, Smouse, and Quattro 1992), which was run in mothur.

Statistics

All statistical tests were run in R (v3.6.1) using the packages stats (R Core Team, 2020) and rstatix (Kassambara, 2020a). We assessed normality of the denitrification and respiration rates based on the Shaprio test (Shapiro & Wilk, 1965). The data was normally distributed, and a two-way ANOVA test was performed. Due to the low degrees of freedom we were unable to calculate interaction of sunlight and drug treatment. None of the data passed the critical p-value of 0.05 so pairwise comparisons were not conducted.

Results

Denitrification and Respiration Rates

There were no significant effects of pharmaceutical treatment ($p=0.921$, $p=0.942$) or sunlight treatment ($p=0.337$, $p=0.552$) on respiration rates on days 4 or 16 (Table 1), and the respiration rates were highly consistent across all treatments (Figures 1 and 2).

Table 1. Statistical Analysis of Treatment Effects on Respiration and Denitrification Rates at Days 4 and 16 Based on Two-Way ANOVA

Function and Date	Treatment	Df	Sum Sq	MeanSq	F-Value	P-Value
Respiration Day 4	Sunlight	1	0.002624	0.002624	1.188	0.337
	Pharmaceuticals	4	0.001843	0.000461	0.209	0.921
Respiration Day 16	Sunlight	1	0.001247	0.001247	0.420	0.552
	Pharmaceuticals	4	0.002028	0.000507	0.171	0.942
Denitrificaion Day 4	Sunlight	1	0.000008	0.000008	0.056	0.824
	Pharmaceuticals	4	0.000345	0.000086	0.630	0.667
Denitrificaion Day 16	Sunlight	1	0.000165	0.000165	6.096	0.069
	Pharmaceuticals	4	0.000667	0.000167	6.175	0.053

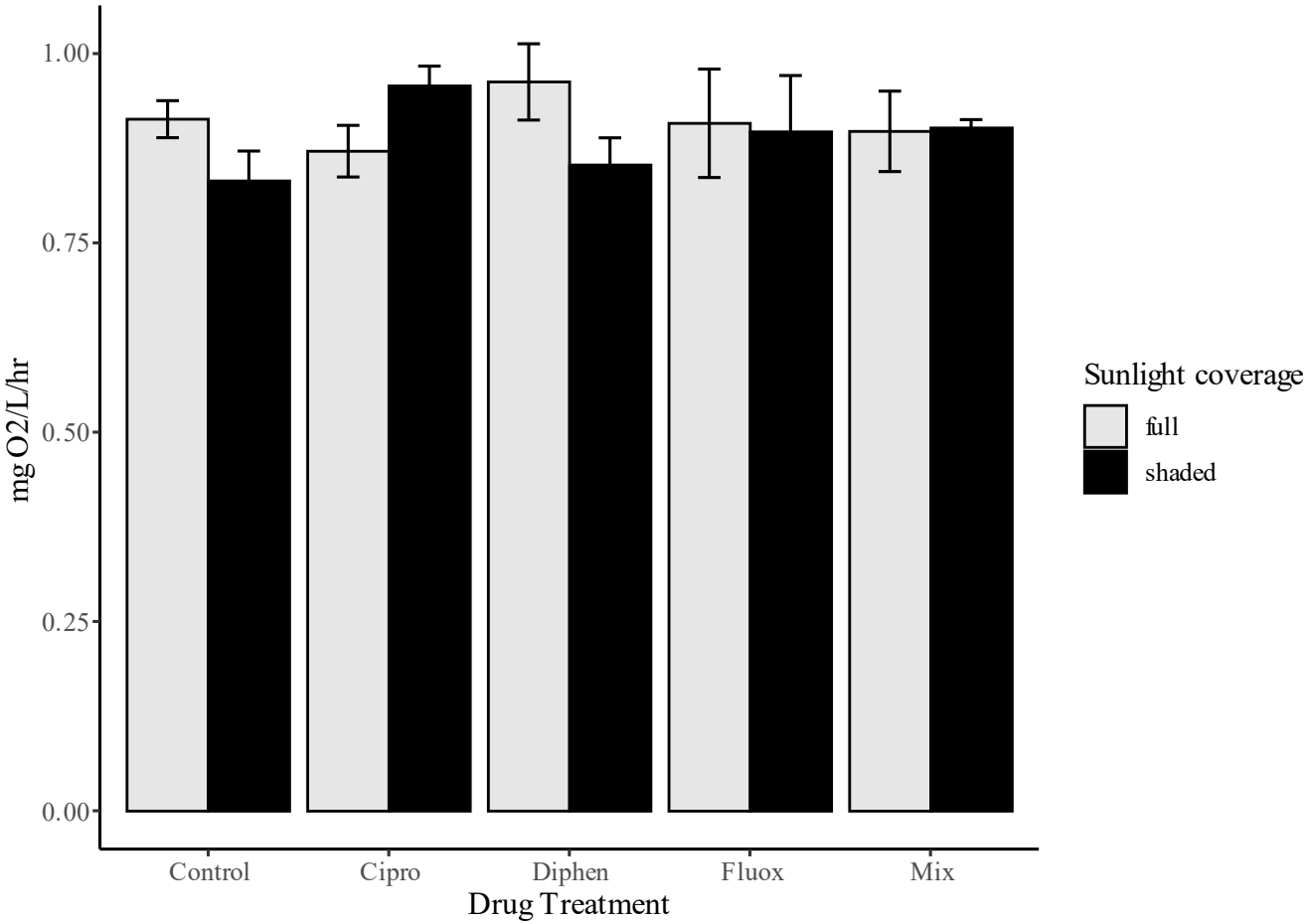

Figure 1. Mean (SE) respiration rate for sediment packs in unshaded conditions and shaded conditions in control, ciprofloxacin (Cipro), diphenhydramine (Diphen), fluoxetine (Fluox), and mixture (Mix) treatments.

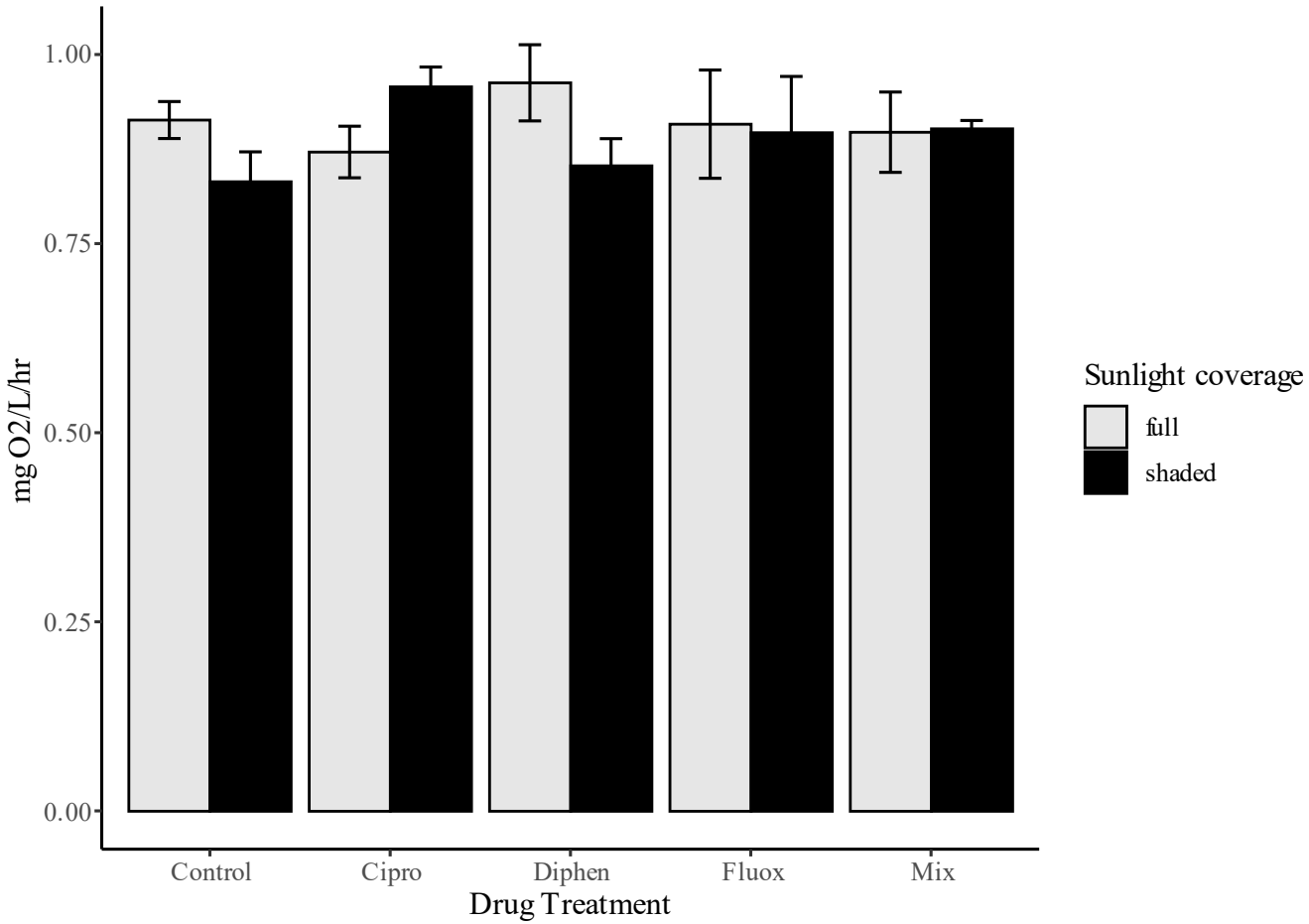

Figure 2. Mean (SE) respiration rate for sediment packs in unshaded conditions and shaded conditions in control, ciprofloxacin (Cipro), diphenhydramine (Diphen), fluoxetine (Fluox), and mixture (Mix) treatments.

Denitrification rates were much more variable across treatments (Figures 3 and 4), but there were still no significant effects of pharmaceutical treatment ($p=0.667$, $p= 0.053$) or sunlight treatment ($p=0.824$, $p=0.069$) on denitrification rates on days 4 or 16 (Table 1).

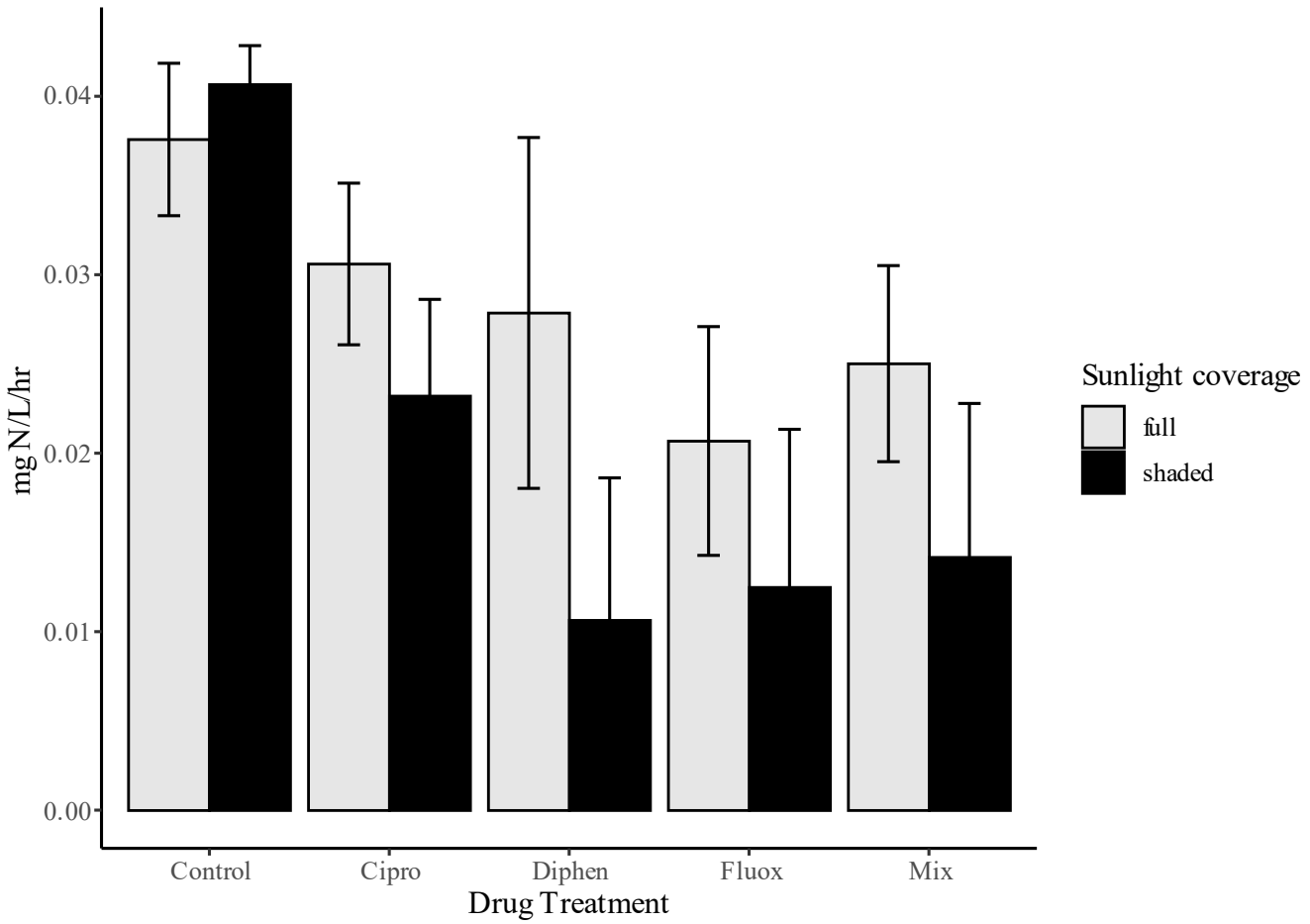

Figure 3. Mean (SE) denitrification rate for sediment packs in unshaded conditions and shaded conditions in control, ciprofloxacin (Cipro), diphenhydramine (Diphen), fluoxetine (Fluox), and mixture (Mix) treatments.

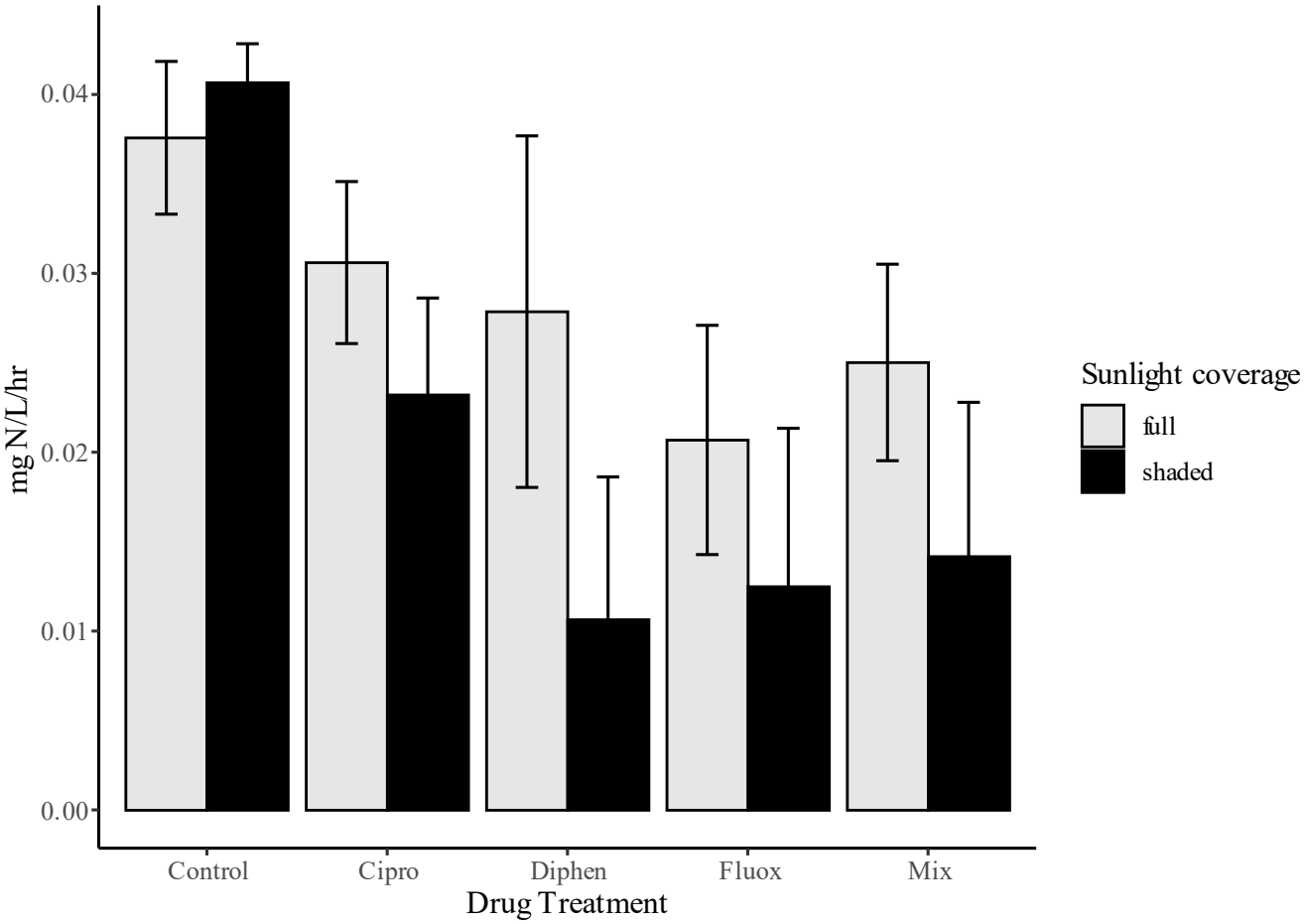

Figure 4. Mean (SE) denitrification rate for sediment packs in unshaded conditions and shaded conditions in control, ciprofloxacin (Cipro), diphenhydramine (Diphen), fluoxetine (Fluox), and mixture (Mix) treatments.

Effect of Pharmaceutical Treatment on Bacterial Community Composition

The pharmaceutical treatments did not have a significant effect on the number of observed bacterial species or Shannon diversity of the bacterial communities in the sediments exposed to full sunlight (Table 2).

Table 2. Bacterial community richness and diversity

Treatment	Observed Species Light (#) [a]	Observed Species Shaded (#) [a]	Shannon Diversity Light (H) [a]	Shannon Diversity Shaded (H) [a]
Control	3526 +/- 230 a	3740 +/- 83 a	6.409 +/- 0.177 a	6.470 +/- 0.117 ab
Cipro	3639 +/- 43 a	3393 +/- 260 ab	6.613 +/- 0.075 a	6.480 +/- 0.058 ab
Diphen	3341 +/- 189 a	3696 +/- 45 ab	6.140 +/- 0.210 a	6.755 +/- 0.059 a
Fluox	3578 +/- 197 a	3284 +/- 20 a	6.487 +/- 0.136 a	6.337 +/- 0.075 b
Mix	3506 +/- 210 a	4010 +/- 114 b	6.422 +/- 0.165 a	6.630 +/- 0.092 ab
ANOVA	p= 0.825	p=0.012	p=0.345	p=0.026

[a] Mean values (n=5) +/- standard error. Different letters within a column indicate significant differences between sites based on Tukey Post-hoc test ($p<0.5$).

However, the pharmaceutical treatments did have a significant effect on the number of observed bacterial species in the sediments that were shaded (Table 2). Specifically, the mixture of all three pharmaceuticals resulted in a significantly higher number of observed bacterial species in the sediments compared to the control, while none of the individual pharmaceuticals affected the number of observed bacterial species as compared to the control (Table 2). Finally, the pharmaceutical treatments also had a significant effect on the Shannon diversity of the bacterial communities in the sediments that were shaded, but the only pairwise difference was between the diphenhydramine and fluoxetine treatments (Table 2).

The pharmaceutical treatment did not affect the relative abundance of any of the most abundant bacterial families in the sediments (Figure 5 and Table 3). Bacteroidales did show a

significant p-value (0.019) but there were no significant pairwise differences between any of the treatments.

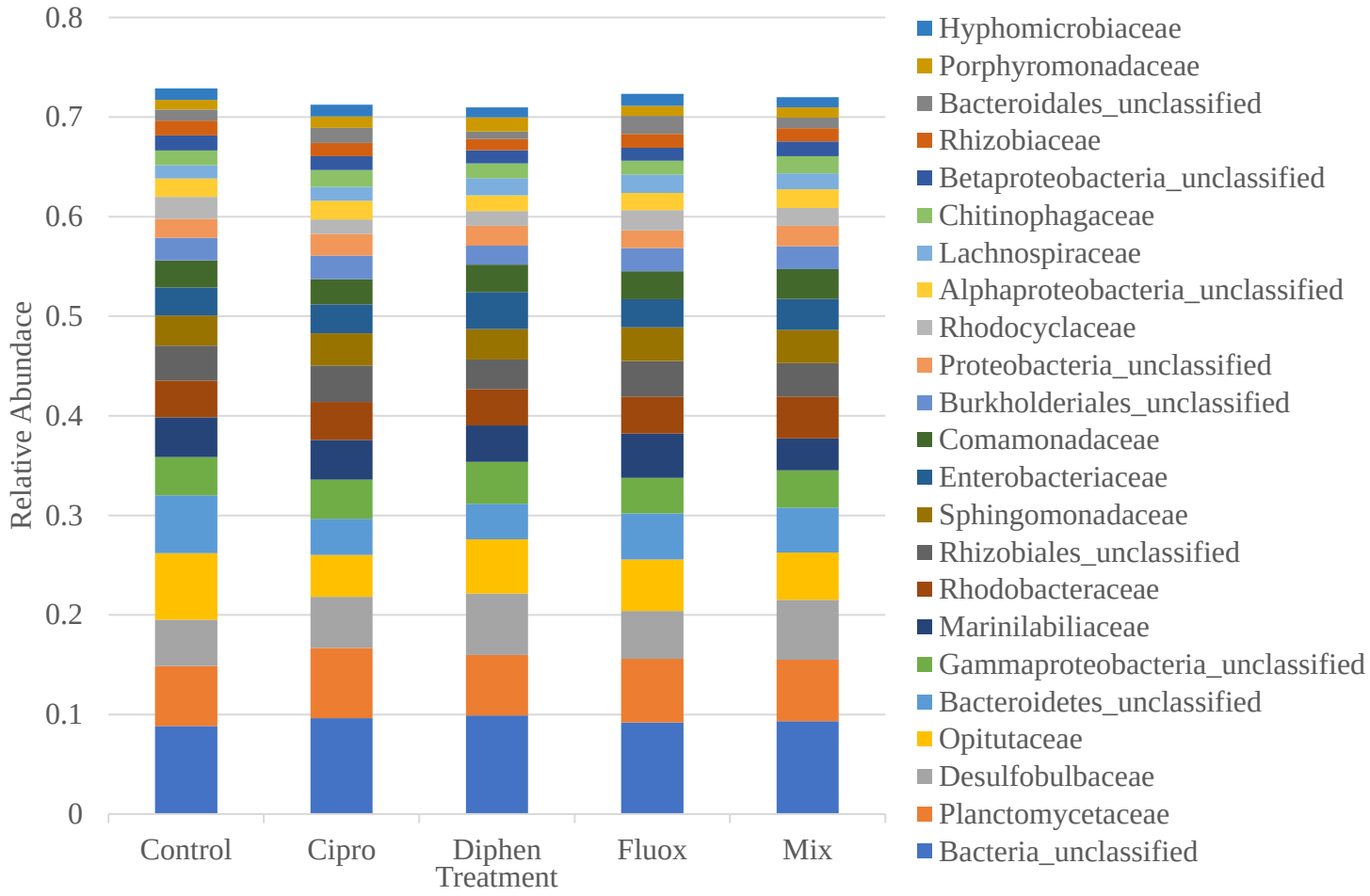

Figure 5. Relative abundance of the 23 most abundant bacterial families in sediment samples from five treatments based on high-throughput amplicon sequencing of partial 16 rRNA genes. Each bar represents the mean (n=8).

Table 3. Relative abundance of the 23 most abundant bacterial families

Taxa	Control	Cipro	Diphen	Fluox	Mix	ANOVA[c] Kruskal-Wallis[d]
Bacteria_unclassified [a]	8.8% a	9.6% a	9.9% a	9.2% a	9.3% a	p=0.572 [c]
Planctomycetaceae [a]	6.0% a	7.0% a	6.1% a	6.4% a	6.2% a	p=0.822 [c]
Desulfobulbaceae [b]	4.7% a	5.1% a	6.2% a	4.8% a	6.0% a	p=0.593 [d]
Opitutaceae [b]	6.7% a	4.2% ab	5.5% ab	5.2% ab	4.8% b	p=0.527 [d]
Bacteroidetes_unclassified [b]	5.8% a	3.6% a	3.6% a	4.6% a	4.5% a	p=0.431 [d]
Gammaproteobacteria_unclassified [b]	3.9% a	3.9% a	4.2% a	3.6% a	3.8% a	p=0.788 [d]
Marinilabiliaceae [b]	4.0% a	4.0% a	3.7% a	4.4% a	3.2% a	p=0.511 [d]
Rhodobacteraceae [a]	3.7% a	3.8% a	3.7% a	3.7% a	4.2% a	p=0.867 [c]
Rhizobiales_unclassified [a]	3.5% a	3.7% a	3.0% a	3.6% a	3.4% a	p=0.773 [c]
Sphingomonadaceae [a]	3.0% a	3.2% a	3.1% a	3.4% a	3.3% a	p=0.930 [c]
Enterobacteriaceae [a]	2.8% a	2.9%a	3.7% a	2.8% a	3.1% a	p=0.565 [c]
Comamonadaceae [b]	2.7% a	2.5% a	2.7% a	2.8% a	3.0% a	p=0.808 [d]
Burkholderiales_unclassified [a]	2.3% a	2.4% a	1.9% a	2.3% a	2.3% a	p=0.540 [c]
Proteobacteria_unclassified [b]	1.9% a	2.2% a	2.0% a	1.8% a	2.1% a	p=0.823 [d]
Rhodocyclaceae [b]	2.2% a	1.5% a	1.5% a	2.0% a	1.8% a	p=0.313 [d]
Alphaproteobacteria_unclassified [b]	1.8% a	1.9% a	1.6% a	1.7% a	1.8% a	p=0.505 [d]
Lachnospiraceae [b]	1.3% a	1.4% a	1.7% a	1.8% a	1.6% a	p=0.886 [d]
Chitinophagaceae [a]	1.5% a	1.7% a	1.5% a	1.4% a	1.7% a	p=0.763 [c]
Betaproteobacteria_unclassified [b]	1.5% a	1.4% a	1.3% a	1.3% a	1.5% a	p=0.456 [d]
Rhizobiaceae [b]	1.5% a	1.3% a	1.1% a	1.4% a	1.3% a	p=0.805 [d]
Bacteroidales_unclassified [b]	1.1% a	1.5% a	0.8% a	1.8% a	1.0% a	p=0.019 [d]
Porphyromonadaceae [b]	1.0% a	1.1% a	1.4% a	1.0% a	1.0% a	p=0.285 [d]
Hyphomicrobiaceae [a]	1.1% a	1.2% a	1.0% a	1.2% a	1.0% a	p=0.597 [c]

[a,b] Mean values (n=8) +/- standard error. Different letters within a row indicate significant differences between sites based on Tukey Post-hoc test ($p<0.5$) [a] or Dunn's Multiple Comparison test ($p<0.05$) [b]

Similarly, there was no separation of samples based on pharmaceutical treatment or sunlight in the PCOA analysis of the theta index (Figure 6) and no significant effect of either treatment (pharmaceutical, sunlight) based on AMOVA of the theta index (p=0.477, p=0.06).

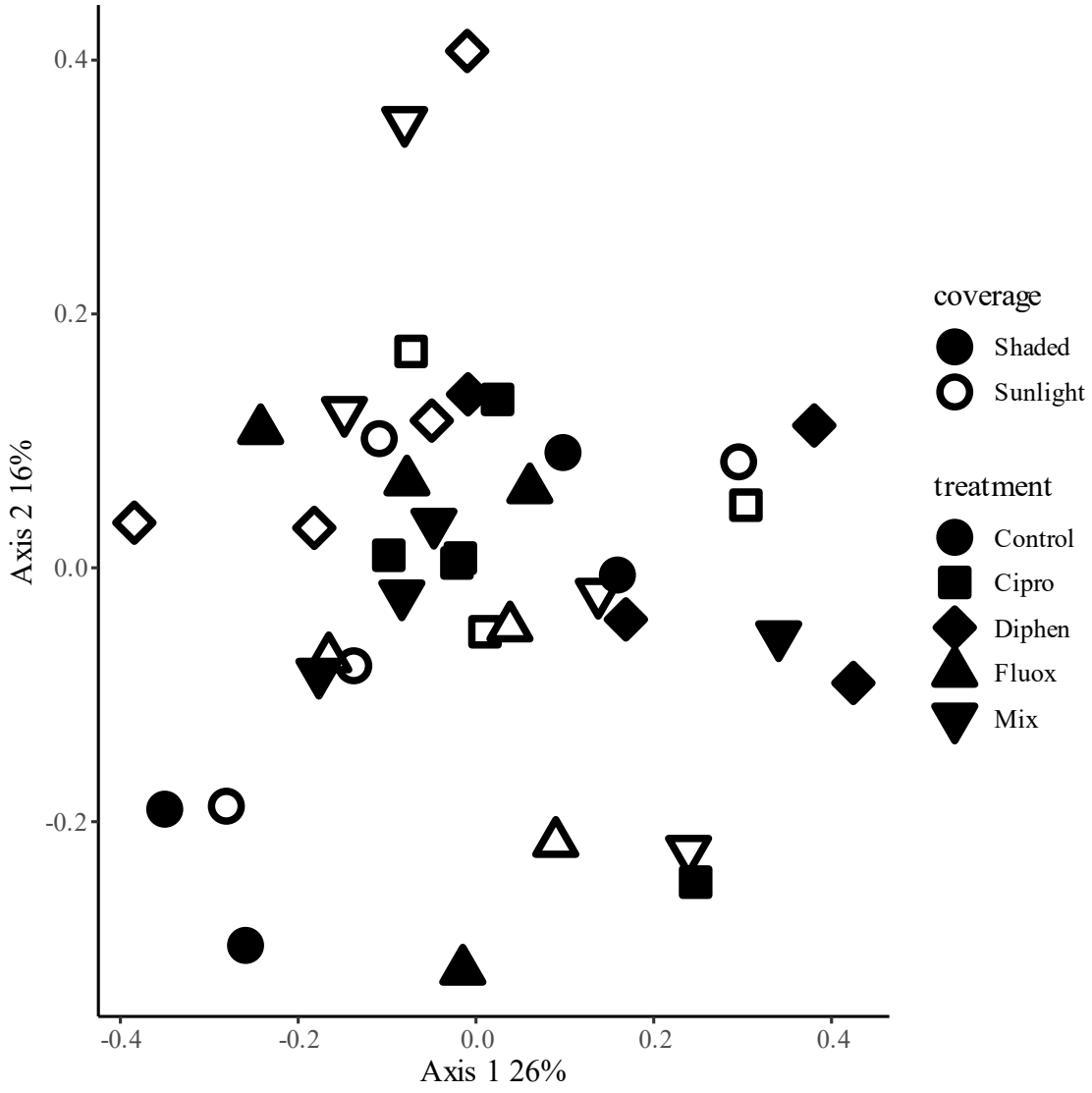

Figure 6. Principal coordinates analysis of sediment bacterial communities from 5 treatments. Community analysis was based on sequencing of partial 16S rRNA genes and ordination is based on the theta index.

Discussion

The addition of three pharmaceuticals (the antibiotic ciprofloxacin, the antihistamine diphenhydramine, and the antidepressant fluoxetine), either individually or in a mixture, at environmentally relevant concentrations to artificial stream mesocosms had almost no significant impact on the metabolic activity (respiration and denitrification rates) or taxonomic composition of microbial communities colonizing sediment packs. This result was somewhat surprising as previous studies had shown that pharmaceuticals, in general, and specifically these three compounds, can have significant impacts on the activity and composition of aquatic microbial communities (Richmond et al., 2019; Rosi et al., 2018; Rosi-Marshall et al., 2013). However, the pharmaceutical concentrations used in the current study were significantly lower than those used in the prior studies cited above. While this increases the environmental relevance of the current study, these concentrations were likely too low to have an effect. The concentrations chosen for this study were mean values based on previously published data (see Robson et al., 2020 for details). However, concentrations of these pharmaceuticals an order of magnitude higher than these mean values have been measured in the field, so the lack of response in our study does not indicate that these compounds might not be having an effect at sites with higher concentrations. In future studies we would suggest using a range of concentrations that encompasses the variation seen in the field. In addition, the prior studies cited above focused on benthic biofilms formed on solid substrates, whereas the current study focused on microbial communities within three-dimensional sediment packs. It is possible that these sediment packs limited the exposure of the microbial communities, especially those microbes in the inner layers of the sediment packs, to the pharmaceuticals dissolved in the water column. This hypothesis is supported by

results of a prior study by our collaborators that used a similar experimental design and the same concentrations of the same pharmaceuticals and reported significant effects on biofilms developing on silica rocks (Robson et al., 2020). Therefore, we would suggest that future studies examine both sediment and biofilm communities to assess differences in their responses.

The only significant effect of the PPCPs observed in this study was that the mixture of all three pharmaceuticals resulted in a slightly higher number of observed bacterial species in the sediments packs compared to the no pharmaceutical control. This specific result was surprising as a previous study by our collaborators demonstrated that ciprofloxacin exposure resulted in a decrease in taxonomic richness for aquatic biofilm communities (Rosi et al., 2018). However, this previous study was based on a higher concentration of ciprofloxacin. The increase in species richness observed in the current study suggests that exposure of microbial communities to a range of PPCPs at low concentrations might provide enhanced opportunities for additional taxa that can interact with these compounds, perhaps as a carbon or energy resource. However, the fact that no broad changes in taxonomic composition were observed for the PPCP mixture, for example in the PCOA ordination, indicates that the observed increase in taxonomic richness was driven by low abundance taxa. These results support the conclusion that additional studies of the effects of low, sub-lethal concentrations of PPCPs are warranted.

CHAPTER 3

EFFECTS OF TREATED WASTEWATER ON BENTHIC MICROBIAL COMMUNITIES

Introduction

Domestic wastewater refers to water released from residences and businesses that has been contaminated through human activities such as restroom usage, washing, bathing, food preparation, and laundry. The release of large volumes of untreated domestic wastewater to the environment can have negative effects on aquatic ecosystems due to the high nutrient content of wastewater and the potential presence of pathogens (Rittmann & McCarty, 2000). Wastewater treatment plants (WWTPs) are the most common method to treat domestic wastewater, serving over 75% of the United States population (EPA 2004), and are designed to reduce the nutrient and pathogen content of wastewater so that it is safe for release to the environment (Rittmann & McCarty, 2000). WWTPs frequently release treated water (i.e. effluent) into surface waters, including streams and rivers (EPA 2004), and WWTP effluent can be a major source of flow in streams, especially in urban areas (Brooks et al., 2006).

Domestic wastewater also contains a range of pharmaceuticals and personal care products (PPCPs) that are used by humans and released into wastewater, including stimulants, analgesics, antibiotics, antiseptics, disinfectants, antihistamines, and other medications (Daughton & Ternes, 1999; Hedgespeth et al., 2012). WWTPs are not designed explicitly to remove PPCPs, although some are removed incidentally. The efficiency of incidental removal of PPCPs by WWTPs varies widely for different compounds (Aga, 2007), so WWTP effluent can be a point source of

many PPCPs to the receiving system (Waiser et al., 2011). For commonly used PPCPs that are not effectively removed, WWTPs continuously deliver these compounds to the environment, resulting in their pseudo-persistence, which has been observed across the United States (Heberer, 2002; Kolpin et al., 2002b).

There is concern about the inputs of PPCPs to surface waters because of the potential for PPCPs to interact with aquatic biota, including microorganisms. Microbes are critical components of stream ecosystems because of their contributions to nutrient cycling and organic matter breakdown, and because they are an important food resource for stream food webs. The effects of PPCPs on stream microbial communities are largely unknown, but recent studies have reported that PPCPs can have effects on microbes that are drastically different than in humans (Rosi-Marshall & Royer, 2012). For example, in artificial streams dosed with amphetamines, a decrease in gross primary production was observed in microbial biofilms (Richmond et al., 2016). A similar effect, a decrease in primary production, was observed in artificial streams dosed with the antidepressants fluoxetine and citalopram (Richmond et al., 2016). Other studies have demonstrated decreases in respiration rates and shifts in bacterial community composition for biofilms experimentally exposed to a range of PPCPs, including antibiotics, antihistamines, and stimulants, using contaminant exposure substrates (Costello et al., 2016; Rosi et al., 2018; Rosi-Marshall et al., 2013). While these studies have identified possible effects of PPCPs on stream bacterial communities, they were based on manipulative experiments using either artificial streams (Richmond et al., 2016) or artificial substrates (Rosi et al., 2018; Rosi-Marshall et al., 2013), and generally included high PPCP concentrations. Field-based studies of native

communities under actual exposure scenarios are lacking in the literature. This study was designed to address this knowledge gap.

The goals of this study were to 1) measure the concentration of PPCPs present in urban streams in the Chicago metro area, and 2) compare benthic bacterial communities in streams with varying PPCP concentrations to assess possible correlations between PPCP exposure and bacterial community composition. To achieve these goals, we collected water and sediment samples from five different sites on three different streams, two of which received effluent from WWTPs. In the two streams receiving effluent we collected samples both upstream and downstream of the effluent input points. We quantified concentrations of a suite of ~30 common PPCPs in water samples from each site, and we analyzed benthic bacterial communities using DNA-based approaches to characterize both their taxonomic composition and their functional (i.e. genomic) potential.

Methods

Study Sites

Nippersink Creek (NPRS) is a woodland stream located in McHenry County, IL which has minimal urbanization in its watershed. NPRS has a drainage area of 5,095 ha that is 7.8% residential, 63.1% agricultural, 2.1% vacant, 20.7% open land and 0.1% industrial (www.nippersink.org). We collected water and sediment samples from one site on NPRS (42.41835, -88.34466) on 11/4/2019. There are no WWTPs or combined sewer overflows (CSOs) on NPRS upstream of the sampling site.

Sites 2 and 3 were located on Springbook Creek, a suburban stream in DuPage County, IL, which receives treated wastewater effluent from the Wheaton Sanitary District WWTP. This

effluent accounts for ~80% of the flow of Springbook Creek downstream of the WWTP (McCormick et al., 2016). Site 2 (USPR; 41.84796, -88.14001) was located on Springbrook Creek 750 meters upstream of the WWTP effluent input point and site 3 (DSPR; 41.84277, -88.14684) was on Springbrook Creek 87 meters downstream of the effluent input point. We collected water and sediment samples from sites 2 and 3 on 11/6/2019.

Sites 4 and 5 were located on Salt Creek, a suburban stream in DuPage County, IL, which receives treated wastewater effluent from the Elmhurst WWTP. This effluent accounts for ~13% of the flow of Salt Creek downstream of the WWTP (McCormick et al., 2016). Site 4 (USLT; 41.88281, -87.95924) was located on Salt Creek 200 meters upstream of the WWTP effluent input point and site 5 (DSLT; 41.87881, -87.95825) was on Salt Creek 256 meters downstream of the effluent input point. We collected water and sediment samples from sites 4 and 5 on 11/6/2019.

Sample Collection

The following sampling was conducted at each of the 5 field sites. We collected 5 replicate 20 mL water samples at each site using a 50 ml syringe, filtered them on-site with 0.2-um syringe filters (Thermo Fisher Scientific, Rockwood, Tennessee), placed them in sterile scintillation vials, stored them on ice in a cooler for transport to the lab, and then stored them in the lab at -20°C for subsequent nutrient chemistry analysis. We collected 3 replicate 1 L water samples at each site in amber glass bottles with Teflon-lined lids, stored them on ice for transport to the lab, and then stored them in the lab at 4°C for subsequent PPCP analysis. We collected 5 replicate sediment samples from each site. For each replicate sample we collected sediment from the stream using a shovel, passed the sediment through a 4mm sieve into a plastic tub,

homogenized the sediment by mixing it in the tub with the shovel, and then collected a 90 mL subsample in a sterile 90 mL plastic specimen cup (Parter Medical, Carson CA). Replicate sediment samples at each site were collected using this same approach from locations at least 1 m from the other replicates. The shovel, sieve, and tub were rinsed with stream water between replicates and were sterilized with ethanol between sites. The specimen cups were stored on ice for transport to the lab and then were stored in the lab at 4°C overnight. The next day five 0.5 ml subsamples of each sediment sample were transferred to 2 mL microcentrifuge tubes and stored at -20°C for subsequent DNA extraction. Approximately 10 g of the remaining sediment was used for quantification of organic matter content. At each field site we measured water temperature, dissolved oxygen, specific conductance, total dissolved solids, salinity, pH, turbidity, chlorophyll a, and phycocyanin concentration using a YSI ProDSS multiparameter water quality meter (YSI Yellow Springs, OH). Replicate readings (n=5) of each of these parameters were taken at each of the field sites.

Nutrient Chemistry and Sediment Organic Matter

We analyzed water samples for soluble reactive phosphorus (SRP), ammonium (NH_4^{+1}), and nitrate (NO_3^{-2}) with an Auto Analyzer 3 (SEAL Analytical,Mequon,Wisconsin). We measured SRP with the antimonyl tartrate technique (Murphy and Riley 1962), NH_4^{+1} with the phenol hypochlorite technique (Solorzano 1969), and NO_3^{-2} with the cadmium reduction technique (Rice et al., 2012). Chemical analyses were completed within 10 weeks of collection. We followed quality control and assurance checks recommended by the manufacturer (Seal Analytical) including equipment blanks, carryover tests, and drift correction. All standard curves

showed $r^2 \geq 0.999$. Organic matter content of the sediment samples was calculated by loss on ignition at 500°C (Bear 1964).

PCPP Analysis

Water samples were loaded onto solid phase extraction (SPE) cartridges within 2 weeks of sample collection. Cartridges were first conditioned with 5-10 mL of methanol and then 5-10 mL of deionized water. Sample water was passed through a 25 mm diameter, 1um pore size glass fiber filter and then through the SPE cartridge at a rate of 1 drop per second until approximately 300 mL was passed through the filter, with the specific volume passed recorded for each sample. Cartridges were stored at -20°C and then sent to the University of Nebraska for analysis using Ultra Efficient Liquid Chromatography.

DNA Extraction and Sequencing

DNA extraction of sediment samples was completed using the Qiagen DNeasy Power Soil Extraction kit (Qiagen, Inc., Hilden, Germany) and successful extraction was confirmed with gel electrophoresis. Kits without samples were run as contamination controls and produced no visible bands on the agarose gels. Extracted DNA was quantified with Nanodrop (Thermo Fisher, Rockland DE).

For metagenome analysis, replicate DNA samples from each of the 5 field sites were pooled and sent to the DNA Services Facility, University of Illinois at Chicago for sequencing. Dual-indexed paired-end libraries were prepared using the Nextera FLEX DNA Prep Kit. Sequencing was conducted in a 2 x 151 paired-end format with the NovaSeq 6000 SP platform (Illumina, San Diego, California). All shotgun sequence data analyzed in this paper can be

downloaded from the National Center for Biotechnology Information Sequence Read Archive with accession number (PRJNA662915).

For 16S amplicon sequencing, polymerase chain reaction (PCR) was performed on each replicate sample from each site (total of 25 samples) using 515F and 806R primers targeting the V4 hypervariable region of the 16S ribosomal RNA gene (Caporaso et al., 2012). Successful amplification was confirmed with gel electrophoresis. No bands were observed for kit controls, confirming the kits were not a source of contamination. Amplicons were sequenced in a 2 x 250 paired-end format with the MiSeq platform (Illumina, San Diego, California; Caporaso et al., 2012) by the DNA Services Facility, University of Illinois at Chicago. All sequence data analyzed in this paper can be downloaded from the National Center for Biotechnology Information Sequence Read Archive with accession number (PRJNA662915).

Statistics

All statistical analyses were run in R (v3.6.1) using the package rstatix (Kassambara, 2020b). We assessed the normality of the sediment organic matter concentrations and water column nitrate, ammonium, and phosphate concentrations based on the Shapiro test (Shapiro & Wilk, 1965). None of these data were normally distributed, so we assessed the effect of site on these data with the Kruskal-Wallis rank sum test (Kruskal & Wallis, 1952) followed by pairwise comparisons with the Dunn's test (Dunn, 1961) when there was a significant main effect ($p<0.05$). We assessed the normality of the YSI data (water temperature, dissolved oxygen, specific conductance, total dissolved solids, salinity, pH, chlorophyll a and phytocyanin concentration, and turbidity) based on the Shapiro test (Shapiro & Wilk, 1965). For normally distributed data, we assessed the effect of site by one-way ANOVA followed by pairwise

comparisons with Tukey's Honestly-Significant-Difference Test (Tukey, 1949) when there was a significant main effect ($p<0.05$). For non-normally distributed data, we assessed the effect of site with the Kruskal-Wallis rank sum test (Kruskal & Wallis, 1952) followed by pairwise comparisons with the Dunn's test (Dunn, 1961) when there was a significant main effect ($p<0.05$).

Analysis of Amplicon Sequence Data

Amplicon sequences were processed with mothur V.1.42.2 (Schloss et al., 2009) following the MiSeq Standard Operating Procedure (Kozich et al., 2013). Briefly, paired reads were assembled and demultiplexed, and any sequences with ambiguities or homopolymers >8 bases were removed from the data set. Sequences were aligned with the SILVA-compatible alignment database available within mothur. Chimeric sequences were identified with UCHIME (Edgar et al., 2011) and removed from the data set. Sequences were classified with the mothur-formatted version of the RDP training set (v.9) and any unknown (i.e., not identified as bacterial), chloroplast, mitochondrial, archaeal, and eukaryotic sequences were removed. Sequences were then clustered into operational taxonomic units (OTUs) based on 97% sequence identity. We randomly subsampled the entire dataset to 58,826 sequences per sample to avoid biases associated with uneven numbers of sequences across samples. Bacterial communities were compared by calculating dissimilarities for each pair of samples based on theta index (Yue & Clayton, 2005) in mothur and visualizing the resulting dissimilarity matrix using principal coordinates analysis (PCOA) run in R (v.3.6.1). Statistical significance of differences in communities between sampling sites based on the theta index was assessed by analysis of molecular variance (AMOVA), a nonparametric analog of traditional analysis of variance

(Excoffier et al., 1992), which was run in mothur. Effect of sampling site on the relative abundance of the 25 most abundant bacterial families was assessed by one-way ANOVA run in R. Metastats analysis (J. R. White et al., 2009) run in mothur was used to identify bacterial genera (OTUs grouped at 95% sequence identity) that were differentially abundant between upstream and downstream sites from both Salt Creek and Springbrook, and ANOVA run in R was used to assess significance of differences in relative abundances of these OTUs. For OTUs that were not identified to the genus level by mothur (i.e. those labeled unclassified) we used mothur to select a representative sequence for that OTU, defined as the sequence with minimum average distance to other sequences within the OTU, and compared these representative sequences to the NCBI 16S rRNA database using Megablast. The results from these searches had percent identities that ranged from 96% to 99%.

Analysis of Shotgun Sequence Data

FastQC was used to evaluate the quality of sequence reads. The average number of reads per sample was 3,422,666 and the average quality score for each sample was 36.23. The shotgun reads were compared to an NCBI database to determine taxonomic classification using Kaiju (Menzel et al., 2016). The abundances were summarized into a human readable format and then plotted as a stacked bar chart using Microsoft Excel.

Results

Site Physical and Chemical Characteristics

There was a significant effect of site on all of the physical and chemical characteristics measured (Tables 4 and 5).

Table 4. Sediment Organic Matter and Water Chemistry at Field Sites

Site	Organic Matter %[a]	NO_3^- (mg N L^{-1})[a]	SRP (mg P L^{-1}) [a]	NH_4^+ (mg N L^{-1}) [a]
Nippersink Creek	0.901 +/- 0.064 a	2.473 +/- 0.036 ab	0.000 +/- 0.000 a	0.147 +/- 0.005 ab
Salt Creek Upstream	3.504 +/- 0.127 b	4.422 +/- 0.084 bd	0.393 +/- 0.018 ac	0.155 +/- 0.002 a
Salt Creek Downstream	2.343 +/- 0.038 bc	4.969 +/- 0.086 cd	0.449 +/- 0.110 bc	0.168 +/- 0.006 a
Springbrook Upstream	2.363 +/- 0.102 b	2.120 +/- 0.012 a	0.000 +/- 0.000 a	0.159 +/- 0.005 a
Springbrook Downstream	1.085 +/- 0.117 ac	12.627 +/- 1.180 c	0.801 +/- 0.070 b	0.090 +/- 0.002 b
Kruskal-Wallis	$p<0.001$	$p<0.001$	$p<0.001$	$p=0.005$

[a] Mean values (n=5) +/- standard error. Different letters within a column indicate significant differences between sites based on Dunn's Multiple Comparison post-hoc test ($p<0.05$).

These results reveal a pattern of significant differences in physical and chemical characteristics between the sites on Springbrook that were upstream and downstream of the WWTP effluent input, indicating a significant effect of the effluent on the physical and chemical characteristics of this stream. In contrast, there were very few differences observed between the sites on Salt Creek that were upstream and downstream of the WWTP effluent input, suggesting less of an effect of the effluent on the physical and chemical characteristics of this stream. Specifically, there was a significant effect of site on sediment organic matter (Table 4), which was significantly lower at the woodland site (Nippersink Creek) than at both of the suburban sites upstream of the effluent inputs (Salt Creek Upstream and Springbrook Upstream). In addition, organic matter was significantly lower downstream of the effluent input compared to upstream of the effluent input on Springbrook, but there was no significant difference in organic

matter between the downstream and upstream sites on Salt Creek. There was a significant effect of site on nitrate concentration (Table 4). Although there were no significant differences in nitrate concentration between Nippersink Creek and the upstream sites, and no significant difference between the upstream and downstream sites on Salt Creek, the downstream site on Springbrook had a significantly higher nitrate concentration than the upstream site. There was a significant effect of site on SRP concentration (Table 4), with SRP showing the same pattern as nitrate. Specifically, there were no significant differences in SRP between Nippersink Creek and the upstream sites, and no significant difference between the upstream and downstream sites on Salt Creek, but the downstream site on Springbrook had a significantly higher SRP concentration than the upstream site. There was a significant effect of site on ammonium concentration (Table 4). There were no significant differences in ammonium concentration between Nippersink Creek and any of the suburban sites, and no significant difference between the upstream and downstream sites on Salt Creek, but the downstream site on Springbrook had a significantly lower ammonium concentration than the upstream site. There was a significant effect of site on water temperature (Table 5). Temperature at the woodland site (Nippersink Creek) was not significantly different than the suburban upstream sites, and there was no significant difference between the upstream and downstream sites on Salt Creek, but the temperature at the downstream site on Springbrook was significantly higher than upstream.

Table 5. Water Characteristics Measured on Site

Site	Temperature [a] °C	Dissolved O_2 [a] %	Specific Conductance [a] uS/cm	Total Dissolved Solids [a] mg/L	Salinity [a] ppt	pH [a]	Turbidity [a] FNU	Chlorophyll [a] RFU	Phycocyanin [b] RFU
Nippersink Creek	6.9 +/- 0.033 a	93.5 +/- 0.033 a	721.0 +/- 0.000 a	469.0 +/- 0.333 a	0.35 +/- 0.000 a	7.89 +/- 0.019 a	12.4 +/- 0.067 a	6.10 +/- 0.058 a	0.90 +/- 0.050 a
Salt Creek Upstream	8.7 +/- 0.000 abc	91.4 +/- 0.000 bc	942.0 +/- 0.000 ac	612.0 +/- 0.000 ac	0.47 +/- 0.000 ac	7.72 +/- 0.006 abc	8.55 +/- 0.782 ac	2.55 +/- 0.050 ac	0.57 +/- 0.033 d
Salt Creek Downstream	9.1 +/- 0.033 bc	92.0 +/- 0.200 abc	948.7 +/- 0.882 abc	617.0 +/- 0.000 abc	0.47 +/- 0.000 ac	7.75 +/- 0.028 ac	6.53 +/- 1.122 bc	2.42 +/- 0.120 abc	0.48 +/- 0.044 d
Springbrook Upstream	7.7 +/- 0.000 ab	72.7 +/- 1.139 b	1102.3 +/- 0.333 b	716.3 +/- 0.667 b	0.55 +/- 0.000 b	7.48 +/- 0.000 bc	7.65 +/- 1.000 abc	1.25 +/- 0.074 bc	0.31 +/- 0.010 b
Springbrook Downstream	15.3 +/- 0.033 c	93.4 +/- 0.088 ac	1056.3 +/- 0.333 bc	686.3 +/- 0.333 bc	0.53 +/- 0.000 bc	7.44 +/- 0.012 b	1.05 +/- 0.026 b	0.57 +/- 0.017 b	0.09 +/- 0.021 c
Kruskal-Wallis[c] / ANOVA[d]	p= 0.008 [c]	p=0.01 [c]	p=0.008 [c]	p=0.008 [c]	p=0.005 [c]	p=0.01 [c]	p= 0.01 [c]	p=0.01 [c]	p=<0.001 [d]

[a,b] Mean values (n=3) +/- standard error. Different letters within a column indicate significant differences between sites based on [a] Dunn's Multiple Comparison post-hoc test ($p<0.05$) or [b] Tukey post-hoc test ($p<0.05$).

There was a significant effect of site on dissolved oxygen (Table 5), with the most notable difference being significantly higher dissolved oxygen on Springbrook downstream of the effluent input as compared to upstream. Specific conductance and total dissolved solids significantly differed based on site (Table 5), with significant differences for both of these parameters between all of the individual sites. Salinity significantly differed by site (Table 5), although there were no significant upstream vs. downstream differences. There were significant but very minor differences in pH based on site, and no significant upstream vs. downstream differences (Table 5). Finally, there were some site-specific differences in turbidity (Table 5), but no significant difference between upstream and downstream sites on either Salt Creek or Springbrook.

Photosynthetic Pigment Concentrations

There was a significant effect of site on phycocyanin concentrations (Table 5). Nippersink Creek had the highest concentration of phycocyanin, almost 2-fold higher than any other site, and both sites on Salt Creek had higher phycocyanin concentrations than the two Springbrook sites. Finally, the phycocyanin concentration was significantly lower downstream vs. upstream for Springbrook, while there was no significant difference upstream vs. downstream for Salt Creek. The pattern for chlorophyll a concentration was virtually identical to phycocyanin, with the highest concentration at Nippersink Creek, followed by Salt Creek and then Springbrook, and with a lower concentration downstream vs. upstream for Springbrook but no difference between downstream and upstream sites on Salt Creek (Table 5). However, the difference in chlorophyll a concentrations between downstream and upstream sites on Springbrook was not statistically significant.

Pharmaceuticals and Personal Care Products

Quantification of pharmaceuticals and personal care products has not yet been completed by our collaborator at the University of Nebraska due to the shutdown of the university for the COVID-19 pandemic. These data will be incorporated into the study prior to submission for publication.

Microbial Community Analysis via 16SrRNA Amplicon Sequencing

There was a significant effect of site on sediment bacterial species richness (total number of observed species) and Shannon diversity assessed via 16S rRNA amplicon sequencing (Table 6).

Table 6. Bacterial Community Richness and Diversity

Site	**Observed Species (#)** [a]	**Shannon Diversity (H)** [b]
Nippersink Creek	16,914 a	8.494 ab
Salt Creek Upstream	19,641 c	8.779 c
Salt Creek Downstream	15,712 d	8.212 b
Springbrook Upstream	18,637 b	8.664 ac
Springbrook Downstream	17,980 b	8.707 c
ANOVA [c] Kruskal-Wallis [d]	$p<0.001$ [c]	$p<0.001$ [d]

[a,b] Mean values (n=5) +/- standard error. Different letters within a column indicate significant differences between sites based on Tukey Post-hoc test ($p<0.5$) [a] or Dunn's Multiple Comparison test ($p<0.05$) [b] .

Specifically, the total number of species observed at the woodland site (Nippersink Creek) was significantly different than at all four of the suburban sites. In addition, the number of observed species upstream of the effluent point on Salt Creek was significantly higher than the number of observed species downstream, but there was no significant difference in number of

observed species at the upstream and downstream sites on Springbrook (Table 6). The pattern for Shannon diversity was similar, with the index score being significantly lower downstream on Salt Creek than upstream, but no significant difference for the upstream and downstream sites on Springbrook (Table 6).

Based on 16S rRNA amplicon sequencing, the most abundant bacterial families identified in the sediments of all sites included *Betaproteobacteria, Gammaproteobactera*, *Burkholderiales, Bacteroidetes*, *Rhizobiales* and *Plantomycetacea* (Figure 7).

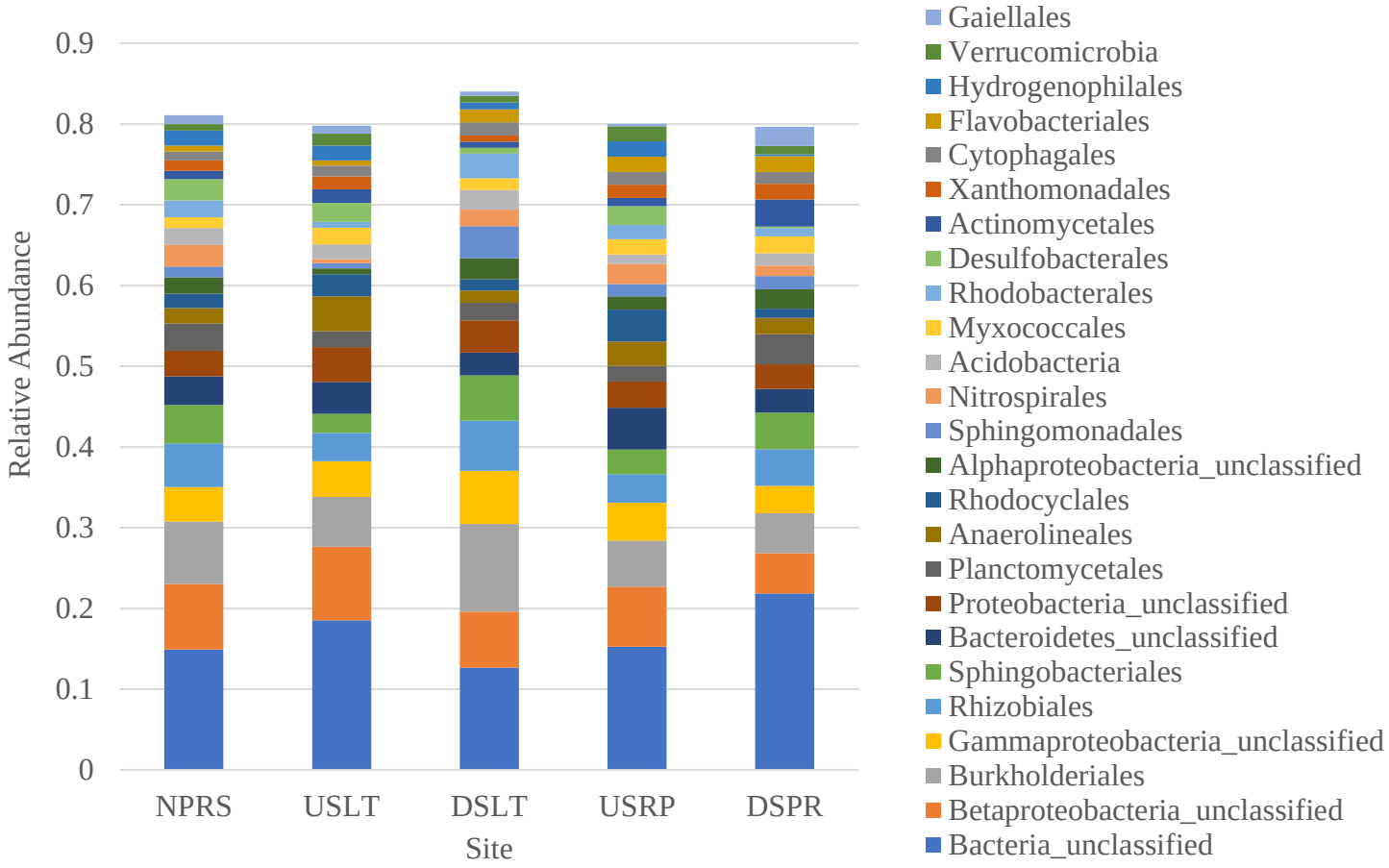

Figure 7. Relative abundance of the 25 most abundant bacterial families in sediment samples from five sites based on high-throughput amplicon sequencing of partial 16 rRNA genes. Each bar represents the mean (n=5).

There was a significant effect of site on the relative abundance of all of the 25 most abundant families (Table 7). One family that differed significantly in relative abundance between upstream and downstream sites on both Salt Creek and Springbrook was *Betaproteobacteira*_unclassified, which was 23% less abundant downstream than upstream on Salt Creek and 32% less abundant downstream than upstream on Springbrook (Table 7).

Table 7. Differences in Relative Abundance of The 25 Most Abundant Bacterial Families Based on Amplicon Sequencing of Partial 16S rRNA Genes

Taxa	Nippersink Creek	Salt Creek Upstream	Salt Creek Downstream	Springbrook Upstream	Springbrook Downstream	ANOVA c Kruskal-Wallis d
Bacteria unclassified [b]	14.9% ab	18.5% a	12.7% b	15.3% a	21.9% b	p=0.0004 [d]
Betaproteobacteria unclassified [a]	8.1% a	9.1% c	7.0% a	7.4% b	5.0% ac	p<0.001 [c]
Gammaproteobacteria unclassified [b]	4.3% a	4.4% c	6.6% ac	4.7% b	3.4% bc	p=0.0005 [d]
Burkholderiales unclassified [b]	5.1% a	3.5% bc	6.3% ac	3.0% b	2.6% ac	p=0.0005 [d]
Bacteroidetes unclassified [a]	3.5% a	4.0% b	2.8% c	5.2% ab	2.9% ab	p<0.001 [c]
Proteobacteria unclassified [b]	3.2% ab	4.3% b	4.0% ac	3.2% c	3.0% ac	p=0.006 [d]
Rhizobiales unclassified [a]	3.9% ab	2.1% c	3.6% c	2.4% ac	2.7% b	p<0.001 [c]
Planctomycetaceae [b]	3.4% a	2.0% ac	2.2% abc	2.0% b	3.8% bc	p=0.001 [d]
Anaerolineaceae [b]	1.9% ab	4.3% b	1.5% ab	3.0% ac	2.0% c	p=0.0004 [d]
Chitinophagaceae [a]	2.6% a	1.3% d	3.8% cd	1.7% b	2.9% bc	p<0.001 [c]
Comamonadaceae [b]	2.2% ab	2.1% a	3.7% c	2.1% a	1.6% bc	p=0.003 [d]
Rhodocyclaceae [b]	1.8% a	2.7% c	1.4% bc	4.0% ab	1.2% c	p=0.0001 [d]
Alphaproteobacteria unclassified [a]	2.0% a	0.7% d	2.6% c	1.6% ab	2.4% bc	p<0.001 [c]
Nitrospiraceae [a]	2.7% ab	0.5% c	2.1% b	2.5% ac	1.3% b	p<0.001 [c]
Acidobacteria [a]	2.1% a	1.8% bd	2.5% c	1.1% bc	1.5% ad	p<0.001 [c]
Rhodobacteraceae [a]	2.1% a	0.7% b	3.0% c	1.8% b	1.0% c	p<0.001 [c]
Sphingomonadaceae [b]	1.2% a	0.5% ac	3.7% b	1.2% bc	1.4% b	p=0.0001 [d]

Taxa	Nippersink Creek	Salt Creek Upstream	Salt Creek Downstream	Springbrook Upstream	Springbrook Downstream	ANOVA [c] Kruskal-Wallis [d]
Hydrogenophilaceae [a]	1.9% a	1.9% b	0.8% b	1.9% a	0.2% b	p<0.001 [c]
Flavobacteriaceae [b]	0.6% a	0.5% a	1.3% a	1.7% a	1.9% a	p=0.001 [d]
Verrucomicrobia [b]	0.8% a	1.5% b	0.8% c	1.9% ab	1.0% ac	p=0.0007 [d]
Myxococcales unclassified [b]	0.8% ab	1.3% a	0.9% ab	0.8% a	1.6% b	p=0.0007 [d]
Gaiellaceae [b]	1.1% ab	1.0% b	0.6% a	0.3% a	2.4% b	p=0.0002 [d]
Desulfobacteraceae [b]	1.8% a	1.3% ab	0.4% b	1.6% ab	0.1% b	p=0.0002 [d]
Sphingobacteriales unclassified [b]	1.5% a	0.7% b	1.2% b	0.7% a	1.2% a	p=0.001 [d]
Deltaproteobacteria unclassified [a]	1.2% a	1.4% c	0.6% b	1.0% b	1.0% bc	p<0.001 [c]

[a,b] Mean values (n=5). Different letters within a row indicate significant differences between sites based on Tukey Post-hoc test (p<0.5) [a] or Dunn's Multiple Comparison test (p<0.05) [b] .

Several other families differed significantly in relative abundance between the upstream and downstream sites of one stream but not the other (Table 7). For example, *Bacteroidetes*_unclassified and *Proteobacteria*_unclassified were both significantly less abundant (29% and 7%, respectively) and *Alphaproteobacteria*_unclassified and *Sphingomonadaceae* were significantly more abundant (3-fold and 7-fold) at Salt Creek downstream compared to upstream, but none of these families were significantly different between Springbrook upstream and downstream (Table 7). In contrast, *Rhizobiales*_unclassified was significantly more abundant (14%) and *Rhodocyclaceae* was significantly less abundant (71%) at Springbrook downstream compared to upstream, but neither of these families differed significantly for Salt Creek upstream and downstream sites (Table 7). Finally, several families including *Comamonadaceae*, *Nitrospiraceae*, and *Rhodobacteraceae* showed opposite trends for the two streams, increasing downstream to upstream at one site and decreasing at the other.

Table 8. Significance of Site Specific Differences in Bacterial Community Composition Based on The Theta Index

Site	**P-Value** [a]
Across All sites	<0.001
Nippersink Creek-Salt Creek Upstream	0.010
Nippersink Creek-Salt Creek Downstream	0.006
Nippersink Creek - Springbrook Upstream	0.007
Nippersink Creek - Springbrook Downstream	0.012
Salt Creek Upstream - Salt Creek Downstream	0.014
Springbrook Upstream - Springbrook Downstream	0.007
Salt Creek Upstream - Springbrook Upstream	0.005
Salt Creek Upstream - Springbrook Downstream	0.005
Salt Creek Downstream - Springbrook Upstream	0.010
Salt Creek Downstream - Springbrook Downstream	0.011

Based on AMOVA [a]

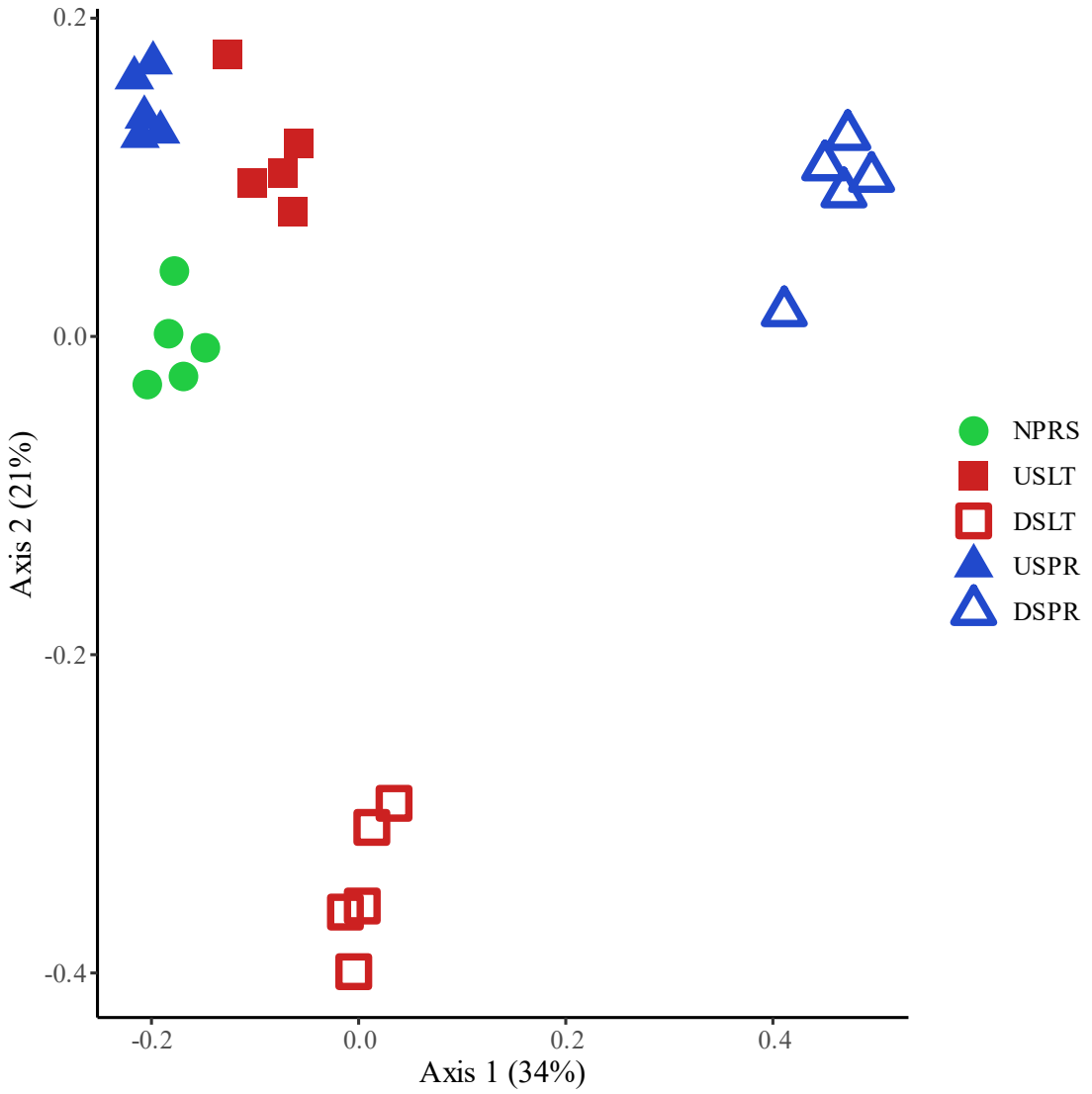

Figure 8. Principal coordinates analysis of sediment bacterial communities from 5 sites. Community analysis was based on sequencing of partial 16S rRNA genes and ordination is based on the theta index.

Comparison of the sediment bacterial communities from each of the sites based on 16S rRNA amplicon sequencing, principal coordinates analysis (Figure 8), and AMOVA (Table 8) indicated significant differences between samples from each of the 5 sites. There is a distinct separation between the bacterial communities from the upstream (USPR, USLT) and the downstream sites (DSLT, DSPR) of both streams (Figure 8). The upstream communities from

both streams (USLT, USPR) are relatively similar to each other and to the community from the woodland site (NPRS), whereas bacterial communities from the downstream sites are highly distinct from the upstream sites and from each other (Figure 8).

Table 9. Bacterial genera with the largest differences in relative abundance between sites upstream and downstream of wastewater treatment plants based on amplicon sequencing of partial 16S rRNA genes

Genus[a]	**Upstream**[b]	**Downstream**[b]	**p-value**[c]
Sphingorhabdus	0.1831% +/- 0.0194%	1.4329% +/- 0.2893%	<0.001
Thiobacillus	1.7569% +/- 0.0683%	0.5095% +/- 0.1052%	<0.001
Burkholderiales unclassified	4.2162% +/- 0.2283%	5.4058% +/- 0.8419%	0.177
Rhodocyclaceae unclassified	1.4036% +/- 0.0627%	0.3704% +/- 0.0303%	<0.001
Bacteroidetes unclassified	1.1376% +/- 0.0278%	0.1610% +/- 0.0337%	<0.001
Bacteria unclassified	0.1328% +/-0.0401%	0.9411% +/- 0.1361%	<0.001
Rhodocyclaceae unclassified	1.1114% +/- 0.1556%	0.3573% +/- 0.0284%	<0.001
Nitrospira	0.2427% +/- 0.0202%	0.9321% +/- 0.0929%	<0.001
Sinobacteraceae unclassified	0.9662% +/- 0.0537%	0.2801% +/- 0.0271%	<0.001
Terrimonas	0.4576% +/- 0.0491%	1.1102% +/- 0.0757%	<0.001
Desulfobacteraceae unclassified	0.7111% +/- 0.0423%	0.1419% +/- 0.0306%	<0.001
Ferruginibacter	0.1095% +/- 0.0102%	0.5977% +/- 0.0537%	<0.001
Nitrospira	1.1165% +/- 0.3104%	0.637% +/- 0.1336%	0.163
Rhodobacteraceae unclassified	0.2111% +/- 0.0155%	0.6858% +/- 0.1639%	0.011
Gammaproteobacteria unclassified	0.1028% +/- 0.0093%	0.5402% +/- 0.1984%	0.033
Desulfuromonas	0.4870% +/- 0.0321%	0.0592% +/- 0.0102%	<0.001
Betaproteobacteria unclassified	0.8153% +/- 0.1091%	0.4048% +/- 0.0981%	0.021
Mycobacterium	0.0656% +/- 0.0118%	0.4352% +/- 0.1438%	0.037
Gaiella	0.1906% +/- 0.0425%	0.5588% +/- 0.1434%	0.024
Bacteria unclassified	0.4286% +/- 0.0733%	0.0627% +/- 0.0168%	<0.001
Methylococcaceae unclassified	0.5744% +/- 0.0569%	0.2341% +/- 0.0143%	<0.001
Betaproteobacteria unclassified	0.4292% +/- 0.1029%	0.092% +/- 0.0069%	0.002
Hydrogenophaga	0.1623% +/- 0.0245%	0.4855% +/- 0.0523%	<0.001
Rhodobacteraceae unclassified	0.8772% +/- 0.1491%	1.2001% +/- 0.2005%	0.178
Sphingomonadaceae unclassified	0.5759% +/- 0.1055%	0.8816% +/- 0.1438%	0.094

[a] Genera are listed in order of decreasing differences in relative abundance between upstream and downstream sites. [b] Mean values +/- standard error (n=10).

[c] Based on one way ANOVA

In order to focus on the effects of WWTP effluent, metastats analysis was used to identify bacterial genera with the largest differences in relative abundance between upstream and downstream sites of both streams (i.e. comparing USLT and USPR to DSLT and DSPR) (Table 9). *Sphingorhabdus*, an unclassified Bacterial genus, and one genus of *Nitrospira* showed the largest increases downstream compared to upstream (8-fold, 7-fold, and 4-fold, respectively), whereas *Thiobacillus*, two unclassified *Rhodocyclaceae* genera, and one unclassified *Bacteroidetes* genus showed some of the largest decreases downstream compared to upstream (71%, 74%, 68%, and 86%, respectively). BLAST analysis indicated that the representative sequence from the unclassified Bacterial genus showed the highest percent identity to multiple species within the genus *Methylobacterium*. The representative sequence from the unclassified *Bacteroidetes* genus showed the highest percent identity to multiple species within the genus *Flavobacterium*. The representative sequences from the unclassified *Rhodocyclaceae* genera showed the highest percent identity to a varied range of taxa, so these OTUs could not be identified more specifically via this approach.

Microbial Community Analysis via Shotgun Sequencing

Generally, the relative abundance of the major taxa at each site based on the shotgun sequence data (Table 10) follows a similar pattern to the amplicon data. The amount of unclassified sequences ranged from 34-40% in each site.

Table 10. Differences in Relative Abundance of the 25 Most Abundant Bacterial Families Based on Shotgun Metagenomic Sequencing

Taxa	Nippersink Creek	Salt Creek Upstream	Salt Creek Downstream	Springbrook Upstream	Springbrook Downstream	ANOVA [c] Kruskal-Wallis [d]
Comamonadaceae [a]	2.0% a	1.6% b	2.6% c	1.8% d	1.3% e	p<0.001 [c]
Nitrospiraceae [a]	1.7% a	0.6% b	1.7% c	1.7% d	1.1% e	p<0.001 [c]
Enterococcaceae [a]	1.6% a	1.4% b	0.7% c	1.8% d	1.7% e	p<0.001 [c]
Rhodocyclaceae [a]	0.8% a	1.0% b	0.7% c	1.1% d	0.0% e	p<0.001 [c]
Rhodobacteraceae [a]	1.2% a	0.7% b	1.8% c	1.1% d	0.9% e	p<0.001 [c]
Planctomycetaceae [a]	1.2% a	0.9% b	1.0% c	0.8% d	1.2% e	p<0.001 [c]
Burkholderiaceae [b]	0.9% a	0.8% a	1% a	0.9% a	0.0% a	p=0.4 [d]
Streptomycetaceae [a]	0.9% a	0.8% b	0.8% c	0.7% d	0.9% e	p<0.001 [c]
Bradyrhizobiaceae [a]	0.8% a	0.9% b	1.4% c	0.8% d	1.0% e	p<0.001 [c]
Sphingomonadaceae [a]	0.7% a	0.0% b	1.4% c	0.8% d	0.8% e	p<0.001 [c]
Desulfobacteraceae [b]	0.6% a	0.6% a	0.0% a	0.6% a	0.0% a	p=0.4 [d]
Hyphomicrobiaceae [b]	0.6% a	0.0% a	0.7% a	0.0% a	0.0% a	p=0.4 [d]
Mycobacteriaceae [a]	0.5% a	0.0% b	0.7% c	0.0% d	1.4% e	p<0.001 [c]
Chitinophagaceae [b]	0.0% a	0.0% a	0.8% a	0.0% a	0.0% a	p=0.4 [d]
Anaerolineaceae [b]	0.0% a	0.7% a	0.0% a	0.6% a	0.0% a	p=0.4 [d]
Flavobacteriaceae [b]	0.0% a	0.5% a	0.0% a	0.7% a	0.0% a	p=0.4 [d]
Methylococcaceae [b]	0.0% a	0.0% a	0.0% a	0.6% a	0.0% a	p=0.4 [d]

[a,b] Different letters within a row indicate significant differences between sites based on Tukey Post-hoc test (p<0.5) [a] or Dunn's Multiple Comparison test (p<0.05) [b] .

The difference is in the level of classification allowed by the shotgun sequence data. In the amplicon data the second most abundant family is Betaproteobacteria, but it is unclassified at a lower level. The shotgun sequence data is able to resolve this to Comamonadaceae which is a member of the Betaproteobacteria class.

Discussion

There were multiple differences in the physical and chemical properties of the sites upstream and downstream of the WWTP on one of the study streams, Springbrook, including

increased water column concentrations of nitrate and SRP, increased water temperature, and decreased sediment organic matter and water column ammonium at the downstream site. WWTP effluent accounts for ~80% of the flow of Springbrook Creek downstream of the WWTP (McCormick et al., 2016b), so it is not surprising that this high level of effluent input would significantly alter the stream physical and chemical properties. Increased nitrate, SRP, and temperature (Waiser et al., 2011; Gucker et al., 2006; Chambers and Prepas 1994; Marti et al., 2004; Spänhoff 2007) and decreased sediment organic matter (Drury et al., 2013) downstream of WWTP inputs have been reported previously at other sites. The high level of effluent input and the accompanying physical and chemical changes at Springbrook would be expected to impact the stream microbial communities, and we observed lower concentrations of phycocyanin and chlorophyll a in the water column at the Springbrook downstream site compared to upstream. Phycocyanin is an accessory pigment that is found in cyanobacteria and chlorophyll a is a photosynthetic pigment found in algae and cyanobacteria. These pigments are commonly used as indicators of the abundances of these organisms in aquatic habitats (Pasztaleniec et al., 2020). The fact that these photosynthetic microorganisms were less abundant in the water column downstream vs. upstream on Springbrook was surprising because the higher concentrations of inorganic nitrogen and phosphorous in the water column as well as the increased water temperature would be expected to increase the abundance of these organisms, suggesting that some other aspects of the effluent were negatively impacting them, such as PPCPs or other pollutants. The decreased abundance of algae and cyanobacteria at the downstream sites on Springbrook is ecologically important because these organisms are key drivers of primary production in stream ecosystems and represent important food resources for higher trophic

levels. In addition to the decreased abundance of algae and cyanobacteria in the water column, there were also significant differences in the composition of sediment bacterial communities between the downstream and upstream sites on Springbrook, further demonstrating a significant effect of the WWTP effluent on this ecosystem.

In contrast to Springbrook, there were very few differences in the physical and chemical properties of the upstream and downstream sites at our other study stream, Salt Creek. WWTP effluent accounts for only ~13% of the flow of Salt Creek downstream of the WWTP (McCormick et al., 2016), so it is not surprising that this lower level of effluent input did not have as much of an impact on Salt Creek as it did on Springbrook. The similar physical and chemical properties of Salt Creek upstream and downstream of the effluent input corresponded with a similar abundance of phototrophic organisms in the water column at these sites, based on the cyanobacterial and algal indicators phycocyanin and chlorophyll a. In contrast, there were significant differences in sediment bacterial community composition between upstream and downstream sites on Salt Creek, including decreases in bacterial species richness and diversity downstream. A previous study by our group conducted on two other Illinois rivers also showed significant decreases in species richness and diversity in sediment bacterial communities downstream of WWTP effluent inputs (Drury, Rosi-Marshall, et al., 2013), suggesting that this may be a generalizable effect of effluent addition. The fact that the differences in sediment bacterial communities observed in our current study were not linked to changes in the physical and chemical properties we measured suggests that some other aspects of the effluent were impacting them, such as PPCPs or other pollutants. Previous work by our group has shown that

experimental exposure to PPCPs can have negative effects of the diversity of aquatic bacterial communities (Drury, Scott, et al., 2013; Rosi et al., 2018).

There were some consistent differences in sediment bacterial community composition between the upstream and downstream sites on both Springbrook and Salt Creek, indicating a consistent effect of the WWTP effluent. These consistent differences with effluent input included increases in the relative abundances of the genera *Sphingorhabdus* and *Methylobacterium* and decreases in the relative abundances of an unclassified *Bacteroidetes* genus. The genus *Sphingorhabdus* is a member of the family *Sphingomonadaceae,* which includes taxa that have been found to degrade anthropogenic pollutants such as mono- and polycyclic aromatic compounds (Heberer, 2002). Some of these aromatic compounds are produced from burning tar, oil, or other organic compounds (Centers for Disease Control and Prevention, 2011). *Sphingomonads* have been detected in soil, surface water, and wastewater (Cavicchioli et al., 1999; D. C. White et al., 1996). Due to their common presence in areas of human pollution *Sphingomondas* have been explored as a component of phytoremediation for polluted environments (Gatheru Waigi et al., 2017). The genus *Methylobacterium* has also been detected in diverse environments including wastewater treatment plants (DeLong and Rosenberg 2014, 321). Therefore, the increased relative abundances of *Sphingorhabdus* and *Methylobacterium* at the downstream sites in our study suggests a link to anthropogenic contaminants and WWTP effluent.

The unclassified *Bacteroidetes* genus that was shown to decrease in abundance at our downstream sites showed the highest percent identity to multiple species within the genus *Flavobacterium*. *Flavobacterium* are Gram-negative bacteria that are widely distributed in

nature, occurring mostly in aquatic ecosystems (Bernardet & Bowman, 2006). Within aquatic habitats the *Flavobacterium* are involved in the metabolism of various plant associated organic compounds, including carbohydrates and polysaccharides (Bernardet & Bowman, 2006). Our data suggest a negative impact of WWT effluent on the relative abundance of *Flavobacterium*, which could have negative implications for nutrient cycling in these stream ecosystems. Several previous studies by our group indicated that experimental exposure of stream bacterial communities to PPCPs resulted in decreased relative abundance of *Flavobacterium* (Rosi et al., 2018; Rosi-Marshall et al., 2013), suggesting that the decrease observed in the current study might be linked to these pollutants.

There is further processing to be completed on the shotgun metagenomic sequence data. Topics to be explored include variations in functional genes and antibiotic resistance genes across the different sampling sites. These analyses will be completed before submitting for publication in a scientific journal.

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